

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

**DETERMINATION OF TRNA-DERIVED
FRAGMENT CONTENT AND EFFECTS IN
EXOSOMES RELEASED FROM POLARIZED
MICROGLIA**

BURAK İBRAHİM ARIÖZ

MOLECULAR BIOLOGY AND GENETICS

Ph.D. THESIS

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Supervisor: Prof. Dr. Şermin GENÇ

(This research is supported by TUBITAK 1001 Grant **122Z019**)

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T.C.
DOKUZ EYLÜL ÜNİVERSİTESİ

İZMİR ULUSLARARASI BİYOTİP VE GENOM ENSTİTÜSÜ



Dokuz Eylül University Izmir International Biomedicine and Genome Institute
Department of Genomics and Molecular Biotechnology, Molecular Biology and
Genetics PhD program student Burak İbrahim ARIÖZ has successfully completed the
PhD thesis entitled “**Determination of tRNA-derived fragment content and effects
in exosomes released from polarized microglia**” on date of 27/06/2024.

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LIST OF ABBREVIATIONS

BP: Base pair

BSA: Bovine Serum Albumin

CNS: Central Nervous System

ELISA: Enzyme-linked Immunosorbent Assay

FBS: Fetal Bovine Serum

GO: Gene Ontology

IL-1 β : Interleukin 1 beta

IL-6: Interleukin 6

KEGG: Kyoto Encyclopedia of Genes and Genomes

lncRNAs: Long Noncoding RNAs

sncRNAs: Small Noncoding RNAs

LPS: Lipopolysaccharide

miRNAs: MicroRNAs

mmu: *Mus Musculus*

ncRNAs: Noncoding RNAs

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

RNA: Ribonucleic Acid

RPMI: Roswell Park Memorial Institute

SDS PAGE: Sodium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis

siRNA: small interfering RNA

TNF: Tumor necrosis factor-alpha

tRF: transferRNA-derived fragment

WB: Western Blotting

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DETERMINATION OF TRNA-DERIVED FRAGMENT CONTENT AND EFFECTS IN EXOSOMES RELEASED FROM POLARIZED MICROGLIA

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ABSTRACT

The resident macrophages of brain, microglia, responsible to react to immunological responses and preserve homeostasis in the central nervous system. Microglia play a critical role in neuroinflammation and neurological diseases. Microglial responses has been linked to conditions like metabolic diseases, neurodegenerative diseases, autoimmune diseases, cancer and many more, demonstrating the broad impact on health.

Extracellular vesicles (EVs) are small, membrane-enclosed particles released by cells into the extracellular environment. They play crucial roles in intercellular communication by transferring various biomolecules between cells. One of their cargo, transfer RNA-derived fragments (tRFs) have recently emerged as key regulators of gene expression and cellular function in various biological contexts. Understanding the content of tRFs in extracellular vesicles released by LPS induced microglia could offer insights into the communication mechanisms relevant to neuroinflammation and associated pathologies. By examining the tRF content in extracellular vesicles from LPS induced microglia, this study aims to advance our comprehension of the regulatory mechanisms governing microglial behavior and their involvement in neuroinflammation.

In this research, I determined tRFs profiles in three different fragments (lysate, EV, and EV-free supernatant) of LPS induced microglia using next generation sequencing. Then, I determined the effects of tRF from LPS induced microglia on microglia cells. this study aims to advance our comprehension of the regulatory mechanisms governing microglial behavior and their involvement in neuroinflammation.

Keywords: Microglia, LPS, Extracellular vesicles, Small non-coding RNAs, tRNA-derived fragments

POLARİZE EDİLMİŞ MİKROGLİALARDAN SALINAN EKZOZOMLARDAKİ tRNA KÖKENLİ FRAGMAN İÇERİĞİNİN VE BUNLARIN ETKİLERİNİN BELİRLENMESİ

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ÖZET

Beynin yerleşik makrofajları olan mikroglia, immünolojik yanıtlara tepki vermekten ve merkezi sinir sistemindeki homeostazı korumaktan sorumludur. Mikroglia, nöroinflamasyon ve nörolojik hastalıklarda kritik bir rol oynamaktadır. Mikroglial polarizasyon, metabolik hastalıklar, nörodejeneratif hastalıklar, otoimmün hastalıklar, kanser ve daha birçok durumla ilişkilendirilmiş ve sağlık üzerindeki geniş etkisini göstermiştir.

Hücre dışı veziküller (EV'ler), hücreler tarafından hücre dışı ortama salınan küçük, zarla çevrili parçacıklardır. Hücreler arasında çeşitli biyomolekülleri aktararak hücreler arası iletişimde önemli roller oynarlar. Kargolarından biri olan transfer RNA türevi fragmanlar (tRF'ler) son zamanlarda çeşitli biyolojik bağlamlarda gen ekspresyonu ve hücre fonksiyonunun temel düzenleyicileri olarak ortaya çıkmıştır. Polarize mikroglialardan salınan EV'lerin içeriğinin, özellikle de tRF'lerin anlaşılması, mikroglia ve diğer beyin hücreleri arasındaki iletişim mekanizmalarına dair içgörüler sunabilir.

LPS verilmiş mikroglia tarafından salınan hücre dışı veziküllerdeki tRNA türevi parçaların içeriğinin anlaşılması, nöroinflamasyon ve ilişkili patolojilerle ilgili iletişim mekanizmalarına dair içgörüler sunabilir. Bu çalışma, LPS verilmiş mikroglialardan salınan hücre dışı veziküllerdeki tRNA türevi fragman içeriğini inceleyerek, mikroglial davranışı yöneten düzenleyici mekanizmalar ve bunların nöroinflamasyondaki rolleri hakkındaki kavrayışımızı ilerletmeyi amaçlamaktadır.

Bu araştırmada, yeni nesil dizileme kullanarak LPS verilmiş mikroglialardan lizat, EVler, ve EV içermeyen süpernatantta tRF profilleri belirlendi. Daha sonra, mikroglialardaki tRF'nin mikroglia hücreleri üzerindeki etkileri belirlendi. Bu çalışma, mikroglial davranışı

yöneten düzenleyici mekanizmaları ve nöroinflamasyona katılımlarını anlamamızı ilerletmeyi amaçlamaktadır.

Anahtar Kelimeler: Mikroglia, LPS, Ekstrasellüler veziküller, Küçük kodlayıcı olmayan RNA'lar, tRNA kökenli fragmanlar



1. INTRODUCTION & AIM

1.1. Aims of the study

Neuroinflammation is the natural immune response that the central nervous system (CNS) develops to limit infections, eliminate pathogens, cell debris or abnormal folded proteins. This innate immune response is mediated by cytokines, chemokines, reactive oxygen products and secondary messengers. Therefore, regulation of neuroinflammation is of great importance and if it is not regulated, it becomes chronic which may participate in the pathogenesis of many diseases by causing neural damage.

Microglia cells play a primary role in neuroinflammation in the CNS. Microglial cells play a role in the maintenance of brain homeostasis through the extensions they have. In the presence of any bacterial, viral or toxic stimulus, microglia are activated, changing its transcriptional profile and releasing inflammatory cytokines and chemokines. Activation causes the migration and phagocytosis abilities of the microglia cell to increase.

It has been demonstrated that tRFs participate in many physiological functions and regulate gene expression, similar to miRNAs, both translation and transcription, depending on the type of the tRNA particle. Our knowledge of the immune function of tRFs is very limited. There is no study on the expression of tRNA particles in microglia cell, which is the immune cell of the CNS, and their possible roles in neuroinflammation.

1. Determination of tRFs in the microglia cell activated by LPS stimulation
2. Determination of the effects of tRFs released from LPS activated microglia on the function of unactivated microglia cells.

1.2. The hypothesis of the study

First hypothesis is that tRFs released from activated microglia play a role in cell-cell communication. Second hypothesis is that LPS-activated tRFs have distinct regulatory roles in microglial immune response.

2. BACKGROUND INFORMATION & LITERATURE

2.1. Microglia

Microglia are the resident immune cells of the CNS that play a crucial role in both physiological and pathological processes (Perry & Teeling, 2013). Microglia were first discovered by Pío del Río-Hortega, a Spanish neuroscientist, in the early 20th century. He identified these cells while working in the laboratory of Santiago Ramón y Cajal, often regarded as the father of modern neuroscience (Perez-Cerda, Sanchez-Gomez, & Matute, 2015). Microglia, being mononuclear phagocytes of mesodermal origin, migrate to the CNS during early embryonic development (Vidal-Itriago et al., 2022).

These dynamic cells constantly survey their environment, interacting with neurons and synapses, and other brain cells (Basilico et al., 2022). Specialized as phagocytes in the CNS, microglia actively survey the brain parenchyma and are involved in engulfing microbes, dying neurons and other cells, contributing to the maintenance of CNS homeostasis (Colonna & Butovsky, 2017). Microglia physically interacting with other cells and shaping the brain structurally (Nebeling et al., 2023). They have been found to mediate synaptic plasticity, impacting processes such as learning and memory (Cornell, Salinas, Huang, & Zhou, 2022). Moreover, they are involved in synaptic pruning during postnatal development, contributing to brain circuit refinement (Schafer et al., 2012). Their dynamic interactions with neurons and synapses underscore their significance in CNS function and highlight their potential as therapeutic targets for various neurological disorders.

2.2. Origin of Microglia

The origin of microglia has been a topic of debate and investigation in the field of neuroscience. Firstly, it was thought that microglia originate from hematopoietic stem cells in the bone marrow, constantly replenishing the CNS throughout life. In addition to this non-yolk sac origins of microglia during early postnatal development, researchers have shed more light on the ontogeny of microglia utilizing fate-mapping and genetic lineage tracing techniques. These studies proved that microglia originate from

primitive erythro-myeloid progenitors from the mesoderm, specifically yolk sac during early embryonic development (Ginhoux et al., 2010).

Groundbreaking evidence highlighted the significant contribution of yolk sac-derived progenitors in seeding the developing brain with microglia during embryogenesis. These early progenitors migrate and colonize the CNS before the establishment of the blood-brain barrier, where they proliferate and differentiate into mature microglia. Embryonic development marks the inception of microglial lineage, where yolk sac hematopoietic progenitors give rise to primitive macrophages, including microglial precursors.

Microglial development continues postnatally, orchestrated by intricate interplay between intrinsic genetic programs and extrinsic signals from the microenvironment. Microglial progenitors undergo dynamic transformations, acquiring region-specific phenotypes and functional properties dictated by their spatial and temporal context within the CNS. This developmental plasticity is governed by a myriad of signaling pathways, including but not limited to, CSF1R, TGF- β , and IL-34, which regulate microglial proliferation, survival, and differentiation.

2.3. Physiological Functions of Microglia

Microglial cells in the CNS act as scavengers and perform vital functions in maintaining homeostasis. Microglial cells also retain other functions throughout the stages of development, adulthood, and aging. Research conducted by Tremblay et al. has demonstrated the essential role of microglia in the development of synapses, as well as in synaptic remodeling and plasticity in the developing brain (Tremblay, Lowery, & Majewska, 2010). They are responsible for carrying out functions such as maintaining homeostasis, promoting tissue healing, and conducting surveillance activities for neurons and tissues (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Sierra et al., 2010). They are also responsible for the elimination of damaged agents, apoptotic cells, and misfolded proteins. According to Marin-Teva et al. (2011), it also plays an active role in initiating developmental apoptosis of Purkinje cells and production of programmed death signals in neurons (Marin-Teva, Cuadros, Martin-Oliva, & Navascues, 2011).

During the course of their work, microglial cells engage in a process known as phagocytosis. There are two distinct kinds of receptors that are connected to the phagocytosis process that is carried out by microglial cells. These are Toll-like receptors (TLR), which permit the detection of pathogens that stimulate primary inflammation, and receptor-2 (TREM2) receptors produced in myeloid cells, which are critical for the anti-inflammatory response (R. Fu, Shen, Xu, Luo, & Tang, 2014). Toll-like receptors are responsible for the recognition of pathogens that stimulate primary inflammation. A pro-inflammatory response is produced when TLRs are stimulated and pathogens are ingested, but an anti-inflammatory response is produced when the apoptotic material is eliminated and identification of phosphatidylserine residues is carried out (Piccio et al., 2007).

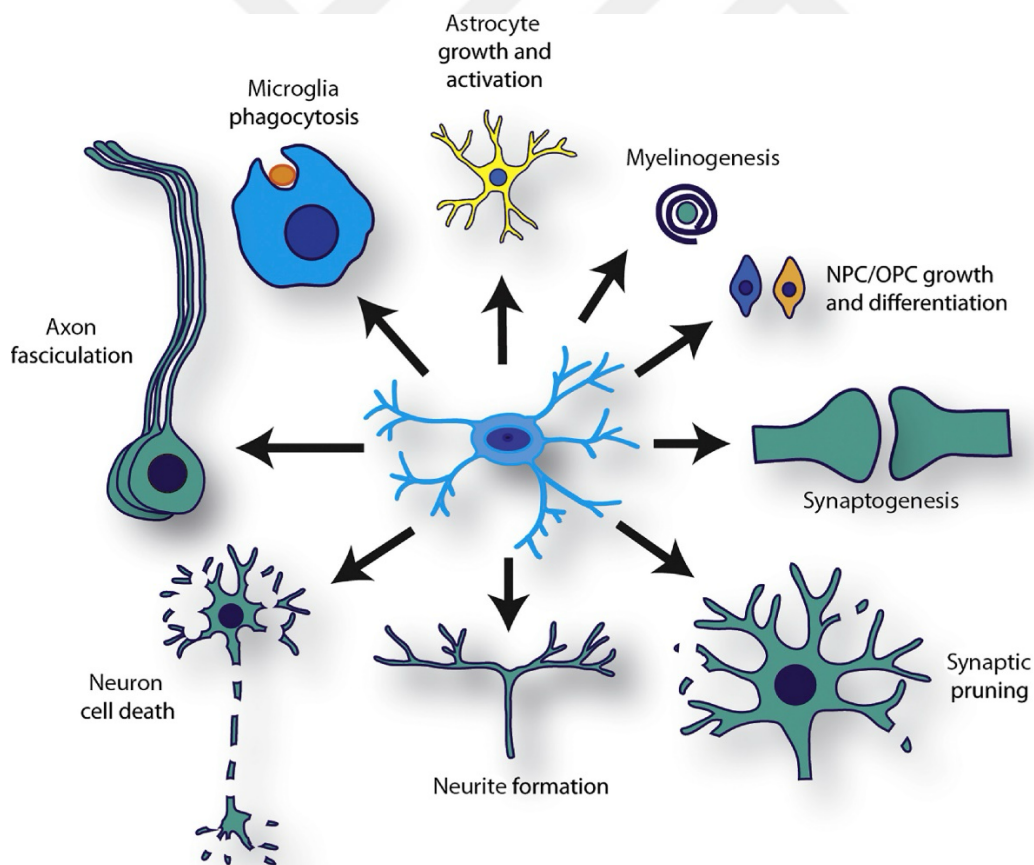


Figure 1. Microglia's roles in the CNS. Adopted from Wright-Jin and Gutmann (Wright-Jin & Gutmann, 2019).

Research that was carried out on an animal model of multiple sclerosis (MS) demonstrated that TREM2 had a role that is protective. It has also been demonstrated that changes in TREM2 considerably increase the likelihood of developing Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Alzheimer's Disease (AD) (Cady et al., 2014; Pottier et al., 2013; Rayaprolu et al., 2013). Microglia are necessary for proper brain function; nevertheless, unregulated or overactive microglia can cause neurotoxicity. Microglia are important for normal brain function. According to Takeuchi et al., the release of glutamate by activated microglia is a factor in the occurrence of extracellular autonomic neuronal injury (Takeuchi et al., 2011). According to Tremblay et al. (2010), activated microglia are capable of producing reactive oxygen species (ROS) via the enzyme nicotinic adenine dinucleotide hydrophosphate (NADPH) oxidase and inducing poor blood brain barrier function (Tremblay et al., 2010). Several neurotrophic factors that are generated by activated microglial cells have an effect on the survival of neurons. These neurotrophic factors include interleukin-3 (IL-3) and IL-10, neurotrophin-4/5, Nerve Growth Factor (NGF), TGF- β , glia-derived neurotrophic factor, and fibroblast growth factor-2 (Nakajima, Tohyama, Maeda, Kohsaka, & Kurihara, 2007). microglial cells are responsible for controlling both synaptic function and synaptic development (Bilimoria & Stevens, 2015). The activity of neural precursor cell pools is controlled by microglial cells. In addition, microglial cells are the ones responsible for getting rid of extra synapses that are formed when neural precursor cells mature into neurons and generate more synapses than are required (Cunningham, Martinez-Cerdeno, & Noctor, 2013). Furthermore, microglial P2X4 receptors are essential for the development of pain hypersensitivity following neuron injury (Beggs, Trang, & Salter, 2012).

2.4. Microglia and neuroinflammation

Microglia are crucial players in inflammation within the CNS, responding promptly to inflammatory signals (Cakir et al., 2022). Neuroinflammation, a normal immune

response arises to protect the CNS against bacterial and viral infections, and to get rid of cell debris and misfolded peptides and proteins (Sochocka, Diniz, & Leszek, 2017).

Despite microglia are integral to immune responses and inflammation, their activation can sustain inflammation through the release of pro-inflammatory and neurotoxic factors, worsening neurotoxicity and neurodegeneration upon chronic activation (Gupta et al., 2018). They serve as initial sensors for inflammatory responses triggered by stimulants such as lipopolysaccharides (LPS) (Kannan et al., 2013) and β -amyloid accumulation (Y. K. Huang et al., 2022). Activated microglia in the vicinity contribute significantly to inflammation and its subsequent pathogenesis through pro-inflammatory responses, resulting in pathological effects like neuronal hyperexcitability, neurotoxicity, and chronic inflammation (Milligan & Watkins, 2009).

2.5. Microglia Phenotypes

Microglia can adapt to the environment and previously believed to change their phenotypes accordingly: previously categorized into 'resting and activated' or 'M1 and M2' (Guo, Wang, & Yin, 2022; Jurga, Paleczna, & Kuter, 2020). Resting microglia, also known as quiescent microglia, are characterized by a ramified morphology with highly motile processes that continuously survey their microenvironment in the brain (Kettenmann et al., 2011). On the other hand, activated microglia can exhibit diverse phenotypes based on their activation status. When activated, microglia can transition into different functional states, with two primary phenotypes being the classically activated M1 phenotype and the alternatively activated M2 phenotype (Guo et al., 2022). The M1 phenotype is considered neurotoxic and pro-inflammatory, while the M2 phenotype is viewed as neuroprotective and anti-inflammatory (Kwon & Koh, 2020). The M1 phenotype is associated with characteristics such as promoting inflammation and neurodegeneration, while the M2 phenotype is linked to functions like regenerative processes, cell growth, and inhibition of inflammation (Guo et al., 2022).

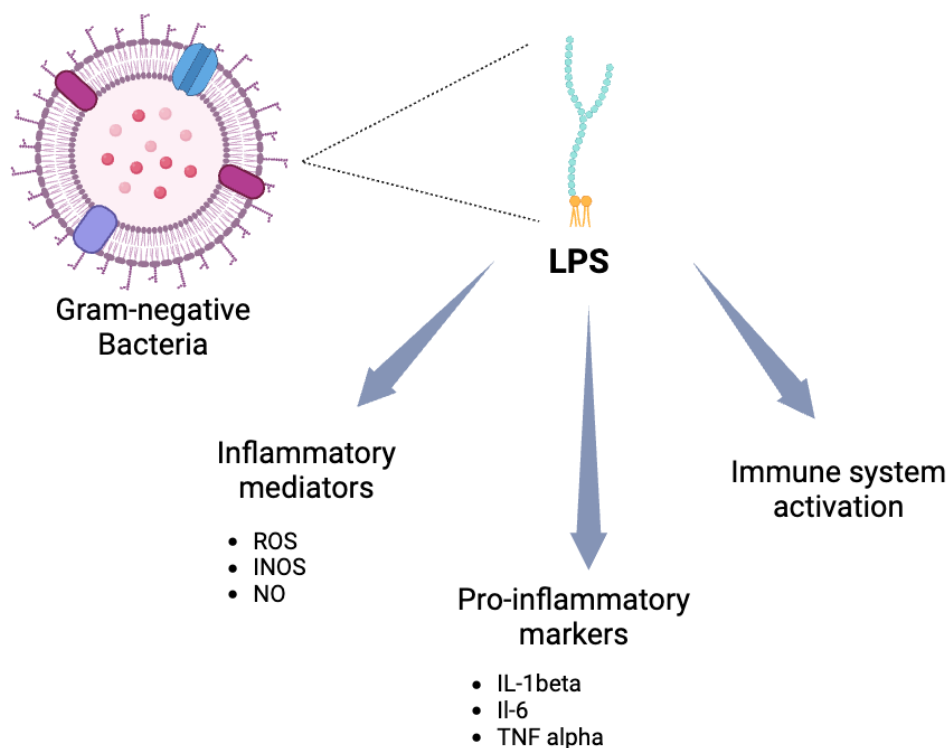
However, a large consensus of microglia focused researchers suggested that Microglia do not change their states in reaction to illness, trauma, injury, infection, or other

difficulties (Paolicelli et al., 2022). They collectively pointed out that microglia are always active and change their reactions and functions in response to various factors such as life stage, CNS region, and health or disease presence. Consequently, they stated that classifying microglia using the terms "resting" and "activated" or any other couldn't reflect their states or phenotypes correctly with our current knowledge. The plasticity of microglia allows them to adapt to various stimuli, highlighting their dynamic nature in maintaining brain homeostasis and responding to pathological conditions (L. Wang et al., 2021). Further understanding the distinct phenotypes of microglia and their functional diversity is essential for developing targeted therapeutic strategies for neuroinflammatory and neurodegenerative diseases. In alignment with this consensus, the name of the microglia population will be mentioned as LPS-induced microglia in this thesis.

2.6. LPS model as pro-inflammatory microglial model

Lipopolysaccharide (LPS) is a well-known pro-inflammatory stimulus for microglial cells, serving as a valuable model for studying neuroinflammation and related conditions. Activation of microglia by LPS results in the release of pro-inflammatory factors, which contribute to neurotoxicity and the progression of inflammatory responses (Ye et al., 2020). Studies have indeed demonstrated the role of LPS in inducing neuroinflammation through microglial activation and subsequent release of pro-inflammatory cytokines (Mizobuchi et al., 2020; Qin et al., 2004). Additionally, LPS has been shown to induce a pro-inflammatory phenotype in microglia, leading to the upregulation of inflammatory mediators and exacerbation of neuroinflammatory processes (Hsieh et al., 2018; Yuan et al., 2016). Moreover, the utilization of LPS-induced microglial models has been crucial in elucidating the mechanisms underlying inflammatory responses in the CNS. For example, LPS has been used to trigger a pro-inflammatory memory-like response in microglia, highlighting its role in promoting a sustained pro-inflammatory state in these cells (J. Wang et al., 2021). Additionally, LPS has been associated with the activation of signaling pathways involved in inflammation, such as NF- κ B and MAPKs, further underscoring its significance as a pro-inflammatory stimulus for microglia (G. Y. Sun et

al., 2015; Yan et al., 2017). Furthermore, LPS-induced microglial activation has been linked to the production of pro-inflammatory enzymes and cytokines, resulting in neuronal damage and neurodegeneration (S. P. Fu et al., 2015). Research has shown that LPS can induce inflammatory neurodegeneration in various brain regions, emphasizing its ability to drive neuroinflammatory processes (Shahraz et al., 2021). Additionally, LPS has been employed to investigate the effects of anti-inflammatory agents in mitigating microglia-induced inflammation and neurotoxicity, highlighting its utility in evaluating potential therapeutic interventions (Bussi et al., 2017). The use of LPS as a pro-inflammatory microglial model offers a valuable tool for exploring the mechanisms of neuroinflammation, understanding the role of microglia in inflammatory responses, and assessing the efficacy of anti-inflammatory compounds in alleviating neuroinflammatory processes.



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Figure 2. Effects of LPS

2.7. Non-coding RNAs

Non-coding RNAs (ncRNAs) are described as small RNA particles with no coding capability but play crucial regulatory roles in various biological processes (Y. M. Sun & Chen, 2020). It can be mainly categorized into two: long non-coding RNAs (lncRNAs) and small non-coding RNAs (sncRNAs). This categorization based on length: small are <200 nucleotides and long are >200 nucleotides long (Ratti et al., 2020). sncRNAs also categorized into many different subtypes, certain well-known and -studied ones are microRNAs (miRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), piwi-interacting RNA (piRNAs), tRNA-derived fragments (tRFs) (Aalto & Pasquinelli, 2012). Their main function is to regulate gene expression via interfering or modifying RNAs, or spliceosome involvement (Aalto & Pasquinelli, 2012). miRNAs are endogenous sncRNAs of about 19-22 nucleotides that regulate gene expression by binding to the 3' untranslated region (UTR) of target mRNAs, leading to mRNA degradation or translational repression (Q. Zhou et al., 2017). miRNAs play pivotal roles in various biological processes, including cell proliferation, differentiation, immune response, and epigenetic regulation (X. Li, Peng, & Yi, 2021). siRNAs are double-stranded sncRNAs of about 20-25 nucleotides that are involved in RNA interference (RNAi) pathways, leading to the degradation of target mRNAs and gene silencing (Taft, Pang, Mercer, Dinger, & Mattick, 2010). siRNAs are essential for regulating gene expression by RNAi and have been investigated for their potential therapeutic applications (Kang et al., 2023). tRFs, the main focus of this study, are emerging as a functional class of sncRNAs that are derived from tRNAs and play regulatory roles in various biological and disease conditions (Q. Chen, Zhang, Shi, Yan, & Zhou, 2021). tRFs have been implicated in stress responses, translation regulation, and disease pathogenesis (Q. Chen et al., 2021). lncRNAs are a heterogeneous group of sncRNAs longer than 200 nucleotides that regulate gene expression at transcriptional, post-transcriptional, and epigenetic levels (Mei & Zhang, 2019). lncRNAs have been shown to participate in various cellular processes, including transcriptional regulation, protein activity modulation, RNA processing, and small RNA precursor generation (Mei &

Zhang, 2019). Overall, ncRNAs including miRNAs, siRNAs, tsRNAs, and lncRNAs are essential regulators of gene expression and play diverse roles in cellular processes, disease pathogenesis, and potential therapeutic interventions.

2.8. tRNA-derived Fragments

Transfer RNA-derived fragments (tRFs) are small non-coding RNA molecules that are generated from mature tRNAs through different nuclease activity (Y. Fu, Lee, Lee, & Bao, 2015). These tRFs have been found to be evolutionarily conserved across different organisms, from bacteria to humans (Kumar, Anaya, Mudunuri, & Dutta, 2014). They are distinct from miRNAs and are known to associate with AGO proteins to recognize specific RNA targets (Kumar et al., 2014). tRFs have emerged as a new category of regulatory noncoding RNAs with significant biological functions in various conditions, including cancers and stress-induced diseases (Yu et al., 2021). They play crucial roles in gene regulation and have been implicated in processes like cell proliferation, cell cycle regulation, and even carcinogenesis (Shao et al., 2017). tRFs can be classified into different types based on their cleavage positions, as 5'-tRF, 3'-tRF, 5'-tRF half, 3'-tRF half, and i-tRF (Pliatsika, Loher, Telonis, & Rigoutsos, 2016). Furthermore, tRFs have been identified as potential biomarkers for various conditions, including osteoarthritis, bladder cancer, colorectal cancer, and even as diagnostic markers for diseases like non-small cell lung cancer and osteoporosis (Deng et al., 2022; Zhou & Cui, 2022; Chen et al., 2022; Zheng et al., 2022). The comprehensive analysis of tRFs has led to the discovery of their roles in different cancers, stress responses, and disease conditions, highlighting their importance as regulatory molecules in various physiological and pathological processes (Li et al., 2020; Olvedy et al., 2016). The identification and characterization of tRFs have opened up new avenues for research into their functional outcomes, diagnostic potential, and therapeutic applications in diverse medical conditions.

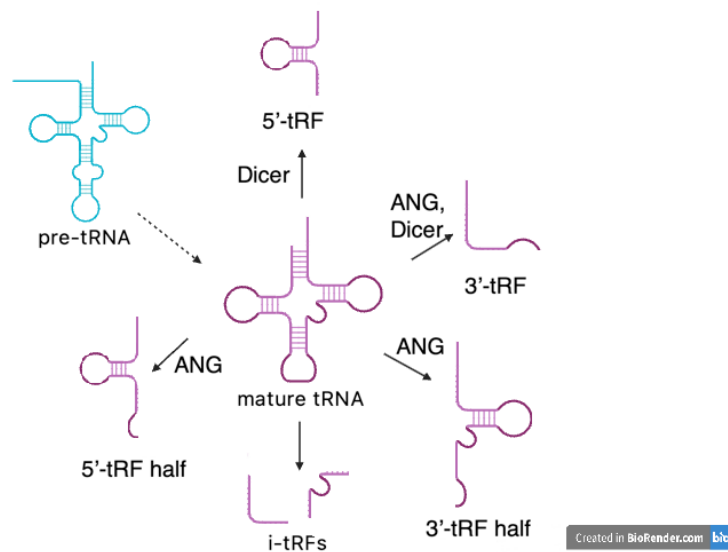


Figure 3. Different types of tRFs produced from mature tRNA.

2.9. tRFs in Central Nervous System

tRFs have been implicated in various roles and functions in the CNS and related diseases. Studies have shown that tRFs can modulate gene expression, proliferation, and the DNA damage response in the CNS, indicating their involvement in maintaining neuronal homeostasis and function (Mathew, Katta, Ludhiadch, Singh, & Munshi, 2023). Specifically, tRFs have been associated with neurodegenerative diseases, metabolic disorders, and immune responses, highlighting their diverse regulatory roles in CNS pathophysiology (Su et al., 2020; Pandey et al., 2021). Furthermore, tRNA fragments have been linked to stress responses and disease development in the CNS, suggesting their potential impact on neurodegenerative conditions and other neurological disorders (Z. Li & Stanton, 2021; Y. Xie et al., 2020). These fragments play crucial roles in RNA processing, cell proliferation, translation suppression, and the modulation of DNA damage response in the CNS, underscoring their significance in neuronal health and disease progression (Avcilar-Kucukgoze & Kashina, 2020; H. Fu et al., 2009). Moreover, tRFs have been shown to regulate transcription and translation, posttranslational modifications, stress responses, and disease outcomes in the CNS, indicating their multifaceted functions in neuronal physiology and pathology (Mathew et al., 2023; Prehn

& Jirstrom, 2020). Overall, tRFs play diverse roles in the CNS, ranging from gene expression regulation to stress responses and disease development. Their involvement in neurodegenerative diseases, metabolic disorders, and immune responses underscores their importance as regulatory molecules in CNS physiology and pathology.

2.10. Neurons

Neurons, also known as nerve cells, are fundamental units of the nervous system responsible for transmitting information through electrical and chemical signals (Ludwig, Reddy, & Varacallo, 2024). They are essential for processing and transmitting information, enabling various functions such as sensory perception, motor control, and cognitive processes (Arshavsky, 2017). Neurons are characterized by their unique structure, which includes dendrites, a cell body (soma), and an axon. Dendrites receive signals from other neurons or sensory receptors, while the axon transmits signals to other neurons, muscles, or glands. The complex morphology and electrochemical properties of neurons allow for the integration and transmission of signals, forming intricate neural networks that underpin brain function and behavior (Kirch & Gollo, 2020). Based on their morphology, neurons are mainly classified into four basic types: multipolar, unipolar, bipolar, and pseudounipolar (Kandel, Koester, Mack, & Siegelbaum, 2021).

Neurons are highly specialized cells crucial for the functioning of the nervous system. Research into the differentiation, classification, and functional properties of neurons has provided valuable insights into their role in physiological processes, as well as their involvement in various neurological conditions.

3. MATERIALS & METHODS

3.1. Type of the study

This research is an experimental study with *in vitro* and *in vivo* parts.

3.2. Research Lab and Time

The experiments were conducted at Genc Laboratory in Izmir Biomedicine and Genome Center with the supervision of Prof. Dr. Şermin Genç from July 2021 to May 2024.

3.3. Research population, sampling, and experimental groups

In this study, N9 mouse microglial cell line and C57BL/6 Wild type mice were utilized. No human material used. In both in vivo and in vitro, experimental groups were formed as Control vs LPS introduced.

3.4. Research materials

The N9 mouse microglial cells (Righi et al., 1989) was provided by Dr. Paola Ricciardi-Castagnoli (Toscana Life Science- Italy).

3.5. Variables of the Study

Pro-inflammatory LPS model is considered as independent variable. Protein/mRNA levels of Pro-inflammatory inflammation markers or cell death rates measured with different assays, are dependent variables.

3.6. Data Collection

3.6.1. Reagents, Equipment, Tools

3.6.1.1. Antibodies and Primers

Table 1. The list of utilized antibodies and primers

	Forward	Reverse
Mouse IL-1beta	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGAT T
Mouse IL-6	TGATGGATGCTACCAAAGTGA	TGTGACTCCAGCTTATCTCT TGG
Mouse TNF	TCACAGACGAATGACTCCAA	GTGCCACTTCATACCAGGA GAA
Mouse GAPDH	GGTTGTCTCCTGCGACTTCA	GGTGGTCCAGGGTTTCTTA CTC

tRF-33- 87R8WP9N1EW JDW	TCCCTGGTGGTCTAGTGGTTAG GATTCG	TTCCTGACCGGGAATCGA ACCCG
CD63	Abcam (ab231975)	
Calnexin	Abcam (ab22595)	

3.6.1.2. Equipment

Table 2. The list of utilized equipment

Biological cabin laminar flow / Thermo Fisher Scientific
Ice Machine / Hoshizaki
CO ₂ incubator / Thermo Fisher Scientific
Precision scales / Sartorius
-150°C liquid nitrogen tanks / Thermo Fisher Scientific
-80°C freezer / Thermo Scientific
Refrigerator (+4°C and -20°C) / Vestel, Ariston
Vortex / Thermo Fisher Scientific
Refrigerated microcentrifuge / Beckman Coulter
Nanopure UV/UF&TOC
Water bath
Autoclave
Inverted microscope / Zeiss
Varioskan ELISA reader / Thermo Fisher Scientific
Nanodrop / Thermo Fisher Scientific

Thermal Cycler
Olympus IX81 Live cell imaging system
LightCycler 480 / Roche
Pipette Set / Eppendorf

3.6.1.3. Consumables, Reagents, and Kits

3.6.1.4. Table 3. The list of utilized consumables, reagents, and kits

RPMI 1640 Cell media / Gibco
DMEM Cell media / Gibco
Glutamax I / Gibco
Fetal Bovine serum (FBS) / Gibco
Trypsin-EDTA / Pan-Biotech
Penicillin-Streptomycin / Pan-Biotech
D-Pbs (Dulbecco Phosphate Buffered Salt Solution) / Gibco
25cm ² filtered flask sterile / Sarstedt
75cm ² filtered flask sterile / Sarstedt
5 mL sterile pipette
10 mL sterile pipette
25 mL sterile pipette
50 mL sterile pipette
50 mL centrifuge tube sterile
15 mL centrifuge tube sterile
Pasteur Pipette
96 well plate sterile
48 well plates
24 well plates
6 well plate sterile

0.22 filter
Stericup 500 ml vacuum filtration system
0.2 ml PCR Tube
0.5 ml microtest tube
1.5 ml microtest tube
2 ml microtest tube
IL-1 β ELISA kit / Elabscience
IL-6 ELISA kit / Elabscience
TNF-alpha ELISA kit / Elabscience
RNA isolation kit
cDNA synthesis kit
SYBR Green Master Mix
PCR primer
PCR plate
tRNA siRNA
Lipofectamine™ RNAiMAX Transfection agent / Thermo Fisher Scientific
LPS (Escherichia coli O55:B5) / Sigma Aldrich
Thermo S1 pipette filter
Syringe 5ml
Bioanalyzer Small RNA Analysis kit
17 ml Ultracentrifuge Tube

3.7. Methods

3.7.1.1. Cell Culture

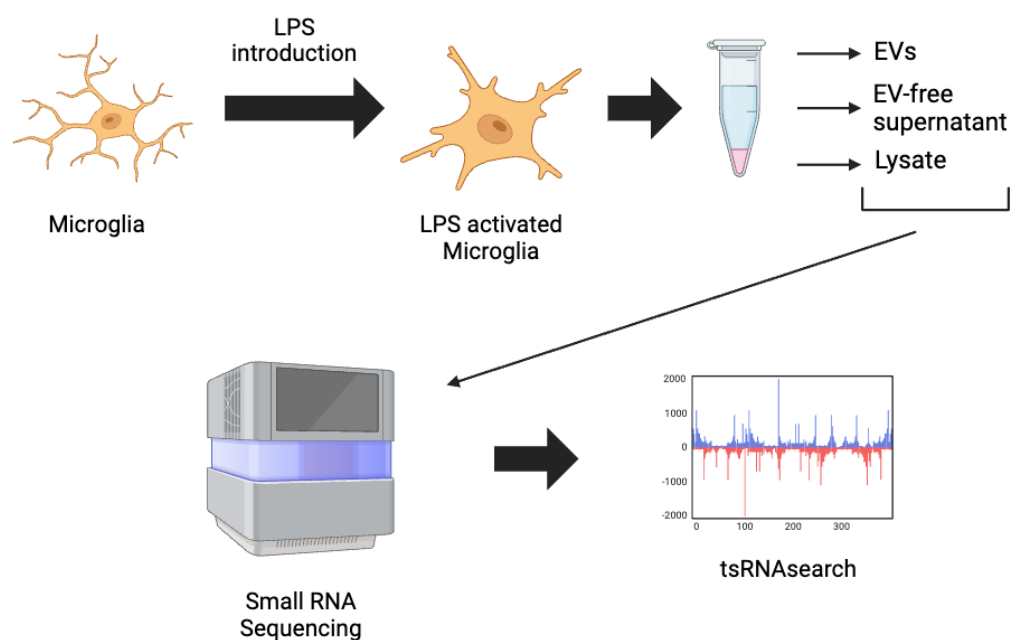
3.7.2. N9 Cell Line

The N9 microglial cells were derived from the mouse brain and immortalized with *v-myc* overexpression (Righi et al., 1989). They show the primary microglial features and characteristics (Weinstein, Swarts, Bishop, Hanisch, & Moller, 2008). RPMI 1640 medium including 10% Fetal Bovine Serum (FBS) (ThermoScientific, USA), 2 mM L-Glutamine (ThermoScientific, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (ThermoScientific, USA) was used for sustaining cells. The cells were passaged in 75 cm² flasks three times per week. The cells were washed using 1x Phosphate Buffered Saline (PBS) (Thermo Scientific, USA) after the medium discarded. Next, the cells were obtained using a 4 ml solution of Trypsin-EDTA (0.25% w/v) (PAN-Biotech, Germany) and, then six ml of 10% FBS RPMI (ThermoScientific, USA) were added to the flask in order to halt the enzymatic reaction. The mixture was put into a 15-ml falcon and centrifuged at 1,500 rpm in order to extract the cells. Following the removal of the medium, the cells were suspended in 2 ml of the medium. Ultimately, 1x10⁶ cells were seeded into a fresh culture flask with 10 ml of fresh medium after the cells were counted using the Thoma slide (Marienfeld, Germany). The cells were grown in an incubator at 37°C with 5% CO₂ and 80% humidity, and all operations were performed in a Class II B2 biosafety cabinet.

3.7.2.1. Pro-inflammatory Activation of N9 Microglial Cells

First objective is to promote pro-inflammatory activation of N9 microglial cells. LPS, an pro-inflammatory agent, was utilized for activation of N9 microglial cells which our lab previously established (Eren et al., 2018). For this, N9 cells will be incubated with 100 ng/ml LPS (E. Coli 055:B5, Sigma) for 24 hours. This selected dose and duration is sufficient to provide microglial activation, as we have shown in our previous studies (Eren et al., 2018). At the end of the experiment, to confirm microglial activation, TNF-α, IL-1β and IL-6 and their release will be examined by ELISA test (Invitrogen, USA) (Lan et al., 2017; Nam et al., 2018). Cell lysate and supernatant will be studied separately to determine intracellular and released tRNA particles (found freely in extracellular vesicles and supernatant) from activated cells. Extracellular vesicle (EV) will be isolated from the

separated supernatant and will be used in our analyzes as EV-free supernatant containing free tRNAs obtained after EV isolation.



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Figure 4. The thesis study's experimental design for LPS activation on microglia fractions and Small RNA sequencing.

3.7.2.2. Total EV isolation

EVs were isolated from N9 microglial cells with and without LPS application (control group). First of all, supernatant was separated after the LPS model applied. Supernatants was subjected serial centrifugation at 4°C and 3500xg, two times at 4500xg (10 min each) and one time at 4°C and 10,000xg for 30 min using low-bind Eppendorf tubes (Cat. Number 0030122283) to remove debris and apoptotic blebs. Then, the supernatant is transferred to ultracentrifugation tubes (Beckman, Cat. Number: 344088) and centrifuged at 100,000xg for 1 h at 4°C. After that, supernatant was removed, and all the remaining pellet was washed with phosphate buffered saline

(PBS) and the ultracentrifugation step was repeated. The pellet was dissolved in 100 µl of PBS and used in the next steps. Adapted from Chatterjee et al. (Chatterjee, Ozdemir et al. 2023).

3.7.2.3. Extracellular Vesicle Confirmation

Isolated EVs will be characterized by Nanoparticle Tracking Analysis (NTA), Western Blot and TEM methods.

3.7.2.4. NTA

NTA is a light emitting technique that is useful for rapid sizing and counting of extracellular vesicles (Gardiner, Ferreira, Dragovic, Redman, & Sargent, 2013). Particles in fluid move in a Brownian motion with minor dispersion characteristics, allowing for the determination of EV concentration and size distribution. To achieve the ideal concentration for detection, samples were diluted with distilled water and then injected five times at a rate of 50 µl/min using a continuous syringe system for 30 seconds each time. NTA will collect data at room temperature and measure it five times. With a minimum predicted particle size of 10 nm, data will be analyzed using NTA 2.2 software (Malvern, UK).

3.7.2.5. Western Blotting

3.7.2.5.1.1. Protein Isolation

EVs will also be characterized using EV marker CD63, and EV exclusion marker Calnexin by immunoblotting method. Firstly, proteins were extracted from cell culture samples. At the end of the incubation, the cells in the flask were removed with a cell scraper and transferred to a 15 ml falcon tube. They were centrifuged at 1500 rpm for 5 minutes and the supernatant of the samples was discarded. 1 ml of cold PBS was added and transferred to the Eppendorf tube. 100-200 µl of lysis buffer was added to the samples. They were mixed in a vortex every 2 minutes (5-20 seconds) and incubated with lysis buffer on ice for a total of 30 minutes. At the end of this time, the samples were centrifuged at 12,000 rpm at +4°C for 15 minutes. After centrifugation, supernatants are

taken and stored at -70°C. An additional 5µl of the supernatant is taken and stored for BCA.

3.7.2.5.1.2. Protein Amount Measurement

Next, Protein levels were measured with BCA protein assay kit – Pierce (Thermo Fisher Scientific, USA). Briefly, 0 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 800 µg/ml, and 1000 µg/ml standards were prepared. A mixture of 200 µl Solution A and 4 µl Solution B (A:B ratio = 50:1) as 200 µl per well was prepared and added. Highest value of BSA standard should reach maximum of 1.6-1.8 values. Spectrophotometric measurement was made at 562 nm wavelength.

3.7.2.5.1.3. Western Blotting

After, resolving and stacking gel were prepared consisting of H₂O, acryl-bisacrylamide mix, TRIS, SDS, ammonium persulfate and TEMED. Samples were loaded to the gels and they were run at 10mAmp. After completion, transfer step was started: The PVDF membrane is treated with methanol for 5 minutes. 1X Towbin transfer was prepared and used consisting of 10X Towbin transfer buffer, distilled water, and methanol. The gel was cut appropriately with a spatula and placed on the membrane. The membrane was kept wet all the time in case of drying. 2 sides were sandwiched using filter paper, and those were sandwiched with sponges. Then, the cassettes were closed and run at 375 mA for 2 hours. Membranes were blocked for 30 mins with blocking solution containing powdered milk or bovine serum albumin (BSA) according to antibody used. After blocking, membranes were treated with primary antibody overnight at +4°C with the desired dilution (1:100-1:1000). After, membranes were washed for three times for 5 mins with the washing solution containing 1X TBST containing 0.1% Tween-20. Then, membranes were treated with secondary antibody for 1 hours at +4°C with the desired dilution (1:1000-1:10000). After, membranes were washed for five times for 5 mins with the washing solution. Then the images were taken with Chemidoc imaging system (Bio-Rad, USA) using ECL solution (Thermo Fisher Scientific, USA). Analysis of images were done with Image Studio software (LI-COR Biosciences, USA).

3.7.2.6. Small RNA Sequencing

3.7.2.7. Library preparation and small RNA-sequencing

For every group, n = 4 was employed for the NGS analysis. A260/A280 ratio >1.8 and RIN value ≥9.0 were accepted as the criteria for RNA content quality. With the help of a Small RNA Library Prep Kit for Illumina, Norgen Biotek (Canada) created a small RNA library. Norgen Biotek (Canada) conducted small RNA-seq analysis using the Illumina NextSeq 500 platform (Illumina Inc., USA). Single-end 100 base-pair reads were performed, with a minimum of 30 million reads per sample.

3.7.2.8. tRF Identification

MINTmap has been used version 2.0-alpha (<https://cm.jefferson.edu/mintmap/>) to identify tRFs. We used an exact test on the unnormalized count values of tRNA fragments (tRFs) obtained from the MINTmap results to identify statistically significant tRNA fragments between the AD and Control groups. The R environment's EdgeR package (v3.40.2) was used to perform this analysis.

For the identification of tRNA fragments, we utilized MINTmap version 2.0-alpha (<https://cm.jefferson.edu/mintmap/>). To discern statistically significant tRNA fragments between AD and Control groups, we applied an exact test on the unnormalized count values of tRNA fragments (tRFs) sourced from the MINTmap results. This analysis was conducted using the EdgeR package (v3.40.2) within the R environment.

3.7.2.9. Target Identification and GO and KEGG Analysis

To identify targets, we used tRFtarget-pipeline (<http://trftarget.net/>) (N. Li, Shan, Lu, & Wang, 2021). To find RNA-RNA interaction sites between target RNAs and tRFs is the aim of tRFtarget-pipeline. It offers binding sites that are anticipated by RNAhybrid and IntaRNA, two different mechanisms. The maximum complementary region and calculated free energy between these two predictions are then produced as consensus results. Every evaluation was made in accordance with the methodology they outline in their paper [28].

DAVID web tool (<https://david.ncifcrf.gov/>) has been used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses and Gene Ontology (GO) enrichment [29]. This tool finds overrepresented target genes of differentially expressed tRNA fragments that are associated with biological processes, molecular functions, and cellular components. Visualization of these data were done using SRPlot online tool (<https://www.bioinformatics.com.cn/srplot>) (Tang et al., 2023).

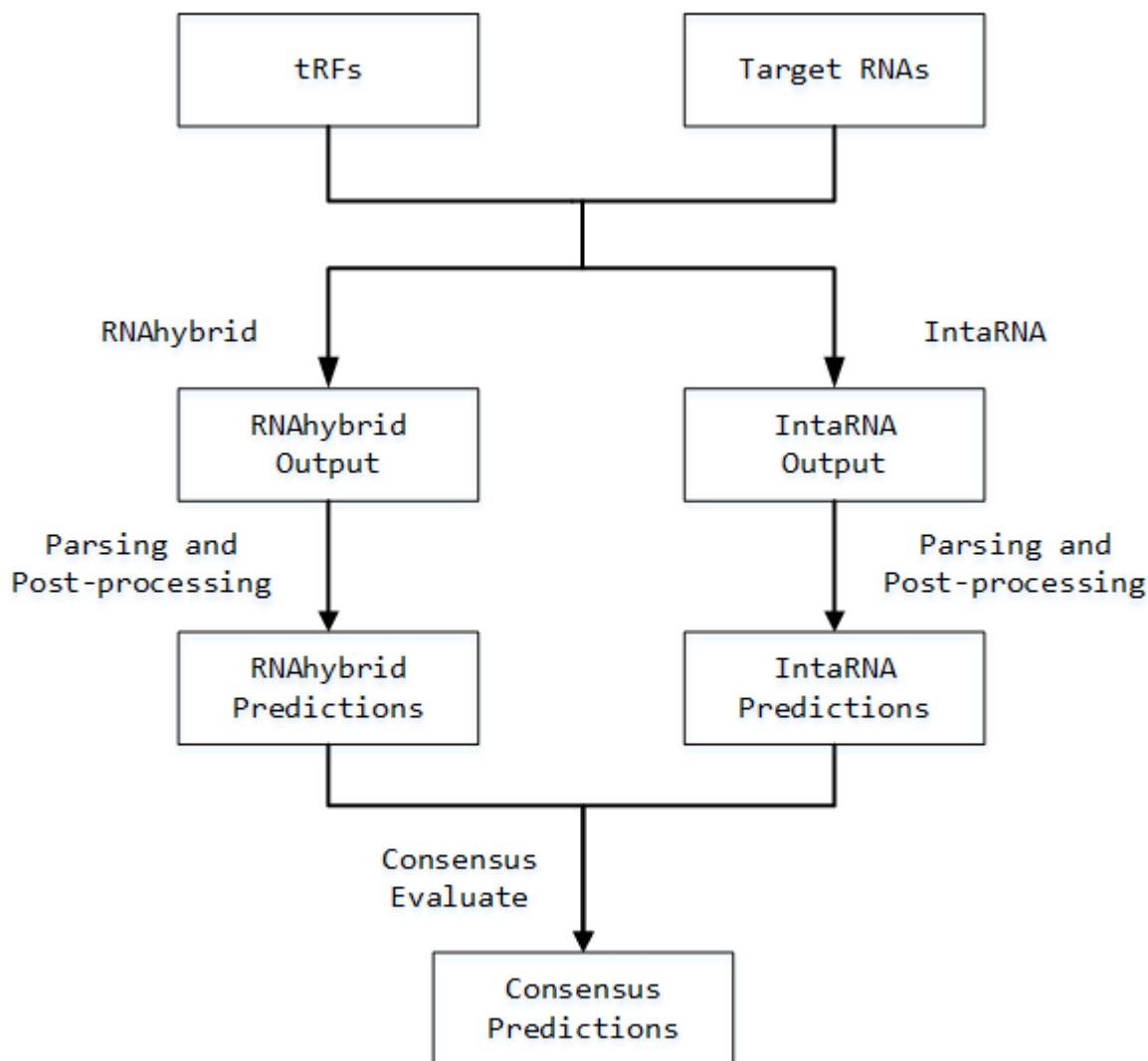
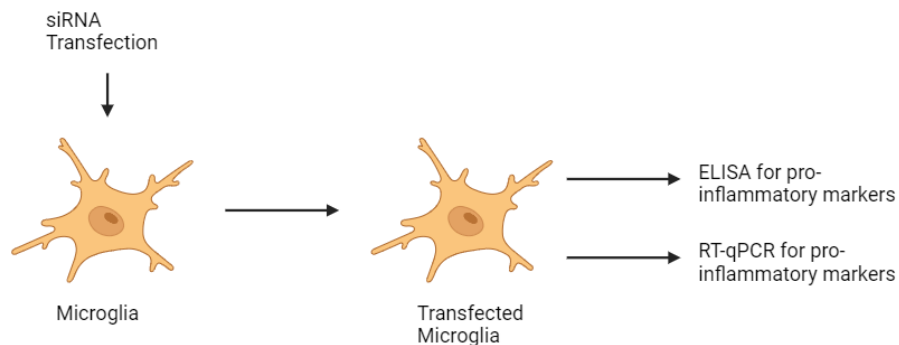


Figure 5. The pipeline of tRFtarget tool. Adopted from Li et al. (N. Li et al., 2021).

3.7.2.10. Transfection with siRNA

Transfection was done using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, USA). After cells were seeded, transfection solutions were prepared. 1.5 μL , 3 μL , or 9 μL Lipofectamine® RNAiMAX Reagent was diluted in 25 μL , 50 μL , or 150 μL medium for 96-well, 24-well, or 6-well plates respectively. 0.5 μL (5 pmol), 1 μL (10 pmol), or 3 μL (30 pmol) tRF-siRNA was also diluted in same amount of medium with Lipofectamine® RNAiMAX Reagent for 96-well, 24-well, or 6-well plates respectively. Mix both diluted Lipofectamine® RNAiMAX Reagent with diluted tRF-siRNA as 1:1 ratio and incubated at room temperature for 5 mins. Same process with exact same amounts were executed for negative control siRNA (mock group) instead of tRF-siRNA. Then, both were applied as 10 μL , 50 μL , or 250 μL for 96-well, 24-well, or 6-well plates, respectively, for 1 days.



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Figure 6. Experimental plan for siRNA transfection of microglia.

3.7.2.11. ELISA

Secreted levels of IL-1beta, IL-6 and TNF-alpha were measured with commercial ELISA kits (Elabscience); This kit is based the Sandwich-ELISA principles. Firstly, serial dilutions of the standard were prepared as 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 0 pg/mL. After, duplicates of 100 µL of each standard and sample were loaded into the wells and sealed the plate using the sealer. Then, left for 90 min at 37°C in the incubator. At the end, all the liquid was removed and 100 µL of Biotinylated Detection Ab working solution was put into each well instantly. Again, sealed the plate using the sealer and left for 60 min at 37°C in the incubator. After that, all the liquid was removed and washed with 350 µL of 1x wash buffer for three times. 100 µL of HRP Conjugate working solution was put into each well instantly and left for 30 min at 37°C in the incubator. After that, all the liquid was removed and washed with 350 µL of 1x wash buffer for five times. Then, 90 µL of Substrate Reagent was added and left for 15 min at 37°C in the incubator. Optical density (OD value) was obtained at 450 nm with Varioskan (Thermo Fisher Scientific, USA).

3.7.2.12. RT-qPCR

3.7.2.13. RNA isolation

RNA isolation was done with Direct-zol Microprep Kit (Zymo USA) kit. At the end of the incubation, the cells in the flask were removed with a cell scraper and transferred to a 15 ml falcon tube. 300 µl of RNA lysis buffer was added to cells. To extract the RNA, the lysed sample in Trizol Lysis Buffer was transferred into a yellow Spin-Away™ Filter into a collection tube and centrifuge. Then, equal volume of 95-100% ethanol to the supernatant and mixed with vortex or pipetting. After, mixture was filtered through Zymo-Spin III CG Column (green) into a collection tube and centrifuged, the flow-through was discarded. 400 µl of Direct-zol RNA PreWash buffer was added to the column and centrifuged for 1 min. This process was repeated after discarded the flow-through. 700 µl of Direct-zol RNA Wash buffer was added to the column and centrifuged for 2 mins to dry. RNA was eluted in DNase/RNase-Free water.

The amount of RNA/DNA samples were measured with the Nanodrop (Thermo Fisher Scientific, USA). First, measurement of blank was done by adding 1 µl of nuclease-free

water. Then, 1 µl of each sample is taken and the DNA/RNA amounts of the samples were measured.

3.7.2.14. cDNA Synthesis

cDNA was synthesized by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). RNA concentration of 1 – 5 µg/ml was utilized. In a tube, reaction mixture was prepared containing 4 µl 5X Reaction Buffer, 2 µl dNTP, 1 µl Ribolock RNase inhibitor, and 1 µl Reverse Transcriptase enzyme. Appropriate amount of RNA was added and completed to 10 µl with dH₂O. In the PCR device, cycling program was set to incubation at 25°C for 5 minutes, then at 42°C for 1 hour, then at 70°C for 5 minutes.

3.7.2.15. RT-qPCR

10 µl of 2X GoTaq® qPCR Master Mix (Promega, USA), Rnase-free H₂O (0-8 µl) 1 µl + 1 µl of Forward and Reverse Primer (total 2 µl), 1 µl of cDNA were mixed and total of 20 µl mixture was obtained. In the PCR device, cycling program was set to 95°C for 10 min, 95°C for 15 s, and 60°C for 60 s.

3.7.2.16. Statistical Analysis

Graphpad Prism 10.0 (Graphpad Software Inc., CA, USA) was utilized for statistical analysis and visualization unless otherwise stated. To analyze nonparametric data for comparing two groups, Mann–Whitney U test was employed. Data were presented as mean ± standard error of the mean. $P < 0.05$ means statistical significant. All in vitro experiments were repeated three times, unless otherwise indicated.

4. RESULTS

4.1. Characterization of sEVs isolated from N9 microglial cells

sEVs isolated from N9 microglial cells was done by western blotting, and nanoparticle tracking analysis (NTA). sEV presence were detected with CD63 expression and absence of Calnexin due to its cellular origin. The results of the western blotting demonstrated that CD63 protein was detected, however no Calnexin protein (**Figure**

6A). The majority of sEVs were detected to be between 50 and 300 nm in diameter, according to NTA (**Figure 6B**).

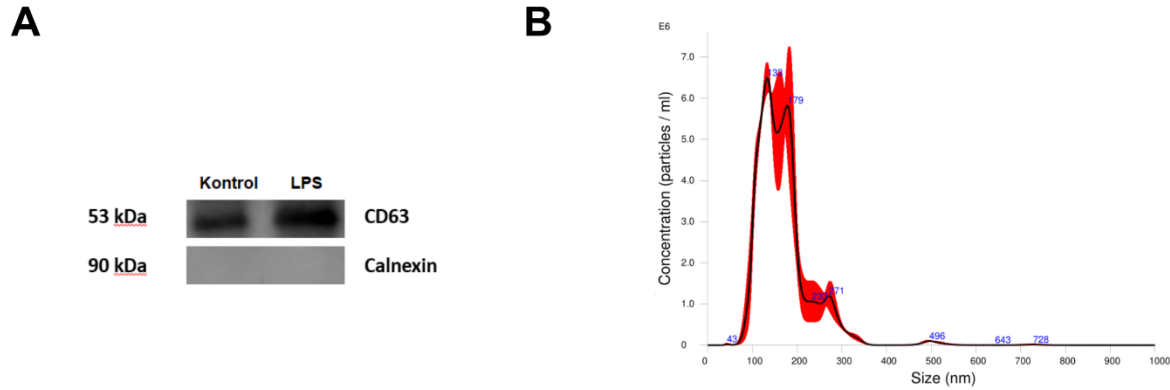


Figure 7. Characterization of EVs with western blotting and NTA. **(A)** The level of these proteins in EVs was detected by Western Blot. The presence of the transmembrane protein tetraspanin CD63, and the absence of the cellular protein Calnexin serve as typical biomarkers for EVs. **(B)** NTA results showed that most EVs were found in the diameter range of approximately 50 and 300 nm.

4.2. Confirmation of pro-inflammatory markers after LPS induction

Pro-inflammatory activation of N9 microglial cells were achieved by 100 ng/ml LPS introduction for 24h. After this period, IL1 β , IL6 and TNF levels were measured with RT-qPCR. For IL1 β , control group level was 0 ± 2.75 and increased to 23.7 ± 0.55 in the LPS group ($p < 0.05$) (**Figure 7A**). For IL16, control group level was 0 ± 2.89 and increased to 26.3 ± 0.63 in the LPS group ($p < 0.05$) (**Figure 7B**). For TNF, control group level was 0 ± 0.96 and increased to 3.53 ± 1.27 in the LPS group ($p < 0.05$) (**Figure 7C**). These results indicate that LPS introduction to the N9 microglial cells successfully induced the pro-inflammatory markers.

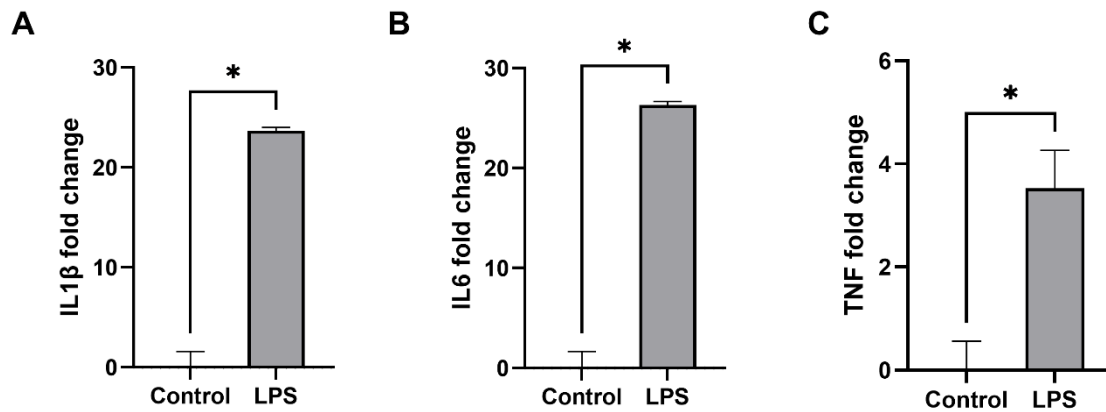


Figure 8. mRNA levels of pro-inflammatory markers were increased with the LPS treatment of N9 microglial cells. Measurement of relative mRNA levels of **(A)** IL1 β , **(B)** IL6, and **(C)** TNF levels. Data are given as mean $\pm$ standard error of the mean. * $p < 0.05$.

4.3. Small RNA sequencing of different fragments of cells

After small-RNA sequencing bioinformatic analysis was done for groups ($n=3$). The sample distance matrix provides information about the similarities and differences among the samples. The division between conditions 1 and 2 is displayed via the Principal Component Analysis (PCA). Because each replicate is represented as a separate data point, this kind of figure is helpful for illustrating the total impact of batch effects and experimental covariates. Venn diagram shows differentially expressed tRNAs obtained from five different methods by tsRNAsearch. **Table 4** indicated the quality control of samples; there are high number of percentages for QC passed. **Table 5** indicated alignment ratios of individual samples to MINTbase database.

Table 4. Quality control of individual samples. QC: Quality Control; Exo: EV group; Lys: Lysate group; Sup: Supernatant group; C: Control group; LPS: LPS group.

Name	Total	QC Passed	QC Passed%
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c1_exo	24807452	21263729	85.72%
c2_exo	22656580	20716456	91.44%
c3_exo	21475551	20181850	93.98%
c4_exo	28496956	26641359	93.49%
c1_lys	23351936	19026552	81.48%
c2_lys	20468116	17680278	86.38%
c3_lys	35260369	30763157	87.25%
c4_lys	39344985	34862443	88.61%
c1_sup	30161716	27002299	89.53%
c2_sup	37620333	32604382	86.67%
c3_sup	41669254	38901268	93.36%
c4_sup	24719527	22910401	92.68%
lps1_exo	12491593	9180873	73.50%
lps2_exo	18733549	16329003	87.16%
lps3_exo	15629521	13999635	89.57%
lps4_exo	16261817	14507433	89.21%
lps1_lys	34901028	26860994	76.96%
lps2_lys	45245182	38306045	84.66%
lps3_lys	31799821	26869276	84.50%
lps4_lys	37398517	32457816	86.79%
lps1_sup	25214409	20189970	80.07%
lps2_sup	21643329	18148486	83.85%
lps3_sup	9404943	7152874	76.05%
lps4_sup	25176735	21880172	86.91%

Table 5. Alignment ratios of individual samples to MINTbase database. Exo: EV group; Lys: Lysate group; Sup: Supernatant group; C: Control group; LPS: LPS group.

Name	%Aligned
c1_exo	4.59%
c2_exo	5.23%
c3_exo	2.68%
c4_exo	3.97%
c1_lys	5.56%
c2_lys	4.97%
c3_lys	4.71%
c4_lys	4.31%
c1_sup	2.87%
c2_sup	2.70%
c3_sup	2.02%
c4_sup	2.09%
lps1_exo	3.82%
lps2_exo	3.99%
lps3_exo	3.08%
lps4_exo	3.20%
lps1_lys	3.68%
lps2_lys	3.39%
lps3_lys	3.64%
lps4_lys	3.28%
lps1_sup	2.31%
lps2_sup	2.27%
lps3_sup	3.10%
lps4_sup	3.21%

4.3.1. Extracellular Vesicles

In the EV group, sample distance matrix showed that the groups are similar within themselves, and different from another, control vs LPS (**Figure 8A**). Number of features identified by each tsRNAsearch method (**Figure 8B**). Total of 463 features were identified; Fisher method identified 108, and DESeq2 identified 88 unique, and 267 shared features. Ratios of all sncRNAs identified for each group, normalized to RPM (**Figure 8C**). Most of total sncRNAs are miRNAs in both groups, and also miRNAs are increased with LPS introduction. snRNAs are also abundant in control group and decreased with LPS introduction. **Figure 8D** indicates the features on individual basis rather than two main groups. In **Figure 8E**, one of the most abundant tRNA in control group is Glycine and decreased in LPS group. Additionally, Lysine is also highly expressed in both groups.

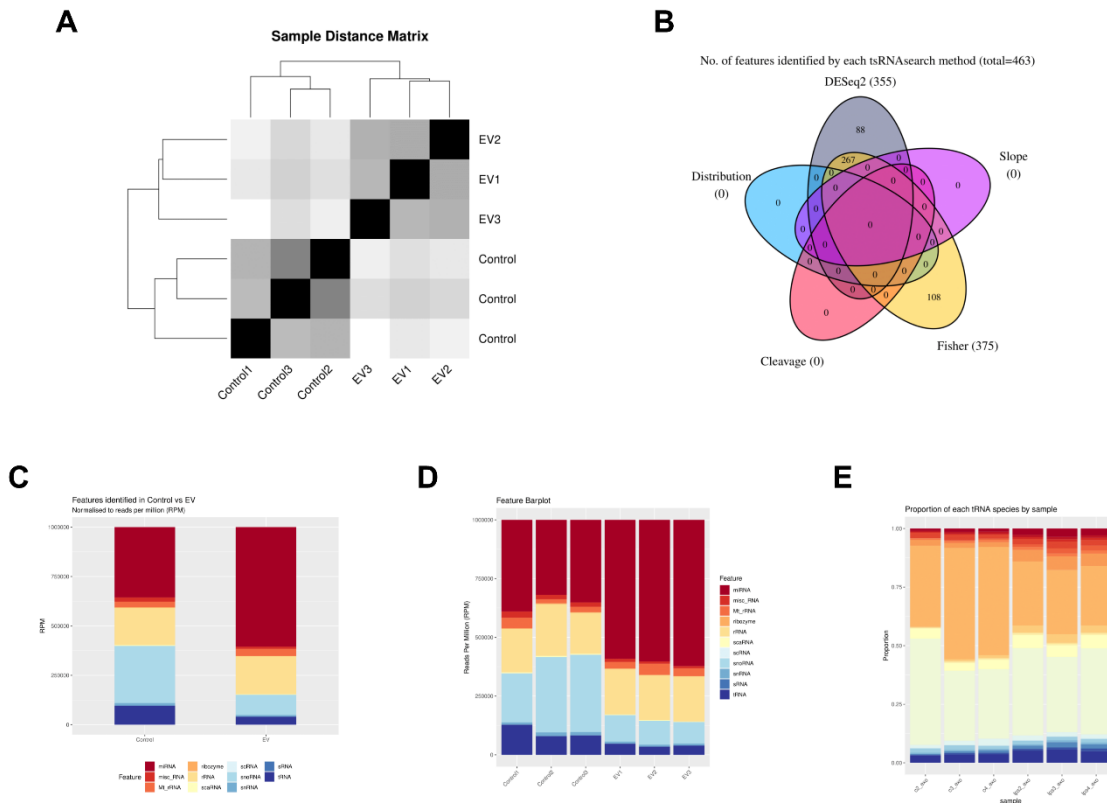


Figure 9. Characterization of small non-coding RNA expression profiles between groups and individuals for EVs. **(A)** Sample distance matrix of samples from EV groups. **(B)** Number of features identified by each tsRNAsearch method. **(C)** Ratios of all sncRNAs identified for each group, normalized to reads per million (RPM). **(D)** Ratios of all sncRNAs identified for each individual sample, normalized to reads per million (RPM). **(E)** Ratios of all tRNAs identified for each group, normalized to reads per million (RPM). i-tRF, internal tRF; miRNA, microRNA; misc_RNA, miscellaneous RNA; scaRNA, Small Cajal body-specific RNA; mt_rRNA, Mitochondrial rRNA; scRNA, small conditional RNA; snoRNA, Small nucleolar RNA; snRNA, Small nuclear RNA; sRNA, Bacterial small RNA; tRNA, Transfer RNA; rRNA, Ribosomal RNA.

4.3.2. Lysate

In the lysate group, sample distance matrix showed that the groups are similar within themselves, and different from another, control vs LPS (**Figure 9A**). Number of features identified by each tsRNAsearch method (**Figure 9B**). Total of 379 features were identified; Fisher method identified 173 unique, DESeq2 identified 99 unique, Cleavage identified one feature, and 106 shared features. Ratios of all sncRNAs identified for each group, normalized to RPM (**Figure 9C**). Most of total sncRNAs are snRNAs in both groups. miRNAs are also abundant in control group and decreased with LPS group. **Figure 9D** indicates the features on individual basis rather than two main groups. In **Figure 9E**, one of the most abundant tRNA in control group is Lysine and decreased in LPS group. Additionally, Glycine and Alanine are also highly expressed in both groups.

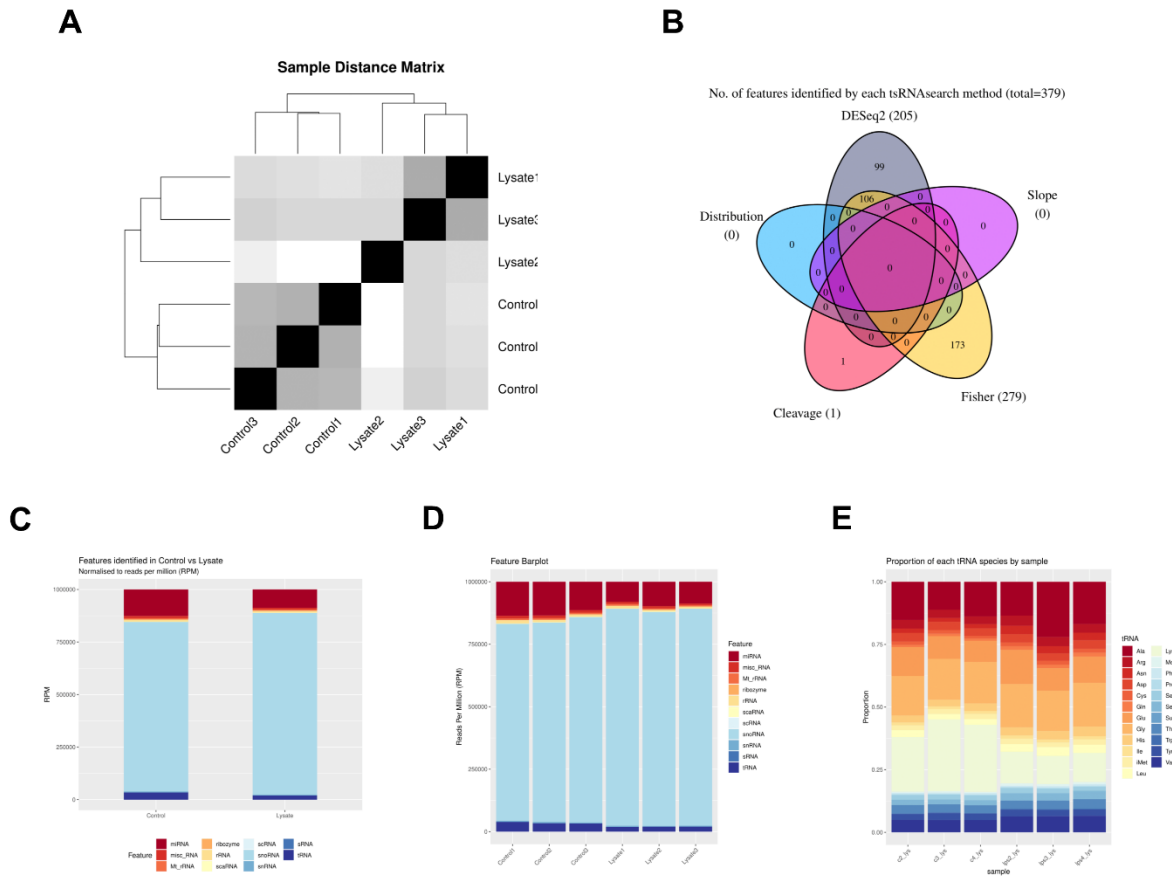


Figure 10. Characterization of small non-coding RNA expression profiles between groups and individuals for lysate samples. **(A)** Sample distance matrix of samples from lysate groups. **(B)** Number of features identified by each tsRNAsearch method. **(C)** Ratios of all sncRNAs identified for each group, normalized to reads per million (RPM). **(D)** Ratios of all sncRNAs identified for each individual sample, normalized to reads per million (RPM). **(E)** Ratios of all tRNAs identified for each group, normalized to reads per million (RPM). i-tRF, internal tRF; miRNA, microRNA; misc_RNA, miscellaneous RNA; scaRNA, Small Cajal body-specific RNA; mt_rRNA, Mitochondrial rRNA; scRNA, small conditional RNA; snoRNA, Small nucleolar RNA; snRNA, Small nuclear RNA; sRNA, Bacterial small RNA; tRNA, Transfer RNA; rRNA, Ribosomal RNA.

4.3.3. Supernatant

In the supernatant group, sample distance matrix showed that the groups are similar within themselves, and different from another, control vs LPS (**Figure 10A**). Number of features identified by each tsRNAsearch method (**Figure 10B**). Total of 361 features were identified; Fisher method identified 229 unique, DESeq2 identified 47 unique, Cleavage identified one feature, and 85 shared features. Ratios of all sncRNAs identified for each group, normalized to RPM (**Figure 10C**). Most of total sncRNAs are miRNAs in both groups. miRNAs are abundant in control group and increased with LPS group. **Figure 10D** indicates the features on individual basis rather than two main groups. In **Figure 10E**, one of the most abundant tRNA in control group is Lysine and decreased in LPS group. Additionally, Glycine are also highly expressed in control group and decreased in LPS groups.

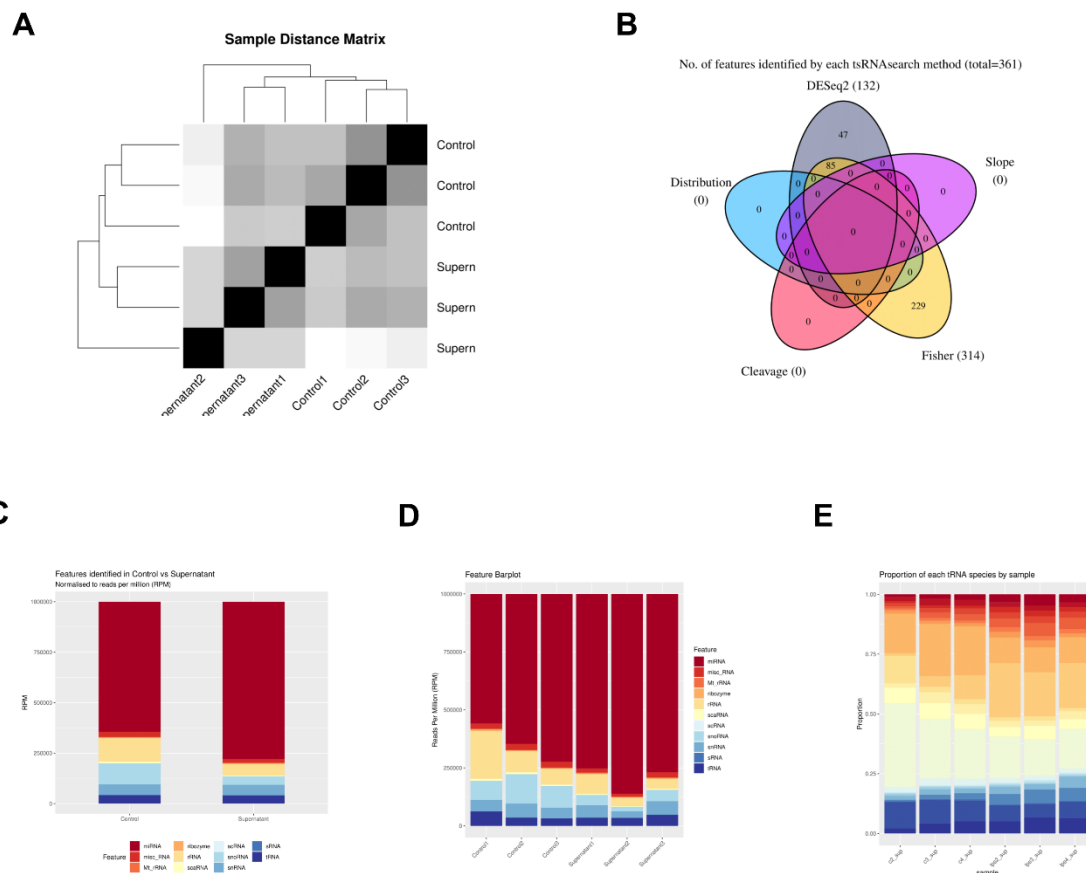


Figure 11. Characterization of small non-coding RNA expression profiles between groups and individuals for supernatant samples. **(A)** Sample distance matrix of samples from supernatant groups. **(B)** Number of features identified by each tsRNAsearch method. **(C)** Ratios of all sncRNAs identified for each group, normalized to reads per million (RPM). **(D)** Ratios of all sncRNAs identified for each individual sample, normalized to reads per million (RPM). **(E)** Ratios of all tRNAs identified for each group, normalized to reads per million (RPM). i-tRF, internal tRF; miRNA, microRNA; misc_RNA, miscellaneous RNA; scaRNA, Small Cajal body-specific RNA; mt_rRNA, Mitochondrial rRNA; scRNA, small conditional RNA; snoRNA, Small nucleolar RNA; snRNA, Small nuclear RNA; sRNA, Bacterial small RNA; tRNA, Transfer RNA; rRNA, Ribosomal RNA.

4.4. Expression Profiles of tRFs from different fragments of cells

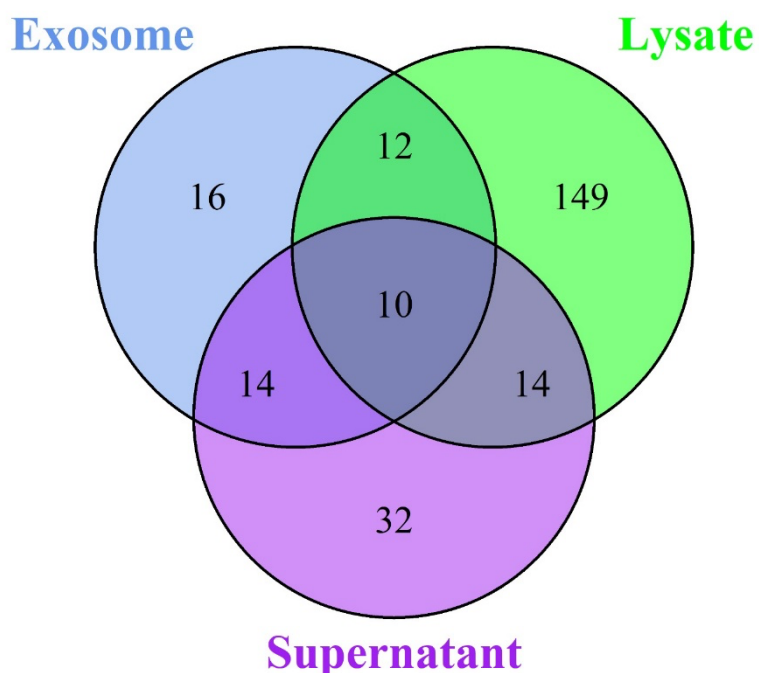


Figure 12. Venn diagram of differentially expressed tRFs for extracellular vesicles, lysate, and supernatant samples.

4.4.1. EVs

tRF profile of EV group using small RNA sequencing. Hierarchical cluster analysis of all DETs from EV group (**Figure 12A**). Dark brown means higher relative expression and light-yellow means lower relative expression. A total of 52 differentially expressed tRFs were detected between groups; 20 increased and 32 decreased. Volcano plot of all detected tRFs; green is increased, red is decreased, and grey is non-significant (**Figure 12B**). The X axis represents the $\log_2(\text{LPS/Control})$ and the Y axis represents $-\log_{10}(\text{adjusted p-value})$. **Figure 12C** demonstrates the PCA analysis of samples, it can be observed that individual samples were clustered together for each group. The cutoff values for differentially altered tRFs are at least 1.5-fold expression change and $p < 0.05$. **Figure 12D** indicates length distribution of differentially expressed tRFs; most tRFs were found at 29-39 range. **Figure 12E** shows the type distribution of differentially expressed tRFs. They belong to 3 groups; 5'-half, 5'-tRF, and i-tRF, from most common to the least.

In the EV group, 5 most upregulated tRFs are RF-19-2VO0SR1Z, tRF-16-Q01LQKB, tRF-32-86J8WPMN1E8YN, tRF-34-87R8WP9I1EWJIQ, and tRF-31-N3R718YKISQID (Table 6). 5 most downregulated tRFs are tRF-36-PS5P4PW3FJHPEZE, tRF-37-HRL7S5QKF1R3WE5, tRF-32-PW5SVP9N15WVN, tRF-38-PNR8YP9LON4VN18, and tRF-16-PJ5830E (Table 7).

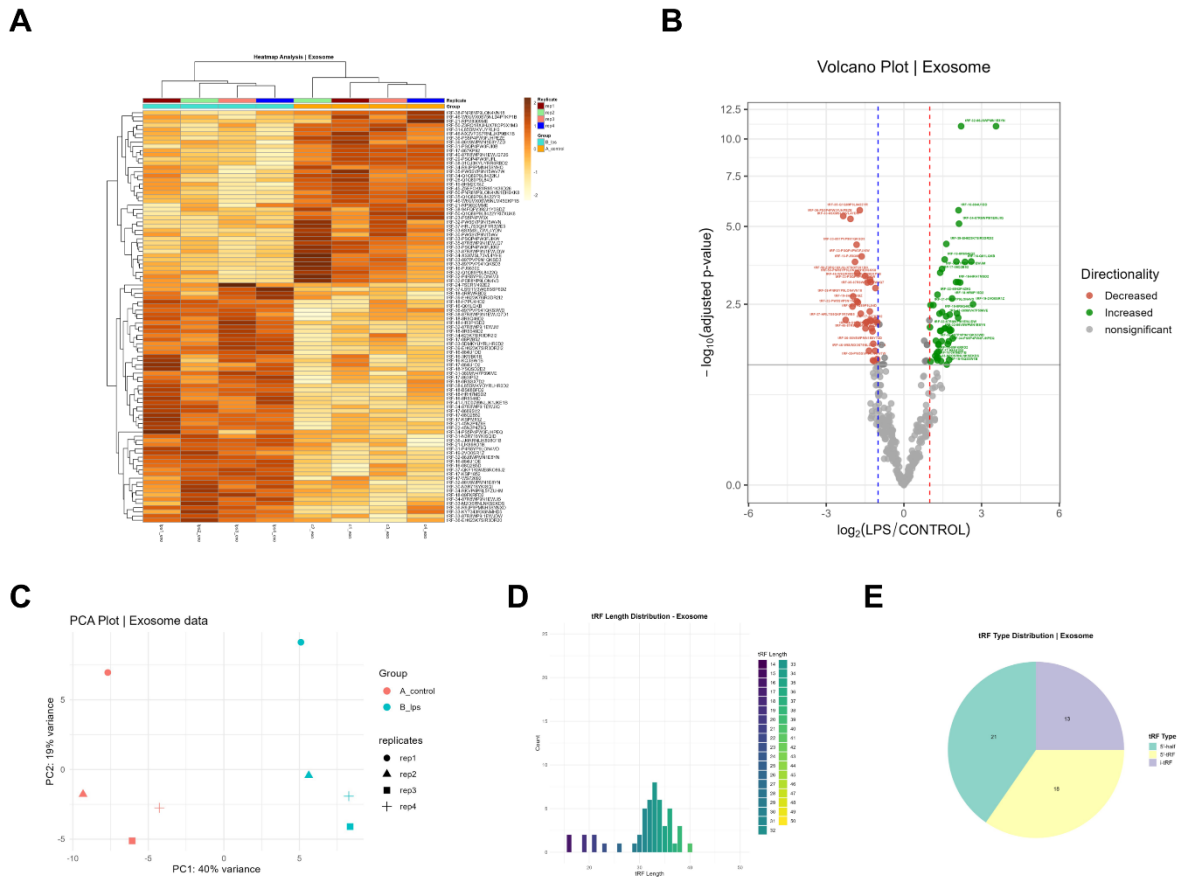


Figure 13. The differential expression profiles of tRFs between LPS and control groups obtained from EV samples. **(A)** Hierarchical cluster analysis of all DETs. Colored bar shows relative expression values; Brown means higher relative expression, and yellow means lower relative expression. The first bar above the graph demonstrates 2 groups, and the second bar above that demonstrates individual samples. **(B)** Volcano plot for all tRFs detected. Red means significantly decreased tRFs, green means significantly increased tRFs, and gray means no significant change according to $\log_2\text{FC}$ and $-\log(\text{adjusted p-value})$. **(C)** Principal component analysis of individual samples. **(D)** Length distribution of differentially expressed tRFs. **(E)** Type distribution of differentially expressed tRFs.

Table 6. Statistically significantly increased tRFs from EV samples. Padj: Adjusted p-value.

tRF Name	log2FoldChange	padj	Sequence	Type
tRF-19-2VO0SR1Z	2,6705	0,003164	CACGCGAAAGGTCCCCGGT	i-tRF
tRF-16-Q01LQKB	2,3611	0,000202	GCGAGAGGTAGCGGGA	i-tRF
tRF-32-86J8WPMN1E8YN	2,2096	9,58E-12	TCCCACATGGTCTAGCGGTTAGGATT CCTGGT	5'-half
tRF-34-87R8WP9I1EWJIQ	2,1290	7,65E-06	TCCCTGGTGGTCTAGTGGCTAGGAT TCGGCGCTT	5'-half
tRF-31-N3R718YKISQID	2,0967	0,006452	CTGGTGGTCTAGTGGTTAGGATTCTG GCGCTC	i-tRF
tRF-30-N3R718YKISQI	2,0477	0,005222	CTGGTGGTCTAGTGGTTAGGATTCTG GCGCT	i-tRF
tRF-36-R9JP9PMNH5SYNXD	1,7684	0,005538	GGTTCCATAGTGTAGCGGTTATCAC GTCTGCTTTAC	5'-tRF
tRF-34-PS5P4PW3FJHPEQ	1,6943	0,02208	GCCCGGATAGCTCAGTCGGTAGAGC ATCAGACTT	5'-half
tRF-31-389MV47P596VE	1,6316	0,006051	CCTGGTGGTCTAGTGGTTAGGATTCTG GGCGCT	i-tRF
tRF-32-87R8WP9I1EWM	1,4698	0,00034	TCCCTGGTGGTCTAGTGGCTAGGAT TCGGCGC	5'-half

tRF-33- KY7343RX6NM HD5	1,4309	0,043 647	CCCTGGTGGTCTAGTGGTTAGGATT CGGCGCTC	i-tRF
tRF-32- 86V8WPMN1E 8YN	1,4306	0,012 19	TCCCATATGGTCTAGCGGTTAGGATT CCTGGT	5'- half
tRF-36- EH623K7SIR3 DR20	1,3347	0,022 08	ACGCGGGAGACCGGGGTTTCGATTCC CCGACGGGGAG	i-tRF
tRF-36- 897PVP941QK S3WD	1,2364	0,019 165	TCCTCGTTAGTATAGTGGTGAGTATC CCCGCCTGTC	5'- tRF
tRF-33- M2OSRNLNKS EKDS	1,0289	0,044 28	CGGGAGACCGGGGTTTCGATTCCCCG ACGGGGAG	i-tRF
tRF-31- P4R8YP9LON4 VD	1,0276	0,003 314	GCATGGGTGGTTCAGTGGTAGAATT CTCGCC	5'- half
tRF-33- 87R8WP9I1EW JDW	1,0077	0,010 381	TCCCTGGTGGTCTAGTGGCTAGGAT TCGGCGCT	5'- half
tRF-38- 87R8WP9N1E WJQ7D1	0.865507	0.042 739	TCCCTGGTGGTCTAGTGGTTAGGATT CGGCGCTCTCAC	5'- tRF
tRF-34- RKVP4PRL5FZ UHM	0.81711	0.043 647	GGGGGTATAGCTCAGGGGTAGAGCA TTTGACTGC	5'- half
tRF-34- 87R8WP9N1E	0.784727	0.019 165	TCCCTGGTGGTCTAGTGGTTAGGATT CGGCGCTC	5'- half

WJI5				
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Table 7. Statistically significantly decreased tRFs from EV samples. Padj: Adjusted p-value.

tRF Name	log2Fold Change	padj	Sequence	Type
tRF-36- PS5P4PW3FJH PEZE	-2,34306	3,55 E-06	GCCCGGATAGCTCAGTCGGTAGAGCAT CAGACTTTT	5'- tRF
tRF-37- HRL7S5QKF1R 3WE5	-2,25028	0,00 7267	ATCCCCGCCTGTCACGCGGGAGACCGG GGTTCGATTC	i-tRF
tRF-32- PW5SVP9N15 WVN	-1,9857	0,00 3587	GCCGTGATCGTATAGTGGTTAGTACTCT GCGT	5'- half
tRF-38- PNR8YP9LON 4VN18	-1,96291	0,00 2008	GCATTGGTGGTTCAGTGGTAGAATTCTC GCCTGCCACG	5'- tRF
tRF-16- PJ5830E	-1,89023	0,00 0202	GCAGCGATGGCCGAGT	5'- tRF
tRF-33- 897PVP9N1QK SD3	-1,82824	5,16 E-05	TCCTCGTTAGTATAGTGGTTAGTATCCC CGCCT	5'- half
tRF-19- 8HM2OSIZ	-1,81886	0,00 269	TCACGCGGGAGACCGGGGT	i-tRF
tRF-50- Z3RQ18XJHJX 78OP3X1M3	-1,80361	0,00 046	TTGGTGGTATAGTGGTGAGCATAGCTGC CTTCCAAGCAGTTGACCCGGGT	i-tRF

tRF-21- RPM8309ME	-1,79923	0,00 9042	GGTAGCGTGGCCGAGTGGTCT	5'- tRF
tRF-31- L85DMKYUYR LH0	-1,77912	0,00 2855	CGCGGGAGACCGGGGTTTCGATTCCCCG ACGG	i-tRF
tRF-35- Q1Q89P9L842 2YR	-1,70521	1,94 E-06	GCGCCGCTGGTGTAGTGGTATCATGCA AGATTCCC	5'- tRF
tRF-23- PS5P4PW3X	-1,66653	0,00 5256	GCCCGGATAGCTCAGTCGGTAGA	5'- tRF
tRF-33- PSQP4PW3FJI KW	-1,63394	0,00 0133	GCCCGGCTAGCTCAGTCGGTAGAGCAT GGGACT	5'- half
tRF-50- PNR8YP9LON 4VN1EH6KK8	-1,49977	0,00 0552	GCATTGGTGGTTCAGTGGTAGAATTCTC GCCTGCCACGCGGGAGGCCCGG	5'- tRF
tRF-32- Q1Q89P9L842 2Q	-1,48364	0,01 0948	GCGCCGCTGGTGTAGTGGTATCATGCA AGATT	5'- half
tRF-33- PSQP4PW3FJI 0W	-1,41328	0,00 0853	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGACT	5'- half
tRF-26- Q1Q89P9L84D	-1,31884	0,00 4672	GCGCCGCTGGTGTAGTGGTATCATGC	5'- tRF
tRF-38- 94FQFY392J1 Y0SDZ	-1,30212	0,00 7313	TGGTGAGCATAGCTGCCTTCCAAGCAGT TGACCCGGGT	i-tRF
tRF-31- PSQP4PW3FJI	-1,28026	0,00 083	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGA	5'- tRF

0B				
tRF-34- Q1Q89P9L842 2KJ	-1,24917	0,01 0948	GCGCCGCTGGTGTAGTGGTATCATGCA AGATTCC	5'- half
tRF-40- 87R8WP9N1E WJQ72S	-1,2115	0,01 2583	TCCCTGGTGGTCTAGTGGTTAGGATTCTG GCGCTCTCACCG	5'- tRF
tRF-30- PW5SVP9N15 WV	-1,19803	0,04 2417	GCCGTGATCGTATAGTGGTTAGTACTCT GC	5'- tRF
tRF-36- 86V8WPMN1E 8Y7ZD	-1,18419	0,02 1796	TCCCATATGGTCTAGCGGTTAGGATTCC TGGTTTTTC	5'- tRF
tRF-21- RP9830MME	-1,15454	0,04 3647	GGTAGTGTGGCCGAGCGGTCT	5'- tRF
tRF-35- 87R8WP9N1E WJQ7	-1,10004	0,00 1216	TCCCTGGTGGTCTAGTGGTTAGGATTCTG GCGCTCT	5'- half
tRF-50- Q1Q89P9L842 2YRI7XUK8	-1,09836	0,00 7376	GCGCCGCTGGTGTAGTGGTATCATGCA AGATTCCCATTCTTGCGACCCGG	5'- tRF
tRF-35- PW5SVP9N15 WV7W	-1,03464	0,04 0534	GCCGTGATCGTATAGTGGTTAGTACTCT GCGTTGT	5'- half
tRF-32- P4R8YP9LON4 V3	-0.95927	0.04 957	GCATGGGTGGTTCAGTGGTAGAATTCTC GCCT	5'- half
tRF-29- PSQP4PW3FJ	-0.94808	0.00 9042	GCCCGGCTAGCTCAGTCGGTAGAGCAT GA	5'- tRF

FL				
tRF-33- 87R8WP9N1E WJDW	-0.86736	0.02 2812	TCCCTGGTGGTCTAGTGGTTAGGATTCTG GCGCT	5'- half
tRF-34- R9JP9PMNH5 SYHQ	-0.82824	0.04 9239	GGTTCCATAGTGTAGCGGTTATCACGTC TGCTTT	5'- half
tRF-33- 897PVP941QK SD3	-0.77183	0.04 9239	TCCTCGTTAGTATAGTGGTGAGTATCCC CGCCT	5'- half

4.4.2. Lysate

tRF profile of lysate group using small RNA sequencing. Hierarchical cluster analysis of all DETs from EV group (**Figure 13A**). Dark brown means higher relative expression and light-yellow means lower relative expression. A total of 185 differentially expressed tRFs were detected between groups; 104 increased and 81 decreased. Lysate Volcano plot of all detected tRFs; green is increased, red is decreased, and grey is non-significant (**Figure 13B**). Compared to other groups, lysate group has the most tRFs detected as it is the biggest fraction amongst all. The X axis represents the $\log_2(\text{LPS/Control})$ and the Y axis represents $-\log_{10}(\text{adjusted p-value})$. **Figure 13C** demonstrates the PCA analysis of samples, it can be observed that individual samples were clustered together for each group. The cutoff values for differentially altered tRFs are at least 1.5-fold expression change and $p < 0.05$. **Figure 13D** indicates length distribution of differentially expressed tRFs; most tRFs were found at 30-36 range. **Figure 13E** shows the type distribution of differentially expressed tRFs. They belong to 3 groups; i-tRF, 5'-half, and 5'-tRF, from most common to the least.

In the lysate group, 5 most upregulated tRFs are tRF-33-87R8WP9I1EWJDW, tRF-34-87R8WP9I1EWJIQ, tRF-35-87R8WP9I1EWJQZ, tRF-31-FU8U76D7M461E, and tRF-34-897PVP941QKS1P (Table 8). 5 most downregulated tRFs are tRF-32-

MIF91SS2P4FI3, tRF-48-Q1Q89P9L8422YRI7XUDJ, tRF-34-LSN3S3RQJVDEJJ, tRF-23-94V4SMIFX, and tRF-32-L85DMKYOYRLH4 (Table 9).

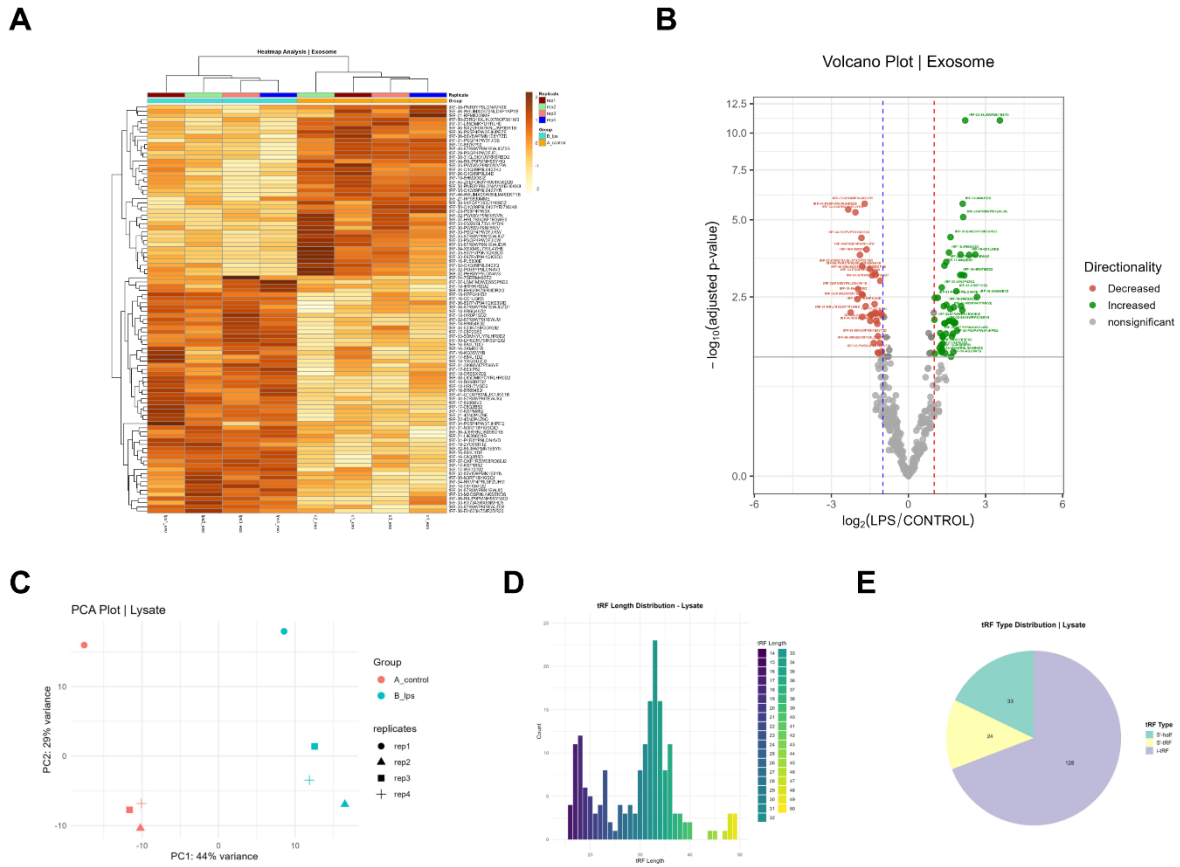


Figure 14. The differential expression profiles of tRFs between LPS and control groups obtained from lysate. **(A)** Hierarchical cluster analysis of all DETs. Colored bar shows relative expression values; Brown means higher relative expression, and yellow means lower relative expression. The first bar above the graph demonstrates 2 groups, and the second bar above that demonstrates individual samples. **(B)** Volcano plot for all tRFs detected. Red means significantly decreased tRFs, green means significantly increased tRFs, and gray means no significant change according to log₂FC and -log(adjusted p-value). **(C)** Principal component analysis of individual samples. **(D)** Length distribution of differentially expressed tRFs. **(E)** Type distribution of differentially expressed tRFs.

Table 8. Statistically significantly increased tRFs from lysate samples. Padj: Adjusted p-value.

tRF Name	log2Fold Change	padj	Sequence	Type
tRF-33-87R8WP9I1E WJDW	4,51008 2549	4,9E -24	TCCCTGGTGGTCTAGTGGCTAGGATTCG GCGCT	5'- half
tRF-34-87R8WP9I1E WJIQ	4,04396 9393	1,9E -13	TCCCTGGTGGTCTAGTGGCTAGGATTCG GCGCTT	5'- half
tRF-35-87R8WP9I1E WJQZ	3,45631 5487	4,5E -12	TCCCTGGTGGTCTAGTGGCTAGGATTCG GCGCTTT	5'- half
tRF-31-FU8U76D7M4 61E	3,31068 8109	5,43 E-08	AGCGATCCGAGTTCAAATCTCGGTGGGA CCT	i-tRF
tRF-34-897PVP941Q KS1P	3,20737 136	7,97 E-13	TCCTCGTTAGTATAGTGGTGAGTATCCCC GCCTG	5'- half
tRF-32-87R8WP9I1E WJM	3,19610 1472	1,04 E-20	TCCCTGGTGGTCTAGTGGCTAGGATTCG GCGC	5'- half
tRF-34-86J8WPMN1 E8Y2Q	2,93064 8809	1,16 E-08	TCCCACATGGTCTAGCGGTTAGGATTCC TGGTTT	5'- half
tRF-31-87R8WP9I1E WJ0	2,51399 1789	8,83 E-08	TCCCTGGTGGTCTAGTGGCTAGGATTCG GCG	5'- tRF
tRF-32-	2,32129	2,1E	AGGCGGCCCGGGTTCGACTCCCGGTGT	i-tRF

1HPSR9O933 7KF	7966	-06	GGGAA	
tRF-33- PY5P4PW3FJ HPW	2,32122 4289	4,22 E-05	GCCTGGATAGCTCAGTCGGTAGAGCATC AGACT	5'- half
tRF-37- 8RPY8HM2O SRNLNJ	2,29323 9989	0,00 0131	TCCCCGCCTGTACGCGGGAGACCGGG GTTCGATTCC	i-tRF
tRF-21- 25QB1MK8E	2,27761 3648	0,00 0187	CACACGCGAAAGGTCCCCGGT	i-tRF
tRF-36- DRFU8U76D7 M461E	2,27568 4524	0,00 5442	AATCCAGCGATCCGAGTTCAAATCTCGG TGGGACCT	i-tRF
tRF-37- L7S5QKF1R3 WE8RL	2,26108 3817	4,81 E-05	CGCCTGTCACGCGGGAGACCGGGGTTC GATTCCCCGA	i-tRF
tRF-30- 93R918VBY9 PY	2,18833 4283	0,02 8303	TGGGTGGTTCAGTGGTAGAATTCTCGCC TG	i-tRF
tRF-18- 3FPD4805	2,15489 8199	0,00 6546	CCGCAGCAACCTCGGTTC	i-tRF
tRF-19- 2VO0SR1Z	2,15101 934	1,44 E-08	CACGCGAAAGGTCCCCGGT	i-tRF
tRF-23- RNLIK898D6	2,14414 8264	0,00 1677	GGGTTCGACTCCCGGTGTGGGAA	i-tRF
tRF-40- PSQP4PW3F JIKE7UM	2,14198 7648	0,00 5237	GCCCGGCTAGCTCAGTCGGTAGAGCATG GGACTCTTAATC	5'- tRF
tRF-18-	2,11522	8,25	CACGCGAAAGGTCCCCGG	i-tRF

2VO0SRD4	6287	E-07		
tRF-32- KY7343RX6N MH3	2,06808 8498	2,17 E-05	CCCTGGTGGTCTAGTGGTTAGGATTCCG CGCT	i-tRF
tRF-36- PY8HM2OSR NLNKS	2,03246 416	0,00 0224	GCCTGTCACGCGGGAGACCGGGGTTTCG ATTCCCCGA	i-tRF
tRF-38- 8RPY8HM2O SRNLNDJ	2,02569 933	0,00 6546	TCCCCGCCTGTCACGCGGGAGACCGGG GTTCGATTCCC	i-tRF
tRF-19- EH623K25	2,01448 2799	0,03 0618	ACGCGGGAGACCGGGGTTTC	i-tRF
tRF-36- 87R8WP9I1E WJQZD	2,01321 0814	0,00 0113	TCCCTGGTGGTCTAGTGGCTAGGATTCCG GCGCTTTC	5'- tRF
tRF-26- M2OSRNLNK SB	1,99669 6048	2,07 E-06	CGGGAGACCGGGGTTTCGATTCCCCGA	i-tRF
tRF-33- 86J8WPMN1 E8Y0E	1,99351 7631	3,53 E-07	TCCCACATGGTCTAGCGGTTAGGATTCC TGGTT	5'- half
tRF-28- N24YDRFU8 U0E	1,97192 4985	0,02 0731	CTGGACTCTGAATCCAGCGATCCGAGTT	i-tRF
tRF-33- 8RPY8HM2O SRNDL	1,94340 9429	0,01 8352	TCCCCGCCTGTCACGCGGGAGACCGGG GTTCGA	i-tRF
tRF-32- R008R959KU	1,90162 0598	2,22 E-07	GGGAGACCGGGGTTTCGATTCCCCGACG GGGAG	i-tRF

MKH				
tRF-33- M2OSRNLNK SEKDS	1,89733 9087	1,41 E-05	CGGGAGACCGGGGTTTCGATTCCCCGAC GGGGAG	i-tRF
tRF-36- 86J8WPMN1 E8Y7ZD	1,89001 1755	3,72 E-05	TCCCACATGGTCTAGCGGTTAGGATTCC TGGTTTTTC	5'- tRF
tRF-18- Q01L7KDD	1,88809 7568	0,00 4682	GCGAGAGGTAGTGGGATC	i-tRF
tRF-33- R9JP9PMNH5 SYDQ	1,88500 0803	3,25 E-06	GGTTCCATAGTGTAGCGGTTATCACGTCT GCTT	5'- half
tRF-20- 2VO0SRMN	1,88388 7744	5,46 E-06	CACGCGAAAGGTCCCCGGTT	i-tRF
tRF-18- LUD3KS0E	1,86480 448	4,31 E-05	CGCGAAAGGTCCCCGGTT	i-tRF
tRF-34- R9JP9PMNH5 SYHQ	1,85853 3124	3,17 E-06	GGTTCCATAGTGTAGCGGTTATCACGTCT GCTTT	5'- half
tRF-33- 87R8WP9N1E WJDW	1,82267 595	1,24 E-06	TCCCTGGTGGTCTAGTGGTTAGGATTCCG GCGCT	5'- half
tRF-35- L85DMKYOY RLHR0	1,78309 2358	0,00 0579	CGCGGGAGACCGGGGTTCAATTCCCCG ACGGGGAG	i-tRF
tRF-22- 9KZR3HJ35	1,77023 6387	0,03 0618	TGGGGTTTCCCCGCGCAGGTTC	i-tRF
tRF-18- 2J1Y0SDZ	1,75698 1821	0,00 8393	CAAGCAGTTGACCCGGGT	i-tRF

tRF-32- 897PVP941Q KSJ	1,75663 0148	2,05 E-07	TCCTCGTTAGTATAGTGGTGAGTATCCCC GCC	5'- half
tRF-35- 8RPY8HM2O SRNLN	1,75302 5196	0,00 1867	TCCCCGCCTGTACGCGGGAGACCGGG GTTCGATT	i-tRF
tRF-33- 897PVP941Q KSD3	1,75105 8574	1,5E -06	TCCTCGTTAGTATAGTGGTGAGTATCCCC GCCT	5'- half
tRF-29- N3R718YKISI M	1,74710 2351	9,61 E-07	CTGGTGGTCTAGTGGTTAGGATTCGGCG C	i-tRF
tRF-34- KS3W2VR008 R9IE	1,74197 2227	0,00 2499	CCCCGCCTGTACGCGGGAGACCGGGG TTCGATT	i-tRF
tRF-26- 897PVP941Q D	1,71604 9703	0,01 944	TCCTCGTTAGTATAGTGGTGAGTATC	5'- tRF
tRF-35- S5QKF1R3W E8RO8	1,71372 1815	4,37 E-05	GTCACGCGGGAGACCGGGGTTCGATTC CCCGACGG	i-tRF
tRF-36- EH6KK87SIR M1DVB	1,70866 7577	0,00 019	ACGCGGGAGGCCCGGGTTCGATTCCCG GCCAATGCA	i-tRF
tRF-24- RPFQRDWWF J	1,69377 6243	0,01 4441	GGTAGAGCATGGGACTCTTAATCC	i-tRF
tRF-44- 9LV470JPN1	1,68616 0537	0,04 4117	TGGTATAGTGGTGAGCATAGCTGCCTTC CAAGCAGTTGACCCGG	i-tRF

Y6FPZD14				
tRF-36- 8RPY8HM2O SRNLND	1,68279 997	0,00 3006	TCCCCGCCTGTCACGCGGGAGACCGGG GTTCGATTC	i-tRF
tRF-36- EH623K7SIR3 DR20	1,67207 6643	2,67 E-05	ACGCGGGAGACCGGGGTTCGATTCCCC GACGGGGAG	i-tRF
tRF-24- SP58309M2S	1,66915 351	0,04 2506	GTCAGGATGGCCGAGTGGTCTAAG	5'- tRF
tRF-50- I89NJ4S2I7L7 P5QK11M3	1,65011 4031	0,00 9711	ATTGGTGGTTCAGTGGTAGAATTCTCGC CTGCCACGCGGGAGGCCCGGGT	i-tRF
tRF-48- I89NJ4S2I7L7 P5QK11D4	1,63811 1565	0,03 9747	ATTGGTGGTTCAGTGGTAGAATTCTCGC CTGCCACGCGGGAGGCCCGG	i-tRF
tRF-34- 8RPY8HM2O SRN17	1,63074 2417	0,00 6546	TCCCCGCCTGTCACGCGGGAGACCGGG GTTCGAT	i-tRF
tRF-17- LUD3KSN	1,62661 8749	1,63 E-06	CGCGAAAGGTCCCCGGT	i-tRF
tRF-31- P4R8YP9LON 4VD	1,60268 9788	4,31 E-07	GCATGGGTGGTTCAGTGGTAGAATTCTC GCC	5'- half
tRF-32- 86J8WPMN1 E8YN	1,60040 441	0,00 0523	TCCCACATGGTCTAGCGGTTAGGATTCC TGGT	5'- half
tRF-31- 897PVP941Q KSD	1,58378 0976	4,47 E-06	TCCTCGTTAGTATAGTGGTGAGTATCCCC GC	5'- tRF

tRF-28- 7343RX6NMH D5	1,58102 1552	0,01 3185	GTGGTCTAGTGGTTAGGATTCGGCGCTC	i-tRF
tRF-39- 897PVP941Q KS3WF8	1,57379 2855	0,03 2745	TCCTCGTTAGTATAGTGGTGAGTATCCCC GCCTGTCACG	5'- tRF
tRF-33- KS3W2VR008 R9D7	1,56939 0369	0,04 5036	CCCCGCCTGTCACGCGGGAGACCGGGG TTCGAT	i-tRF
tRF-32- P4R8YP9LON 4V3	1,56211 7485	4,25 E-06	GCATGGGTGGTTCAGTGGTAGAATTCTC GCCT	5'- half
tRF-33- PS5P4PW3FJ HPW	1,55315 7853	2,67 E-05	GCCCGGATAGCTCAGTCGGTAGAGCATC AGACT	5'- half
tRF-33- 8HM2OSRNL NKS8	1,54518 0976	0,00 1356	TCACGCGGGAGACCGGGGTTTCGATTCCC CGACG	i-tRF
tRF-33- KY7343RX6N MHD5	1,53580 9916	0,00 6234	CCCTGGTGGTCTAGTGGTTAGGATTCGG CGCTC	i-tRF
tRF-31- KY7343RX6N MHD	1,53155 6701	0,00 2535	CCCTGGTGGTCTAGTGGTTAGGATTCGG CGC	i-tRF
tRF-17- DF7UK8N	1,51565 1977	0,00 7888	AAGCAGTTGACCCGGGT	i-tRF
tRF-36- S5QKF1R3W E8RO80	1,50938 4363	0,00 5442	GTCACGCGGGAGACCGGGGTTTCGATTC CCCGACGGG	i-tRF

tRF-30- KY7343RX6N MH	1,48423 2144	0,03 0771	CCCTGGTGGTCTAGTGGTTAGGATTCCGG CG	i-tRF
tRF-28- N3R718YKIS D8	1,48174 7181	0,02 3581	CTGGTGGTCTAGTGGTTAGGATTCCGGCG	i-tRF
tRF-31- 623K7SIR3D R20	1,47693 8452	0,00 1395	GGAGACCGGGGTTTCGATTCCCCGACGG GGAG	i-tRF
tRF-32- PNR8YO9LO N4V3	1,47300 1469	0,00 3127	GCATTGGTGGTTCAATGGTAGAATTCTCG CCT	5'- half
tRF-30- N3R718YKIS QI	1,46653 6469	0,00 0412	CTGGTGGTCTAGTGGTTAGGATTCCGGCG CT	i-tRF
tRF-40- PSQP4PW3F JI0E7UM	1,43253 1291	0,00 6245	GCCCGGCTAGCTCAGTCGGTAGAGCATG AGACTCTTAATC	5'- tRF
tRF-34- QKF1R3WE8 RO8IS	1,43189 1385	0,00 1739	GCGGGAGACCGGGGTTTCGATTCCCCGA CGGGGAG	i-tRF
tRF-36- KS3W2VR008 R959D	1,41332 918	0,04 5036	CCCCGCCTGTCACGCGGGAGACCGGGG TTCGATTCC	i-tRF
tRF-29- EH623K7SIR1 L	1,40579 6895	0,00 4576	ACGCGGGAGACCGGGGTTTCGATTCCCC GA	i-tRF
tRF-32- 3HPSR95933	1,39536 7714	0,03 394	CCGCGGCCCGGGTTCGATTCCCGGTCA GGGAA	i-tRF

JKF				
tRF-18- XEKJ5RDS	1,39450 8967	0,00 0972	TGAATCCAGCGATCCGAG	i-tRF
tRF-37- 2VR008R959 KUMKH	1,38698 824	0,00 4195	CACGCGGGAGACCGGGGTTTCGATTCCC CGACGGGGGAG	i-tRF
tRF-32- 87R8WP9N1E WJM	1,37077 56	0,00 0799	TCCCTGGTGGTCTAGTGGTTAGGATTCTG GCGC	5'- half
tRF-31- 8HM2OSRNL NKSB	1,34789 7446	0,00 9761	TCACGCGGGAGACCGGGGTTTCGATTCCC CGA	i-tRF
tRF-30- P4R8YP9LON 4V	1,31847 7108	0,00 1408	GCATGGGTGGTTCAGTGGTAGAATTCTC GC	5'- tRF
tRF-18- Q01LQKDD	1,25643 1134	0,03 4298	GCGAGAGGTAGCGGGATC	i-tRF
tRF-32- R9JP9PMNH5 SY3	1,22318 1074	0,01 375	GGTTCCATAGTGTAGCGGTTATCACGTCT GCT	5'- half
tRF-33- 86V8WPMN1 E8Y0E	1,19705 7921	0,03 8425	TCCCATATGGTCTAGCGGTTAGGATTCCT GGTT	5'- half
tRF-29- P4R8YP9LON HK	1,19685 0964	0,00 6245	GCATGGGTGGTTCAGTGGTAGAATTCTC G	5'- tRF
tRF-31- 87R8WP9N1E WJ0	1,17960 2373	0,00 0839	TCCCTGGTGGTCTAGTGGTTAGGATTCTG GCG	5'- tRF

tRF-34- 87R8WP9N1E WJI5	1,17696 7218	0,00 5442	TCCCTGGTGGTCTAGTGGTTAGGATTCTG GCGCTC	5'- half
tRF-35- 897PVP941Q KS3W	1,17206 6296	0,00 602	TCCTCGTTAGTATAGTGGTGAGTATCCCC GCCTGT	5'- half
tRF-31- 389MV47P59 6VE	1,14362 4951	0,00 393	CCTGGTGGTCTAGTGGTTAGGATTCTGGC GCT	i-tRF
tRF-34- 8HM2OSRNL NKSE4	1,10901 7012	0,00 392	TCACGCGGGAGACCGGGGTTCTGATTCCC CGACGG	i-tRF
tRF-27- QKF1R3WE8 RL	1,08464 5711	0,04 7516	GCGGGAGACCGGGGTTCTGATTCCCCGA	i-tRF
tRF-30- MIF91SS2P4 FI	1,05978 8423	0,02 6489	CGGCTAGCTCAGTCGGTAGAGCATGAGA CT	i-tRF
tRF-30- 897PVP941Q KS	1,04774 1036	0,00 2499	TCCTCGTTAGTATAGTGGTGAGTATCCCC G	5'- tRF
tRF-38- 8HM2OSRNL NKSEKDS	1,04074 3746	0,03 0572	TCACGCGGGAGACCGGGGTTCTGATTCCC CGACGGGGAG	i-tRF
tRF-17- DRFU8UN	1,03352 2916	0,03 5974	AATCCAGCGATCCGAGT	i-tRF
tRF-33- PW5SVP9N1 5WV0E	1,02166 5557	0,04 7524	GCCGTGATCGTATAGTGGTTAGTACTCT GCGTT	5'- half

tRF-30- PS5P4PW3FJ HP	0.98864 8	0.00 7306	GCCCGGATAGCTCAGTCGGTAGAGCATC AG	5'- tRF
tRF-33- P4R8YP9LON 4VDP	0.96600 7	0.00 2008	GCATGGGTGGTTCAGTGGTAGAATTCTC GCCTG	5'- half
tRF-36- 87R8WP9N1E WJQ7D	0.90520 1	0.00 3561	TCCCTGGTGGTCTAGTGGTTAGGATTCG GCGCTCTC	5'- tRF

Table 9. Statistically significantly decreased tRFs from lysate samples. Padj: Adjusted p-value.

tRF Name	log2Fold Change	padj	Sequence	Type
tRF-32- MIF91SS2P4FI 3	-3,22129	4,31 E-07	CGGCTAGCTCAGTCGGTAGAGCATGAG ACTCT	i-tRF
tRF-48- Q1Q89P9L8422 YRI7XUDJ	-2,60424	0,00 0101	GCGCCGCTGGTGTAGTGGTATCATGCA AGATTCCCATTCTTGCGACCC	5'- tRF
tRF-34- LSN3S3RQJVD EJJ	-2,56747	0,00 117	CGCCGCTGGTGTAGTGGTATCATGCAA GATTCCC	i-tRF
tRF-23- 94V4SMIFX	-2,505	0,00 3692	TGGTGTAGTGGTATCATGCAAGA	i-tRF
tRF-32- L85DMKYOYRL H4	-2,4259	0,01 425	CGCGGGAGACCGGGGTTCAATTCCCCG ACGGG	i-tRF

tRF-34- PSQP4PW3FJI 0E5	-2,34132	1,57 E-06	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGACTC	5'- half
tRF-23- L85J3KYU05	-2,3392	0,00 1835	CGCGGGAGGCCCGGGTTCGATTC	i-tRF
tRF-34- PSQP4PW3FJI KE5	-2,3257	3,23 E-05	GCCCGGCTAGCTCAGTCGGTAGAGCAT GGGACTC	5'- half
tRF-17- M1M3WE5	-2,30505	0,01 1902	CGGCCCCGGGTTCGATTC	i-tRF
tRF-33- PSQP4PW3FJI 0W	-2,27221	2,62 E-08	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGACT	5'- half
tRF-26- RX6NMH490VD	-2,25789	0,00 0187	GGTTAGGATTCGGCGCTCTCACCGCC	i-tRF
tRF-33- PV94V4SMIFF NDJ	-2,23146	3,94 E-05	GCCGCTGGTGTAGTGGTATCATGCAAG ATTCCC	i-tRF
tRF-27- PV94V4SMIFH	-2,19055	0,00 9154	GCCGCTGGTGTAGTGGTATCATGCAAG	i-tRF
tRF-16-I7L7P50	-2,1635	0,00 7888	ATTCTCGCCTGCCACG	i-tRF
tRF-45- 94V4SMIFFNK QWYL1M3	-2,13596	0,02 0731	TGGTGTAGTGGTATCATGCAAGATTCCC ATTCTTGCGACCCGGGT	i-tRF
tRF-23- 4QD7OKW4Z	-2,11021	0,00 4845	CTCATAATCTGAAGGTCGTGAGT	i-tRF
tRF-33- PSQP4PW3FJI	-2,10391	8,88 E-07	GCCCGGCTAGCTCAGTCGGTAGAGCAT GGGACT	5'- half

KW				
tRF-22- RX6NMH491	-2,04889	0,00 1497	GGTTAGGATTCGGCGCTCTCAC	i-tRF
tRF-21- 40KK5Y93E	-1,96861	0,00 2009	CTAAGCCAGGGATTGTGGGT	i-tRF
tRF-39- H3RUSMKVNM EH6214	-1,90093	0,02 9062	ATAGTGGTGAGTATCCCCGCCTGTCAC GCGGGAGACCGG	i-tRF
tRF-20- 8OP3X1M3	-1,83446	0,02 4189	TCCAAGCAGTTGACCCGGGT	i-tRF
tRF-49- LSN3S3RQJVD E86YZQD14	-1,8032	0,00 042	CGCCGCTGGTGTAGTGGTATCATGCAA GATTCCCATTCTTGCGACCCGG	i-tRF
tRF-17- D0SR9N2	-1,76811	0,02 0591	AAGAGGTCCCTGGTTCA	i-tRF
tRF-18- 1SS2PMX	-1,76538	0,00 1215	AGTCGGTAGAGCATCAGA	i-tRF
tRF-34- PV94V4SMIFF N12	-1,7405	0,02 6627	GCCGCTGGTGTAGTGGTATCATGCAAG ATTCCCA	i-tRF
tRF-19- 392J1YEJ	-1,73221	0,00 0981	CCTTCCAAGCAGTTGACCC	i-tRF
tRF-22- 8XF6RE98N	-1,7239	0,04 9376	TCCTAAGCCAGGGATTGTGGGT	i-tRF
tRF-35- PSQP4PW3FJI KE7	-1,70618	0,00 012	GCCCGGCTAGCTCAGTCGGTAGAGCAT GGGACTCT	5'- half
tRF-17- S8V0J6L	-1,67891	9,94 E-05	GTCGGTAGAGCATCAGA	i-tRF

tRF-49- JY7383RPD9W 1X6L85J14	-1,65613	0,03 6466	CATTGGTGGTTCAGTGGTAGAATTCTCG CCTGCCACGCGGGAGGCCCGG	i-tRF
tRF-17- 73V2Y8K	-1,6439	0,00 266	GTGGTTAGGATTCGGCG	i-tRF
tRF-18- 73V2Y8DM	-1,60283	0,00 0494	GTGGTTAGGATTCGGCGC	i-tRF
tRF-34- 4WVLV470VR3 1KD	-1,58736	0,02 6231	CTCGTTAGTATAGTGGTGAGTATCCCCG CCTGTC	i-tRF
tRF-20- 73V2Y8L9	-1,56862	0,00 8146	GTGGTTAGGATTCGGCGCTC	i-tRF
tRF-33- 389MV47P596V 03	-1,55703	0,00 1441	CCTGGTGGTCTAGTGGTTAGGATTCGG CGCTCT	i-tRF
tRF-16- 1SS2PMB	-1,55491	0,00 2535	AGTCGGTAGAGCATCA	i-tRF
tRF-30- 6XQ6S8V0JUO 9	-1,55016	0,02 3581	GGCTAGCTCAGTCGGTAGAGCATGAGA CTC	i-tRF
tRF-23- ZLB6R3LU0E	-1,54658	0,01 5407	TTGTAAACCAGGGGTCGCGAGTT	i-tRF
tRF-50- MY73H3RUPLQ VNRDF7UK8	-1,52242	0,00 7693	CGTTGGTGGTATAGTGGTGAGCATAGC TGCCTTCCAAGCAGTTGACCCGG	i-tRF
tRF-47- 3IRW18V6XO5 9KNNVOR4	-1,51185	0,02 7321	CCGCTGGTGTAGTGGTATCATGCAAGA TTCCCATTCCTTGCGACCCGG	i-tRF
tRF-17-	-1,49508	0,00	CGGCCCGGGTTCGACTC	i-tRF

M1M3WD5		6847		
tRF-21- 6NMH490VD	-1,48948	0,01 4873	GGATTCGGCGCTCTCACCGCC	i-tRF
tRF-17- 9N1EWJM	-1,47054	0,00 4279	TGGTTAGGATTCGGCGC	i-tRF
tRF-32- L85DMKYUYRL H4	-1,46033	0,00 0731	CGCGGGAGACCGGGGTTTCGATTCCCC GACGGG	i-tRF
tRF-16- W3FJHPB	-1,45144	0,00 044	TCGGTAGAGCATCAGA	i-tRF
tRF-23- 9N1EWJQ7D1	-1,41884	0,00 3627	TGGTTAGGATTCGGCGCTCTCAC	i-tRF
tRF-35- PSQP4PW3FJI 0E7	-1,40817	0,00 0845	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGACTCT	5'- half
tRF-34- 389MV47P596V J5	-1,38937	0,00 6245	CCTGGTGGTCTAGTGGTTAGGATTCGG CGCTCTC	i-tRF
tRF-22- R29P4P9LL	-1,38714	0,03 0851	GGGGATGTAGCTCAGTGGTAGA	5'- tRF
tRF-20- 94V4SMIF	-1,37367	0,00 7003	TGGTGTAGTGGTATCATGCA	i-tRF
tRF-32- 3IRW18V6XO5 9J	-1,37085	0,03 3453	CCGCTGGTGTAGTGGTATCATGCAAGA TTCCC	i-tRF
tRF-33- IK9NJ4S2I7L7D V	-1,36028	0,00 8393	ATGGGTGGTTCAGTGGTAGAATTCTCG CCTGCC	i-tRF
tRF-50-	-1,35795	0,00	GCGCCGCTGGTGTAGTGGTATCATGCA	5'-

Q1Q89P9L8422 YRI7XUK8		2069	AGATTCCCATTCTTGCGACCCGG	tRF
tRF-16- 9N1EWJ0	-1,35601	0,00 7332	TGGTTAGGATTCGGCG	i-tRF
tRF-26- V47P596VW6D	-1,34274	0,04 593	TAGTGGTTAGGATTCGGCGCTCTCAC	i-tRF
tRF-35- 389MV47P596V W6	-1,34076	0,01 6674	CCTGGTGGTCTAGTGGTTAGGATTCGG CGCTCTCA	i-tRF
tRF-18- 83ML5F02	-1,331	0,00 6248	TCAGTCGGTAGAGCATCA	i-tRF
tRF-31- K84J83ML5FX2 D	-1,32502	0,02 8794	CCCGGCTAGCTCAGTCGGTAGAGCATG AGAC	i-tRF
tRF-48- 3IRW18V6XO5 9KNNVORDY	-1,28635	0,03 9097	CCGCTGGTGTAGTGGTATCATGCAAGA TTCCCATTCTTGCGACCCGGG	i-tRF
tRF-36- R9JP9PMNH5S YNXD	-1,24359	0,04 9153	GGTTCATAGTGTAGCGGTTATCACGTC TGCTTTAC	5'- tRF
tRF-20- 40KK5Y93	-1,23076	0,00 2891	CTAAGCCAGGGATTGTGGGT	i-tRF
tRF-23- R29P4P9LDS	-1,22271	0,02 6484	GGGGATGTAGCTCAGTGGTAGAG	5'- tRF
tRF-18- 7L73HQDU	-1,21881	0,02 3498	GTGTAGTGGTATCATGCA	i-tRF
tRF-19- 1SS2P4IR	-1,21392	0,01 5566	AGTCGGTAGAGCATGGGAC	i-tRF
tRF-33-	-1,21052	0,00	CACGCGGGAGACCGGGGTTTCGATTCCC	i-tRF

2VR008R959K UD4		4133	CGACGG	
tRF-25- R9JP9PMNH5	-1,19749	0,03 0618	GGTTCATAGTGTAGCGGTTATCAC	5'- tRF
tRF-32- PSQP4PW3FJI 01	-1,1947	0,00 1288	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGAC	5'- half
tRF-19- JMRPFQFX	-1,17382	0,00 6345	CAGTCGGTAGAGCATCAGA	i-tRF
tRF-17- W3FJHP1	-1,16624	0,00 8393	TCGGTAGAGCATCAGAC	i-tRF
tRF-17- 1SS2PMH	-1,12662	0,04 5036	AGTCGGTAGAGCATCAG	i-tRF
tRF-23- 73V2Y8L902	-1,11976	0,03 0634	GTGGTTAGGATTCGGCGCTCTCA	i-tRF
tRF-18- 9N1EWJDW	-1,11907	0,04 231	TGGTTAGGATTCGGCGCT	i-tRF
tRF-38- 87R8WP9N1E WJQ7D1	-1,11661	0,03 1417	TCCCTGGTGGTCTAGTGGTTAGGATTC GGCGCTCTCAC	5'- tRF
tRF-28- QKF1R3WE8R DR	-1,10962	0,04 3253	GCGGGAGACCGGGGTTTCGATTCCCCG AC	i-tRF
tRF-27- W3FJI0E7UM5	-1,10757	0,04 7899	TCGGTAGAGCATGAGACTCTTAATCTC	i-tRF
tRF-49- Q1Q89P9L8422 YRI7XU1K	-1,08139	0,01 2859	GCGCCGCTGGTGTAGTGGTATCATGCA AGATTCCCATTCTTGCGACCCG	5'- tRF
tRF-19-	-1,02028	0,03	AGTGGTTAGGATTCGGCGC	i-tRF

18YKISIM		9401		
tRF-34- Q1Q89P9L8422 KJ	-0.99881	0.00 2077	GCGCCGCTGGTGTAGTGGTATCATGCA AGATTCC	5'- half
tRF-33- L85J3KYUYR66 D0	-0.9218	0.00 3018	CGCGGGAGGCCCGGGTTCGATTCCCG GCCAATG	i-tRF
tRF-33- 941QKS3W2VR 0V	-0.90916	0.00 3711	TGGTGAGTATCCCCGCCTGTCACGCGG GAGACC	i-tRF
tRF-31- PS5P4PW3FJH PB	-0.90178	0.00 1542	GCCCGGATAGCTCAGTCGGTAGAGCAT CAGA	5'- tRF

4.4.3. Supernatant

tRF profile of lysate group using small RNA sequencing. Hierarchical cluster analysis of all DETs from EV group (**Figure 14A**). Dark brown means higher relative expression and light-yellow means lower relative expression. A total of 70 differentially expressed tRFs were detected between groups; 22 increased and 48 decreased. Volcano plot of all detected tRFs; green is increased, red is decreased, and grey is non-significant (**Figure 14B**). The X axis represents the $\log_2(\text{LPS}/\text{Control})$ and the Y axis represents $-\log_{10}(\text{adjusted p-value})$. **Figure 14C** demonstrates the PCA analysis of samples, it can be observed that individual samples were clustered together for each group. The cutoffs for differentially altered tRFs are at least 2-fold expression change and $p < 0.05$. The cutoff values for differentially altered tRFs are at least 1.5-fold expression change and $p < 0.05$. **Figure 14D** indicates length distribution of differentially expressed tRFs; most tRFs were found at 30-36 range. **Figure 14E** shows the type distribution of differentially expressed tRFs. They belong to 3 groups; i-tRF, 5'-half, and 5'-tRF, from most common to the least.

In the supernatant group, 5 most upregulated tRFs are tRF-16-Q01LQKB, tRF-16-QB1MK8E, tRF-35-8RPY8HM2OSRNLN, tRF-32-86J8WPMN1E8YN, and tRF-19-Q01LQKFK (Table 10). 5 most downregulated tRFs are tRF-16-0P5830E, tRF-16-PJ5830E, tRF-22-PJ60MV9JJ, tRF-33-897PVP9N1QKSD3, and tRF-23-LPMBQ4NKDJ (Table 11).

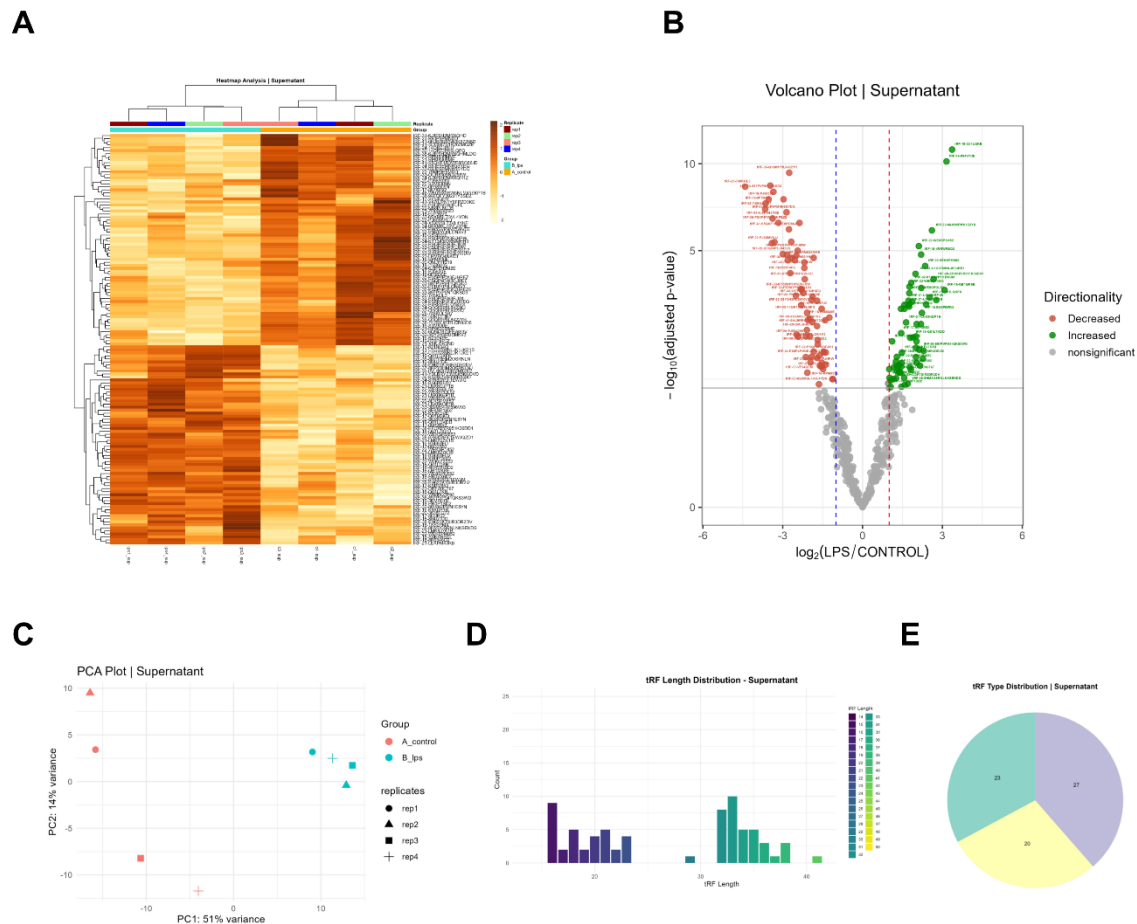


Figure 15. The differential expression profiles of tRFs between LPS and control groups obtained from supernatant samples. **(A)** Hierarchical cluster analysis of all DETs. Colored bar shows relative expression values; Brown means higher relative expression, and yellow means lower relative expression. The first bar above the graph demonstrates 2 groups, and the second bar above that demonstrates individual samples. **(B)** Volcano plot for all tRFs detected. Red means significantly decreased tRFs, green means

significantly increased tRFs, and gray means no significant change according to log2FC and -log(adjusted p-value). **(C)** Principal component analysis of individual samples. **(D)** Length distribution of differentially expressed tRFs. **(E)** Type distribution of differentially expressed tRFs.

Table 10. Statistically significantly increased tRFs from supernatant samples. Padj: Adjusted p-value.

tRF Name	log2FoldC hange	padj	Sequence	Type
tRF-16- Q01LQKB	3,362932	7,07E -12	GCGAGAGGTAGCGGGA	i-tRF
tRF-16- QB1MK8E	3,081282	0,000 277	GCGAAAGGTCCCCGGT	i-tRF
tRF-35- 8RPY8HM2OS RNLN	2,772005	0,000 565	TCCCCGCCTGTCACGCGGGAGACCG GGTTTCGATT	i-tRF
tRF-32- 86J8WPMN1E8 YN	2,604927	1,23E -06	TCCCACATGGTCTAGCGGTTAGGATT CCTGGT	5'-half
tRF-19- Q01LQKFK	2,522527	0,000 473	GCGAGAGGTAGCGGGATCG	i-tRF
tRF-37- 8RPY8HM2OS RNLNJ	2,266629	0,015 071	TCCCCGCCTGTCACGCGGGAGACCG GGTTTCGATTCC	i-tRF
tRF-18- Q01LQKDD	2,20443	0,000 238	GCGAGAGGTAGCGGGATC	i-tRF
tRF-36-	2,162991	0,020	CCCCGCCTGTCACGCGGGAGACCGG	i-tRF

KS3W2VR008R 959D		555	GGTTCGATTCC	
tRF-36- 897PVP941QK S3WD	2,124095	0,009 86	TCCTCGTTAGTATAGTGGTGAGTATCC CCGCCTGTC	5'-tRF
tRF-16- Q01L7KB	2,031459	0,002 548	GCGAGAGGTAGTGGGA	i-tRF
tRF-33- 389MV47P596V 03	2,026309	0,006 538	CCTGGTGGTCTAGTGGTTAGGATTCTG GCGCTCT	i-tRF
tRF-17- 821NQ4Q	2,00821	0,008 992	TCAGAAGGTTGCGTGTT	i-tRF
tRF-18- Q01L7KDD	1,889047	0,005 332	GCGAGAGGTAGTGGGATC	i-tRF
tRF-32- 87R8WP9I1EW JM	1,827803	0,000 162	TCCCTGGTGGTCTAGTGGCTAGGATT CGGCGC	5'-half
tRF-17- Q01M3KQ	1,814008	0,024 143	GCGAGAGGTCCCGGGTT	i-tRF
tRF-34- 87R8WP9I1EW JIQ	1,776135	0,022 012	TCCCTGGTGGTCTAGTGGCTAGGATT CGGCGCTT	5'-half
tRF-38- 8HM2OSRNLN KSEKDS	1,695779	0,042 699	TCACGCGGGAGACCGGGGTTTCGATT CCCCGACGGGGAG	i-tRF
tRF-38- 87R8WP9I1EW JQZD1	1,56441	0,033 98	TCCCTGGTGGTCTAGTGGCTAGGATT CGGCGCTTTCAC	5'-tRF
tRF-32-	1,462847	0,018	TCCCATATGGTCTAGCGGTTAGGATT	5'-half

86V8WPMN1E8 YN		299	CCTGGT	
tRF-23- DRFU8U767	1,434118	0,027 605	AATCCAGCGATCCGAGTTCAAAT	i-tRF
tRF-18- SBKKRSDL	1,391874	0,027 605	GTAAACCAGGGGTCGCGA	i-tRF
tRF-18- 1SS2PMX	1,257816	0,034 562	AGTCGGTAGAGCATCAGA	i-tRF

Table 11. Statistically significantly decreased tRFs from supernatant samples. Padj: Adjusted p-value.

tRF Name	log2FoldC hange	padj	Sequence	Type
tRF-16- 0P5830E	-3,64611	4,55E -08	ACCAGGATGGCCGAGT	5'-tRF
tRF-16- PJ5830E	-3,52909	2,61E -08	GCAGCGATGGCCGAGT	5'-tRF
tRF-22- PJ60MV9JJ	-3,40581	4,77E -06	GCAGCGGAAGCGTGCTGGGCCC	i-tRF
tRF-33- 897PVP9N1QK SD3	-3,35148	9,01E -09	TCCTCGTTAGTATAGTGGTTAGTATCCC CGCCT	5'-half
tRF-23- LPMBQ4NKDJ	-3,30769	4,29E -06	CGCAGCGGAAGCGTGCTGGGCCC	i-tRF
tRF-36- PS5P4PW3FJ HPEZE	-3,16325	5,2E- 07	GCCCGGATAGCTCAGTCGGTAGAGCAT CAGACTTTT	5'-tRF
tRF-32-	-2,99684	1,37E	GCGCCGCTGGTGCTAGTGGTATCATGCA	5'-half

Q1Q89P9L842 2Q		-05	AGATT	
tRF-21- RPM8309ME	-2,96987	2,61E -08	GGTAGCGTGGCCGAGTGGTCT	5'-tRF
tRF-33- R9JP9PMNH5 SYDQ	-2,85744	1,45E -07	GGTTCCATAGTGTAGCGGTTATCACGT CTGCTT	5'-half
tRF-35- Q1Q89P9L842 2YR	-2,75655	4,64E -10	GCGCCGCTGGTGTAGTGGTATCATGCA AGATTCCC	5'-tRF
tRF-16- R29P4PE	-2,71882	7,46E -05	GGGGATGTAGCTCAGT	5'-tRF
tRF-34- Q1Q89P9L842 2KJ	-2,67042	1,98E -05	GCGCCGCTGGTGTAGTGGTATCATGCA AGATTCC	5'-half
tRF-20- 0P58309N	-2,50663	0,000 415	ACCAGGATGGCCGAGTGGTT	5'-tRF
tRF-41- V470JPN1Y6F PZD3KE	-2,47734	0,004 576	TAGTGGTGAGCATAGCTGCCTTCCAAG CAGTTGACCCGGGT	i-tRF
tRF-33- PSQP4PW3FJI 0W	-2,438	1E-05	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGACT	5'-half
tRF-20- PJ58309N	-2,4161	0,005 133	GCAGCGATGGCCGAGTGGTT	5'-tRF
tRF-21- RP9830MME	-2,3705	5,25E -07	GGTAGTGTGGCCGAGCGGTCT	5'-tRF
tRF-32- PNR8YO9LON	-2,31986	0,000 256	GCATTGGTGGTTCAATGGTAGAATTCTC GCCT	5'-half

4V3				
tRF-32- 3SY3H3RUSM KV3	-2,30791	0,000 794	CCTCGTTAGTATAGTGGTGAGTATCCCC GCCT	i-tRF
tRF-33- 87R8WP9N1E WJDW	-2,29512	0,000 272	TCCCTGGTGGTCTAGTGGTTAGGATTC GGCGCT	5'-half
tRF-33- PSQP4PW3FJI KW	-2,18909	4,82E -05	GCCCGGCTAGCTCAGTCGGTAGAGCAT GGGACT	5'-half
tRF-34- R9JP9PMNH5 SYHQ	-2,18462	2,96E -05	GGTTCATAGTGTAGCGGTTATCACGT CTGCTTT	5'-half
tRF-35- 87R8WP9N1E WJQ7	-2,17357	0,000 121	TCCCTGGTGGTCTAGTGGTTAGGATTC GGCGCTCT	5'-half
tRF-18- I1539U0E	-2,13568	0,014 017	ATGCCGAGGTTGTGAGTT	i-tRF
tRF-20- OMJHHS19	-2,09377	0,003 573	GAATCCAGCGATCCGAGTTC	i-tRF
tRF-35- PS5P4PW3FJ HPEZ	-2,04734	0,002 018	GCCCGGATAGCTCAGTCGGTAGAGCAT CAGACTTT	5'-half
tRF-21- ZH0SR959D	-1,9593	0,017 735	TTGCGACCCGGGTTTCGATTCC	i-tRF
tRF-34- PS5P4PW3FJ HPEQ	-1,91396	0,013 856	GCCCGGATAGCTCAGTCGGTAGAGCAT CAGACTT	5'-half
tRF-33-	-1,91303	0,000	GCCGTGATCGTATAGTGGTTAGTACTCT	5'-half

PW5SVP9N15 WV0E		565	GCGTT	
tRF-33- 897PVP941QK SD3	-1,8785	0,000 442	TCCTCGTTAGTATAGTGGTGAGTATCCC CGCCT	5'-half
tRF-21- RPM830MME	-1,83983	1,98E -05	GGTAGCGTGGCCGAGCGGTCT	5'-tRF
tRF-23- 79MP9P9MDD	-1,83666	0,010 869	GTTTCCGTAGTGTAGTGGTCATC	5'-tRF
tRF-20- S3M8309N	-1,7065	0,014 599	GTAGTCGTGGCCGAGTGGTT	5'-tRF
tRF-32- P4R8YP9LON4 V3	-1,6889	0,006 104	GCATGGGTGGTTCAGTGGTAGAATTCT CGCCT	5'-half
tRF-38- 94FQFY392J1 Y0SDZ	-1,6791	0,007 572	TGGTGAGCATAGCTGCCTTCCAAGCAG TTGACCCGGGT	i-tRF
tRF-22- NB8PLML35	-1,61566	0,012 973	CTGAATCCAGCGATCCGAGTTC	i-tRF
tRF-16- S5R830E	-1,60423	0,020 721	GTCACGGTGGCCGAGT	5'-tRF
tRF-23- R9JP9PMNDD	-1,5612	0,021 222	GGTTCCATAGTGTAGCGGTTATC	5'-tRF
tRF-19- R29P4PJZ	-1,53993	0,001 005	GGGGATGTAGCTCAGTGGT	5'-tRF
tRF-34- KY7343RX6NM HH3	-1,53269	0,021 177	CCCTGGTGGTCTAGTGGTTAGGATTCT GCGCTCT	i-tRF
tRF-21-	-1,4832	0,023	TGAATCCAGCGATCCGAGTTC	i-tRF

XEKJ5R5ND		792		
tRF-35- PSQP4PW3FJI 0E7	-1,46484	0,010 869	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGACTCT	5'-half
tRF-33- OUR8309NDJ5 8W	-1,4605	0,011 306	GACGAGGTGGCCGAGTGGTTAAGGCG ATGGACT	5'-half
tRF-29- PSQP4PW3FJ FL	-1,40609	0,002 047	GCCCGGCTAGCTCAGTCGGTAGAGCAT GA	5'-tRF
tRF-32- PSQP4PW3FJI 01	-1,38081	0,011 306	GCCCGGCTAGCTCAGTCGGTAGAGCAT GAGAC	5'-half
tRF-33- PS5P4PW3FJ HPW	-1,38057	0,019 565	GCCCGGATAGCTCAGTCGGTAGAGCAT CAGACT	5'-half
tRF-16- S3M830E	-1,24782	0,001 735	GTAGTCGTGGCCGAGT	5'-tRF
tRF-16- RPM830E	-1,11279	0,035 745	GGTAGCGTGGCCGAGT	5'-tRF

4.5. Effect of Silencing tRF-33-87R8WP9N1EWJDW on Microglia

siRNA transfection was utilized to N9 microglia cells in order to knock down the tRF-33-87R8WP9N1EWJDW to evaluate the impact on pro-inflammatory markers on both protein and mRNA level. Firstly, to evaluate the effect of the tRF-33-87R8WP9N1EWJDWsiRNA knock down, tRF-33-87R8WP9N1EWJDWexpression was investigated with RT-qPCR method. **Figure 15A** indicates that LPS application increased tRF-33-87R8WP9N1EWJDWlevels and siRNA transfection decreased that. Additionally, only siRNA and negative control (NC) groups were similar to control group.

Next, it has been observed that LPS introduction statistical significantly increased IL1beta (**Figure 15B**), IL6 (**Figure 15C**), and TNF (**Figure 15D**) mRNA levels compared to their respective control groups. Then, all these three mRNA levels are downregulated in siRNA + LPS group compared to only LPS group, all changes were statistically significant (Figure 15A-D). Moreover, LPS introduction statistical significantly increased IL6 (Figure 16A), and TNF (Figure 16B) secreted protein levels compared to their respective control groups. Then, all these three mRNA levels are downregulated in siRNA + LPS group compared to only LPS group, all changes were statistically significant (Figure 16 A - B).

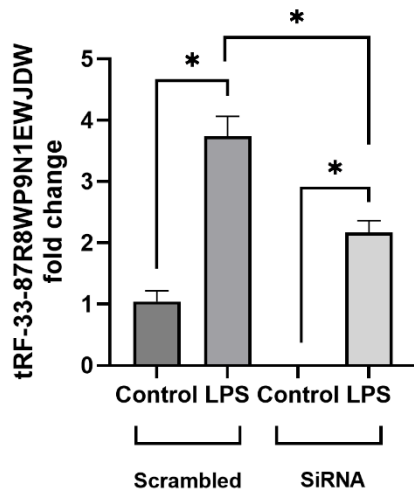
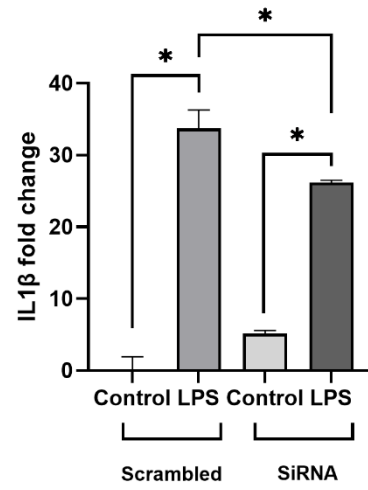
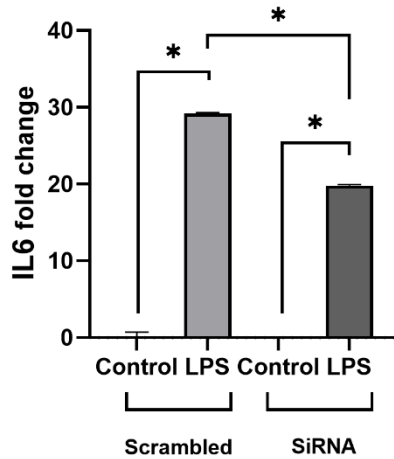
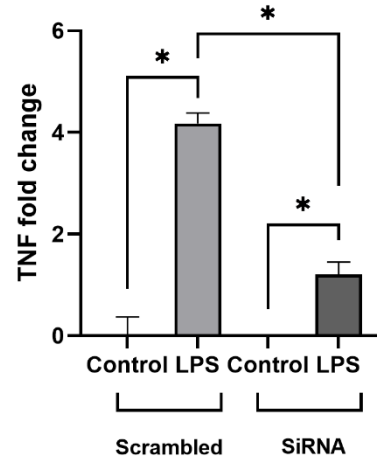
A**B****C****D**

Figure 16. mRNA levels of pro-inflammatory markers and tRF-33-87R8WP9N1EWJDW were decreased with the siRNA addition after the LPS treatment of N9 microglial cells. Measurement of relative mRNA levels of (A) tRF-33-87R8WP9N1EWJDW, (B) IL1 β , (C) IL6, and (D) TNF levels. Data are given as mean $\pm$ standard error of the mean. * $p < 0.05$.

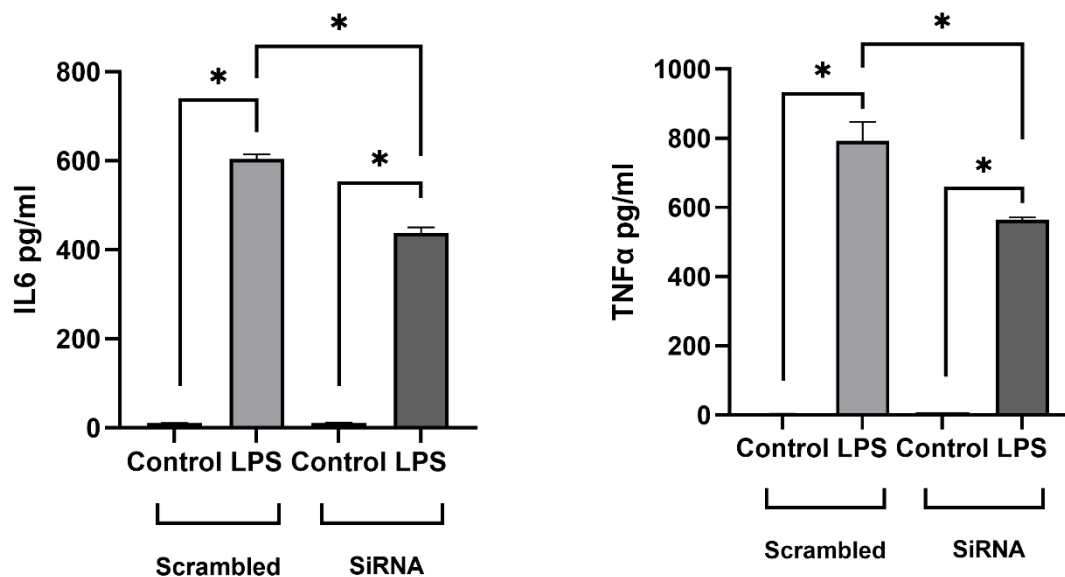


Figure 17. Protein levels of pro-inflammatory markers were decreased with the siRNA addition after the LPS treatment of N9 microglial cells. Measurement of secreted protein levels of (A) IL6, and (B) TNF levels. Data are given as mean $\pm$ standard error of the mean. * $p < 0.05$.

4.6. KEGG and GO Analysis

4.6.1. Lysate

KEGG pathway and GO analysis of top 10 tRFs emerged from lysate samples, only top 20 terms were included (**Figure 17**). 'Metabolic pathways' is the most gene count having term with 169 counts (**Figure 17A**); this may indicate the involvement of tRFs in regulation of metabolic processes. Further, 'Lysine degradation' and 'Glycine, serine and threonine metabolism' terms may indicate that these tRFs are related to tRNAs. More specifically, 'Neurotrophin signaling pathway', 'Axon guidance', and 'Apoptosis' terms are directly related to neurons and neuronal health, thus pointing out the involvement of these tRFs. **Figure 17B-D** shows GO terms for Biological Process (BP), Molecular Function (MF), and Cell Compartment (CC). In BP, 'Nervous system development' and 'Positive regulation of neuron projection development' terms were detected related to

neuron growth and development which indicates possible roles of tRFs **Figure 17B**. Additionally, 'Cell adhesion', 'Cell migration', 'Protein phosphorylation', 'Cell differentiation', 'ECM-receptor interaction', and 'Intracellular signal transduction' terms were also enriched.

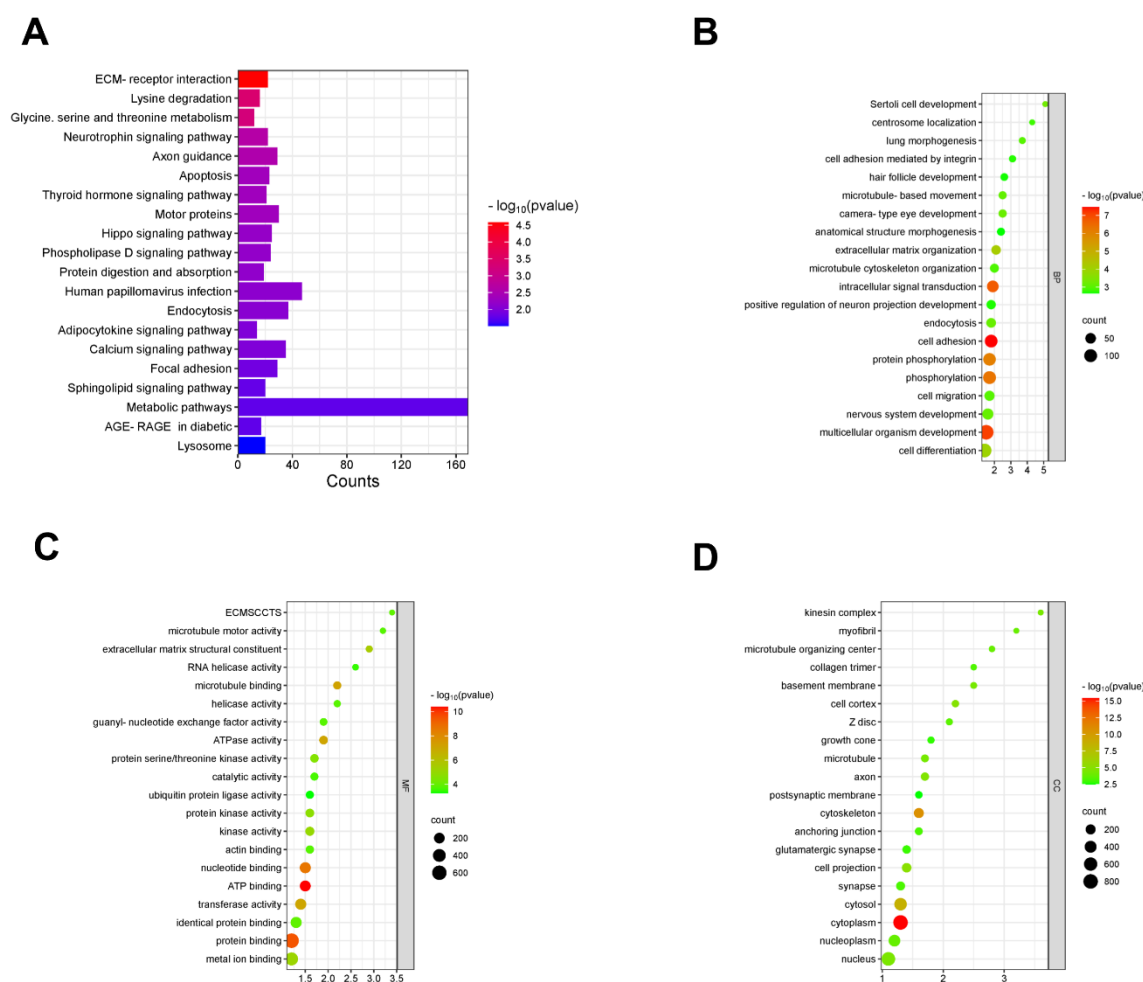


Figure 18. KEGG and GO analysis of lysate samples. **(A)** KEGG pathway analysis of lysate samples. BP **(B)**, MF **(C)**, and CC **(D)** terms of GO term analysis of lysate samples. Terms having $p < 0.05$ were selected. Only 10 tRFs (increased and decreased combined) were shown and only top 20 terms were included. KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: Biological process; MF: Molecular function; CC: Cell compartment; FDR: False Discovery Rate; GO: Gene Ontology.

4.6.2. Extracellular Vesicles

KEGG pathway and GO analysis of top 10 tRFs emerged from EV samples, only top 20 terms were included (**Figure 18**). 'Metabolic pathways' is the most gene count having term with 180 counts (**Figure 18A**); this may indicate the involvement of tRFs in regulation of metabolic processes. Further, 'Lysine degradation' and 'Glycine, serine and threonine metabolism' terms may indicate that these tRFs are related to tRNAs. These are exact same with the results of lysate samples. More specifically, 'Neurotrophin signaling pathway', 'Pathways of neurodegeneration - multiple diseases', and 'Apoptosis' terms are directly related to neurons and diseases. **Figure 18B-D** shows GO terms for Biological Process (BP), Molecular Function (MF), and Cell Compartment (CC). In the EV samples, 'Neuromuscular process', 'Neuromuscular process controlling balance', 'Dendrite morphogenesis', 'Axon guidance', 'Nervous system development', 'Dendrite morphogenesis', and 'Synapse organization' terms were detected related to neurons and diseases for BP (**Figure 18B**). Three different neuromuscular related terms draw the attention from these results. Further, other crucial terms like 'Actin cytoskeleton organization', 'Intracellular signal transduction', 'Extracellular matrix organization', 'Cell adhesion', and 'Negative regulation of MAPK cascade' were enriched.

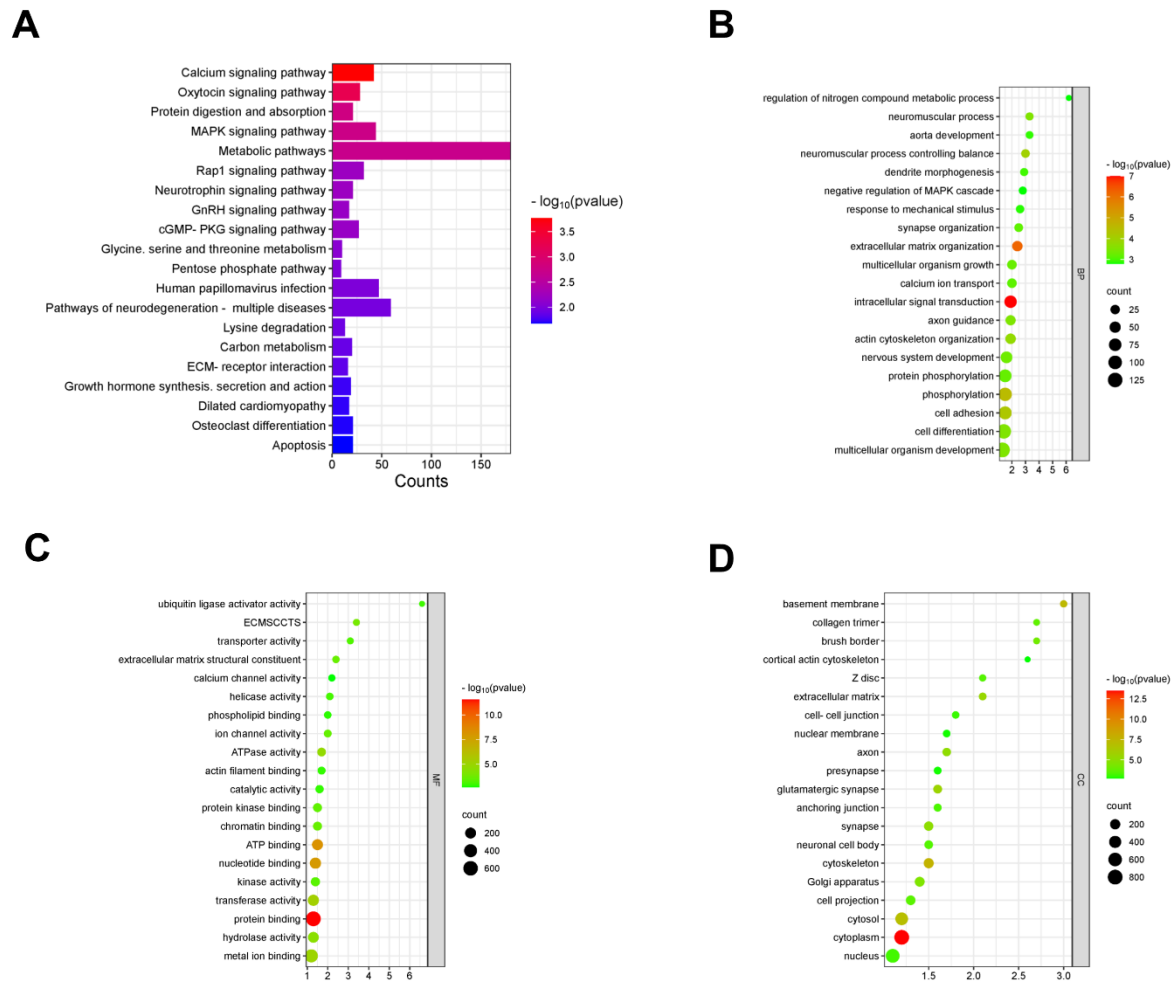


Figure 19. KEGG and GO analysis of EV samples. **(A)** KEGG pathway analysis of EV samples. BP **(B)**, MF **(C)**, and CC **(D)** terms of GO term analysis of EV samples. Terms having $p < 0.05$ were selected. Only 10 tRFs (increased and decreased combined) were shown and only top 20 terms were included. KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: Biological process; MF: Molecular function; CC: Cell compartment; FDR: False Discovery Rate; GO: Gene Ontology.

4.6.3. Supernatant

KEGG pathway and GO analysis of top 10 tRFs emerged from supernatant samples, only top 20 terms were included (Figure 19). 'Autophagy' is the most gene count having

term with 30 counts (**Figure 19A**); this may indicate the involvement of tRFs in regulation of autophagy. No directly tRNA related terms were enriched in supernatant group as in other two groups. More particular, the only GO term that is specifically related to neurons is 'Serotonergic synapse'. Additionally, 'mTOR signaling pathway', 'Fc epsilon RI signaling pathway', 'TNF signaling pathway', 'TGF-beta signaling pathway', 'AMPK signaling pathway', 'Autophagy', and 'FoxO signaling pathway' pathways are enriched in KEGG (**Figure 19A**). These may point out crucial pathways for involvement of supernatant tRFs. **Figure 19B - D** shows GO terms for Biological Process (BP), Molecular Function (MF), and Cell Compartment (CC). In the supernatant samples, 'Brain development', and 'Positive regulation of neuron projection' terms were detected related to neurons and diseases for BP (**Figure 19B**). Further, other crucial terms like 'Positive regulation of protein kinase B signaling', 'potassium ion transmembrane transport', 'Protein phosphorylation', 'Transmembrane transport', 'transmembrane tyrosine kinase signaling pathway', and 'Ion transport' were enriched.

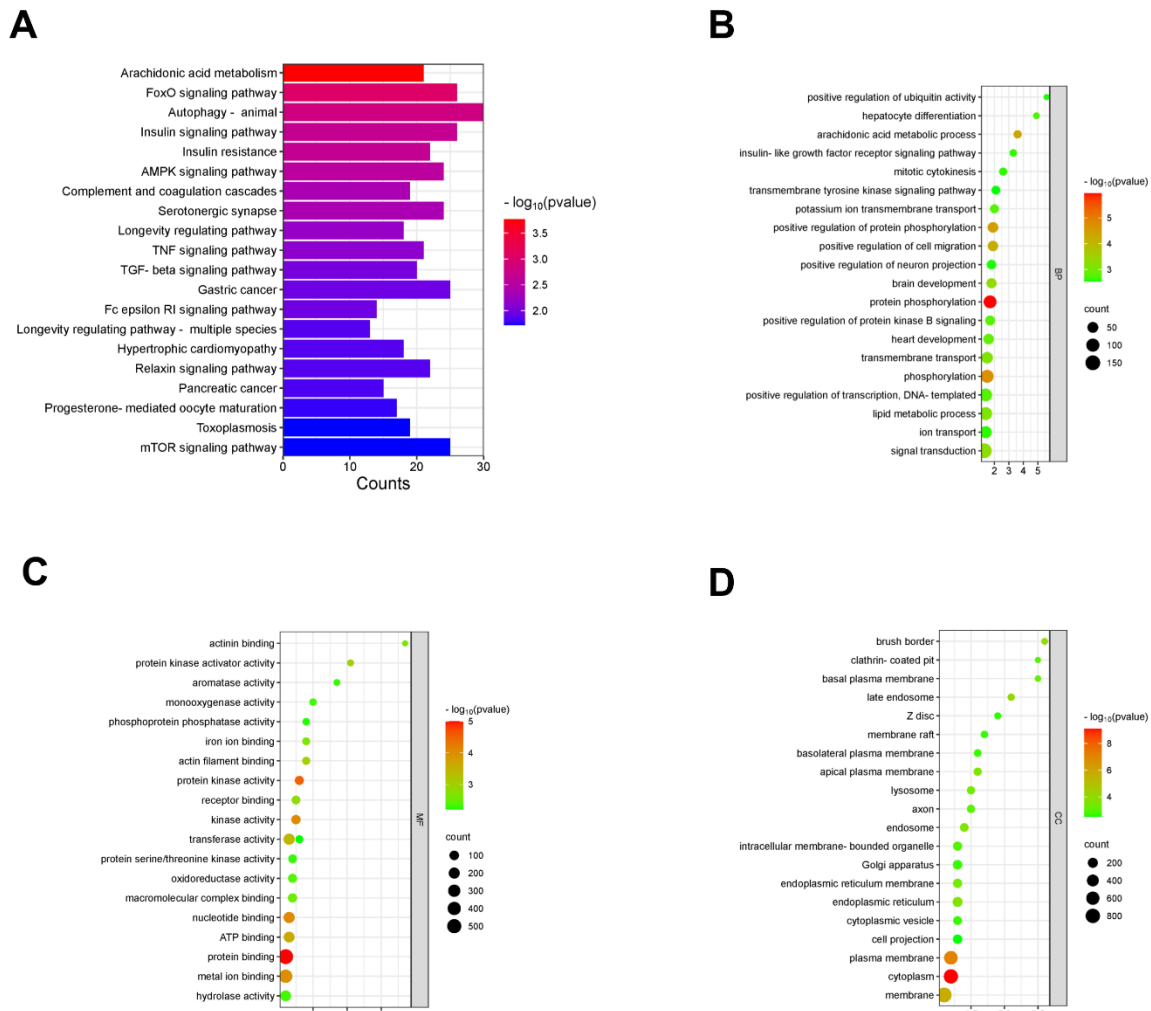


Figure 20. KEGG and GO analysis of supernatant samples. **(A)** KEGG pathway analysis of supernatant samples. BP **(B)**, MF **(C)**, and CC **(D)** terms of GO term analysis of supernatant samples. Terms having $p < 0.05$ were selected. Only 10 tRFs (increased and decreased combined) were shown and only top 20 terms were included. KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: Biological process; MF: Molecular function; CC: Cell compartment; FDR: False Discovery Rate; GO: Gene Ontology; Positive regulation of ubiquitin activity: positive regulation of ubiquitin-protein transferase activity; Positive regulation of neuron projection: positive regulation of neuron projection development; Transmembrane tyrosine kinase signaling pathway: transmembrane receptor protein tyrosine kinase signaling pathway.

4.6.4. tRF-33-87R8WP9I1EWJDW

KEGG pathway and GO analysis of tRF-33-87R8WP9I1EWJDW, only top 20 terms were included (**Figure 20**). 'Metabolic pathways' is the most gene count having term with 170 counts (**Figure 20A**); this may indicate the involvement of tRFs in regulation of metabolic processes. Further, 'Purine metabolism' term may indicate that tRF-33-87R8WP9I1EWJDW may involve in purine metabolism related to tRNAs. 'Calcium signaling pathway' is indirectly related to neurons. 'Endocytosis', 'Cellular senescence', 'cAMP signaling pathway', and 'cGMP-PKG signaling pathway' are other terms enriched in KEGG. **Figure 20B-D** shows GO terms for Biological Process (BP), Molecular Function (MF), and Cell Compartment (CC). For tRF-33-87R8WP9I1EWJDW, 'Neuron differentiation', 'Neuron fate specification', 'Learning', and 'Associative learning' terms were detected related to neurons and diseases for BP (**Figure 20B**). Other crucial terms like 'extracellular matrix organization', 'organelle organization', 'protein phosphorylation', and 'intracellular signal transduction' were enriched.

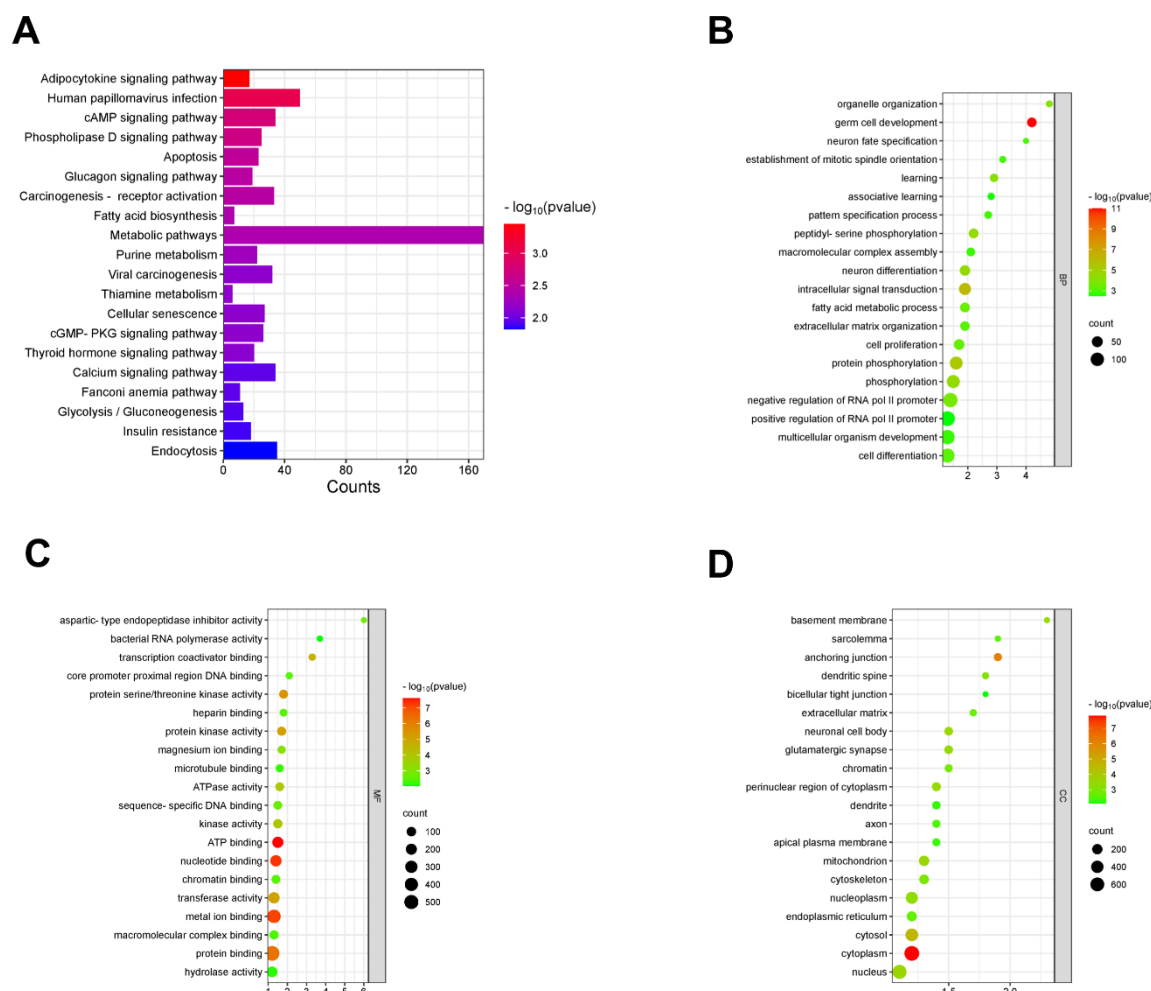


Figure 21. KEGG and GO analysis for only tRF-33-87R8WP9I1EWJDW. **(A)** KEGG pathway analysis for only tRF-33-87R8WP9I1EWJDW. BP **(B)**, MF **(C)**, and CC **(D)** terms of GO term analysis for only tRF-33-87R8WP9I1EWJDW. Terms having $p < 0.05$ were selected. KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: Biological process; MF: Molecular function; CC: Cell compartment; FDR: False Discovery Rate; GO: Gene Ontology; Positive regulation of RNA pol II promoter: positive regulation of transcription from RNA polymerase II promoter; Negative regulation of RNA pol II promoter: Negative regulation of transcription from RNA polymerase II promoter

5. DISCUSSION

Using the Lipopolysaccharide (LPS) model in vitro is a well-established model in research to simulate inflammatory responses and study various diseases and conditions. LPS, a component of the cell wall of Gram-negative bacteria, is known for inducing robust inflammatory reactions (G. Li et al., 2019). By utilizing LPS in in vitro models, researchers can create controlled environments to investigate the effects of inflammation on different cell types and tissues without the complexity of in vivo systems. This method allows for a focused examination of cellular responses to inflammatory stimuli and provides insights into the underlying mechanisms of diseases such as arthritis, sepsis, acute lung injury, and neurodegenerative disorders like Parkinson's disease (Agorreta, Zulueta, Montuenga, & Garayoa, 2005; Jemmett et al., 2008; Tao et al., 2016).

One significant advantage of using the LPS model in vitro is the ability to establish well-defined experimental conditions that can be easily replicated and manipulated. Studies have shown that in vitro models using LPS stimulation of cells, such as chondrocytes, macrophages, and adipocytes, can effectively mimic the inflammatory processes observed in vivo (Ai et al., 2021; Pizzicannella et al., 2021; Z. Xie, Guo, & Liu, 2018). These models provide a platform for testing the efficacy of anti-inflammatory drugs, investigating signaling pathways involved in inflammation, and exploring potential therapeutic interventions for various inflammatory conditions (X. P. Li et al., 2021; Peng, Li, Lu, & Hu, 2020). Moreover, in vitro models utilizing LPS stimulation have been instrumental in elucidating the role of specific pathways in inflammatory responses. For instance, studies have demonstrated that LPS-induced inflammation can activate signaling pathways such as NF- κ B, mTOR, and TLR-4, leading to the production of pro-inflammatory cytokines and the modulation of gene expression (B. Huang et al., 2017; Kunzmann, Hutten, Ottensmeier, Kramer, & Fehrholz, 2022; Yang et al., 2022). By dissecting these molecular mechanisms in cell culture systems, researchers can uncover novel targets for drug development and gain a deeper understanding of the pathophysiology of inflammatory diseases. Furthermore, the use of LPS in in vitro models has enabled researchers to study the crosstalk between different cellular

components in the context of inflammation. Co-culture systems involving macrophages, neurons, and epithelial cells have been employed to investigate the interplay between immune responses, neuronal damage, and barrier integrity in various disease models (Liu & Bing, 2011; Pizzicannella et al., 2018; R. Zhou et al., 2024). These complex in vitro setups provide a more holistic view of the inflammatory process and allow for the exploration of multi-cellular interactions that contribute to disease progression. Additionally, in vitro models utilizing LPS stimulation have been pivotal in evaluating the efficacy of potential therapeutic agents in mitigating inflammatory responses. By exposing cells to LPS and then treating them with candidate compounds, researchers can assess the anti-inflammatory properties of drugs, natural compounds, or genetic interventions (Agorreta, Garayoa, Montuenga, & Zulueta, 2003; Cho, Shanbhag, Hong, Baran, & Goldring, 2002; Kotsuka et al., 2022). These studies not only validate the effectiveness of novel interventions but also offer valuable insights into the mechanisms underlying their anti-inflammatory effects, paving the way for the development of new treatment strategies.

The utilization of the LPS model in vitro has significantly advanced inflammation research by providing a versatile and controlled platform to study inflammatory processes, investigate disease mechanisms, and test potential therapeutics. Through the establishment of cell culture systems that mimic in vivo inflammatory conditions, researchers have made significant strides in understanding the complexities of inflammatory diseases and identifying novel targets for intervention. The wealth of knowledge generated from in vitro studies using the LPS model continues to drive advancements in the development of anti-inflammatory therapies and holds promise for improving the management of various inflammatory disorders.

Inflammation and microglia are intricately linked in various physiological and pathological processes within the CNS. Microglia, as the resident immune cells of the brain, play a pivotal role in the regulation of inflammation and immune responses (Ponomarev, Veremeyko, & Weiner, 2013). Studies have shown that in conditions of systemic inflammation, such as in lupus erythematosus, microglia can migrate to the cerebral

vasculature in a CCR5-dependent manner, affecting blood-brain barrier permeability (Haruwaka et al., 2019). This migration of microglia highlights their dynamic response to inflammatory stimuli and their involvement in maintaining the integrity of the brain environment. Furthermore, the relationship between microglia and inflammation extends to neurodegenerative diseases like Alzheimer's and Parkinson's. In Alzheimer's disease, there is a close interplay between inflammation, particularly mediated by microglia, and the accumulation of misfolded proteins like tau and amyloid beta (Ruan, Kang, Pei, & Le, 2009; Zilka et al., 2012). Activated microglia release proinflammatory cytokines and reactive oxygen species, contributing to neuroinflammation and neuronal damage in these conditions (Qian, Flood, & Hong, 2010). Similarly, the activation of microglia and the resulting neuroinflammation are key players in the pathogenesis of the disease, leading to the degeneration of dopaminergic neurons in Parkinson's disease (He, Wang, Yuan, & Wang, 2017; Qian et al., 2010). Moreover, microglia become activated and release both pro- and anti-inflammatory mediators, influencing the inflammatory response in the brain during ischemic stroke (Lee et al., 2014). This activation of microglia underscores their involvement in the acute phase of inflammation post-stroke and their potential as therapeutic targets to modulate the inflammatory cascade. Interestingly, the relationship between inflammation and microglia is not limited to acute conditions but also extends to chronic pain and traumatic brain injuries. Prolonged inflammation and activation of microglia have been implicated in the sensitization of the pain system, contributing to the development of chronic pain following brain injuries (Salberg, Sgro, Brady, Noel, & Mychasiuk, 2020). Additionally, in conditions like multiple sclerosis, the complex interplay between microglial inflammation and neurodegeneration in the cortical grey matter highlights the multifaceted nature of the relationship between inflammation and microglia (Yates et al., 2022). Additionally, the molecular mechanisms underlying the crosstalk between inflammation and microglia involve various signaling pathways and molecules. For instance, the activation of the $\alpha 7$ nicotinic acetylcholine receptor in microglia has been shown to regulate neuroinflammation and oxidative stress, providing neuroprotection in conditions like brain ischemia (Parada et al., 2013). Additionally, the p38 MAPK pathway has been identified as a key regulator of microglial

activation in the context of retinal inflammation, emphasizing the intricate signaling networks involved in modulating microglial responses (J. Chen et al., 2023).

In this thesis, I showed that LPS introduction increased inflammatory markers in N9 cells as shown in literature before (Eren et al., 2018). Specifically, inflammatory markers IL1beta, IL6, and TNF were shown to be increased both on protein and mRNA level. After the tRF-33-87R8WP9N1EWJDW siRNA application, they were shown to be decreased. First of all, these results indicates that LPS-induced inflammation model activation was achieved in microglia. Second, downregulating tRF-33-87R8WP9N1EWJDW could be a regulatory approach for reducing inflammation in microglia.

tRFs, a member of the non-coding RNA family, are a family of RNAs that are ubiquitously expressed and conserved across species (S. Li, Xu, & Sheng, 2018). Studies have revealed that tRNA fragments are not merely by-products of tRNA degradation but are distinct entities with specific functions (Cole et al., 2009). These fragments, generated through precise processing of mature tRNAs and play crucial roles in cell physiology and have implications for human health and disease (Anderson & Ivanov, 2014). The existence of abundant tRFs, particularly those derived from mature tRNAs, has been confirmed through bioinformatic analysis of deep sequenced RNA (Cole et al., 2009). This highlights the significance of these fragments as potential regulators in cellular processes. For many years, it was thought that the function of tRFs was limited to translation only. However, studies conducted in recent years have revealed that tRNAs have functions that regulate gene expression (Krishna, Raghavan, DasGupta, & Palakodeti, 2021).

tRFs are a relatively recent discovery in the field of RNA biology, and as such, they have not been as extensively studied as other small non-coding RNAs like miRNAs or siRNAs. Thus, the biological significance of tRFs has only recently started to become apparent and attract research interest. So, tRF to work further choice was not done purely on high expression and p-value of our RNA sequencing data. We consulted literature for a commonly studied tRFs, thus we choose tRF-33-87R8WP9N1EWJDW.

An RNA sequencing study conducted in 2020 by Jehn et al. showed that the most tRFs in brain tissue were expressed in the hippocampus region of primates and one of the most commonly expressed tRF is tRF-33-87R8WP9N1EWJDW (Jehn et al., 2020). Winek et al. demonstrated that tRF-33-87R8WP9N1EWJDW was detected in monocytes, B-, T- and NK cells group and serum (Winek et al., 2020). Dunaeva et al. showed that tRF-33-87R8WP9N1EWJDW was increased in the serum of rheumatoid arthritis patients compared to controls (Dunaeva et al., 2024). All these knowledges suggest that tRF-33-87R8WP9N1EWJDW could be potentially involved in microglial processes, thus we selected it for further investigation.

It has been shown that tRFs demonstrate their distinct expression patterns and functions in the development of various cancers (Zeng et al., 2020). These fragments have been identified as potential diagnostic and prognostic biomarkers, indicating their clinical relevance in disease management. Moreover, tRFs have been found to regulate gene expression under stressful conditions, suggesting their involvement in cellular stress responses (Huh et al., 2021). The precise processing of tRNAs to generate tRFs has been linked to their ability to modulate gene expression and impact cellular functions (Zhu et al., 2021). In addition to their roles in cancer and stress responses, tRFs have been implicated in various other physiological processes. For instance, these fragments have been shown to inhibit angiogenesis in the context of tissue ischemia, shedding light on their involvement in vascular biology (Q. Li et al., 2016). Furthermore, tRFs have been associated with promoting vascular smooth muscle cell proliferation, indicating their regulatory functions in cell growth and tissue homeostasis (Zhu et al., 2021). The diverse biological functions of tRFs underscore their versatility and importance in different cellular contexts.

It has been proved that tRFs involved in translational regulation and stress responses related to inflammation. tRFs have been implicated in microbial pathogenesis, where they are secreted in microbial extracellular vesicles to suppress the host immune response, facilitating chronic infections (Z. Li & Stanton, 2021). Furthermore, studies have linked tRNA fragments to stress responses, with evidence showing that stress or

infection can trigger cellular endonucleases to cleave tRNAs, producing tRFs that are involved in cellular stress responses (Manning et al., 2024). The association of tRFs with stress-induced responses and their ability to modulate translation highlight their regulatory roles in cellular adaptation to environmental cues; Huh et al demonstrated that tyrosine fragments regulate based stress responses via translation to restrain cell growth (Huh et al., 2021). Zhang et al. showed that tRF-3023b inhibited inflammation in RAW264.7 macrophage cells via Cul4a/ NF- κ B axis (Zhang et al., 2024). In an in vivo mice study, Wang et al. demonstrated that extracellular vesicle-derived tRF-22-8BWS7K092 from alveolar macrophages triggers the Hippo signaling pathway to cause ferroptosis in acute lung injury (Wang et al., 2022). In a study on human monocytes, 18 tRFs have been proved to be altered with LPS introduction (Lu et al., 2019).

In my thesis, I showed the expression profiles of tRFs in different fractions of N9 microglial cells for the first time. These fractions are lysate, supernatant and small extracellular vesicles. They all have different expression profiles from each other when compared to their corresponding control groups. These results indicates that tRFs may participate in regulation of cellular processes in different locations of cells, even between cells. The use of tRFs in research has unveiled a new layer of complexity in RNA studies. These fragments, derived from tRNAs through specific processing, exhibit diverse functions ranging from gene regulation to disease pathogenesis. Their roles in cancer, stress responses, and various physiological processes underscore their significance as key players in cellular homeostasis. The identification of tRFs as potential biomarkers and regulators in disease highlights their translational potential for diagnostic and therapeutic applications. Further exploration of tRFs will help us to fully elucidate their mechanisms of action and exploit their therapeutic potential in various pathological conditions.

6. CONCLUSION, LIMITATIONS & FUTURE DIRECTIONS

This thesis research examined the tRF expression profiles of three different fractions of murine microglial cells and function of one tRF, tRF-33-87R8WP9N1EWJDW, on neuronal health. This study highlights the significant role of tRFs in three different

fractions from LPS-polarized microglia, expanding our understanding of the molecular mechanisms underlying neuroinflammation and microglial communication. Through next-generation sequencing, we successfully characterized the tRF profiles in the EVs of LPS activated microglia, unveiling distinct patterns that are likely reflective of the microglial pro-inflammatory markers. These findings underscore the importance of tRFs as critical regulators of gene expression and cellular function, especially in the context of neuroinflammation.

In this study, only one database used for tRF detection due to another database not working anymore.

Building on the findings of this study, several future research directions can be pursued to further elucidate the roles of tRFs and EVs in neuroinflammation and microglial communication. Firstly, further investigation into the specific molecular mechanisms by which tRFs influence neuronal cells and other brain cells is necessary. Understanding the pathways and interactions involved will provide deeper insights into how tRFs modulate cellular functions and contribute to neuroinflammatory processes. Secondly, conducting *in vivo* studies using animal models of neuroinflammation and neurodegenerative diseases will help to validate the functional roles of tRFs observed *in vitro*. These studies can also explore the therapeutic potential of modulating tRFs in EVs to alleviate neuroinflammatory conditions.

7. REFERENCES

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8. APPENDIX

8.1. ARBIS CV



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Kişisel Bilgiler

İletişim Bilgileri

İletişim Adresi

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İnternet Sayfası

Öğrenim Bilgileri

26 Şubat 2019 - Şu Anda (5 yıl 5 ay)

Doktora, Doktora, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE
İZMİR ULUSLARARASI BİYOTİP VE GENOM ENSTİTÜSÜ, MOLEKÜLER BİYOLOJİ VE
GENETİK (DR) (İNGİLİZCE)

Ağırlıklı Genel Not Ortalaması: 3.5 / 4.0

28 Ocak 2016 - 06 Temmuz 2017 (1 yıl 6 ay)

Yüksek Lisans, Tezli Program, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE
SAĞLIK BİLİMLERİ ENSTİTÜSÜ, TEMEL SINIR BİLİMLER (YL) (TEZLİ)

Diploma Numarası: 201707230

Ağırlıklı Genel Not Ortalaması: 3.5 / 4.0

31 Ağustos 2009 - 20 Haziran 2014 (4 yıl 10 ay)

Lisans, Anadal/Normal Öğretim, İZMİR YÜKSEK TEKNOLOJİ ENSTİTÜSÜ, TÜRKİYE
FEN FAKÜLTESİ, MOLEKÜLER BİYOLOJİ VE GENETİK PR. (İNGİLİZCE)

Diploma Numarası: 2401

Ağırlıklı Genel Not Ortalaması: 2.71 / 4.0

Deneyim / İşyeri Bilgileri

Şubat 2019 - Şu Anda (5 yıl 6 ay) (Tam Zamanlı)

MİSAFİR ARAŞTIRMACI, İZMİR BİYOTİP VE GENOM MERKEZİ

Yabancı Dil Bilgileri

İNGİLİZCE (Okuma: İyi, Yazma: İyi, Konuşma: İyi)

Bilimsel Teknolojik Faaliyet Alanları

Bilimsel Teknolojik Faaliyet Alanı Bilgileri

Sağlık Bilimleri -- Tıp -- Dahili Tıp Bilimleri -- Nöroloji

Temel Bilimler -- Yaşam Bilimleri -- Nörobiyoloji -- Diğer

Temel Bilimler -- Yaşam Bilimleri -- Moleküler Biyoloji ve Genetik -- Diğer

Anahtar Kelimeler

Mikroglia

Yeni Nesil RNA Dizileme

Sinirbilim

RNA Ekspresyon Profili

Nöron

Ar-Ge Yetkinlik

Makaleler

B. C. DEMİRDOĞEN, V. M. COSTA, S. KALYONCU, B. İ. ARIÖZ, I. P. TROUGAKOS, M. MICKAEL, M. SANTOS, T. W. KENSLER, A. CUADRADO & B. İ. ARIÖZ et al., Biomarkers of NRF2 signalling: Current status and future challenges, REDOX BIOLOGY, 2024, 2213-2317, 72, 9.

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Bildiriler

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B. İ. ARIÖZ, Dimethyl Fumarate ameliorates NLRP3 inflammasome activation in murine microglia, Poster Sunumu, The XIV European Meeting on Glial Cells in Health and Disease, 08 Temmuz 2019, 10 Temmuz 2019.

Ödüller

Diğer, Ulusal, 3. En iyi poster, Poster Ödülü, Ödül Alınan Kurum: KOÇ ÜNİVERSİTESİ, TÜRKİYE, 29 Şubat 2020.

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Ulusal, 100/2000 Doktora Bursu, Doktora Bursu, Ödül Alınan Kurum: yok (Sistemde kayıtlı olmayan kuruluş), TÜRKİYE, 25 Şubat 2019.

TÜBİTAK Burs ve Destekleri

Proje Bilgileri

23AG012, Nöron Hasarı İlişkili Hastalıkların Tanı, İzlem Ve Tedavisine Yönelik Biyobelirteç Ve İleri Teknolojik Uyarı Sistemlerinin Geliştirilmesi, Burslu, Yürürlükte, KAMAG - Kamu Araştırmaları Destek Grubu, 1004 - Mükemmeliyet Merkezi Destek Programı, ARDEB, Proje Katılma/Ayrılma Tarihleri: 01.11.2023 - Devam ediyor, Proje Başlangıç/Bitiş Tarihleri: 01.05.2023 - 01.05.2027.

122Z019, Aktive Mikroglialdan Salınan Trna Parçacıklarının Mikroglia-Nöron Etkileşimindeki Rolü, Burslu, Yürürlükte, KBAG - Kimya Biyoloji Araştırma Destek Grubu, 1001 - Araştırma, ARDEB, Proje Katılma/Ayrılma Tarihleri: 01.12.2022 - 31.10.2023, Proje Başlangıç/Bitiş Tarihleri: 01.05.2022 - 01.05.2024.

219S939, Mikrogliyal NLRP3 İnflamazom Aktivasyon Regülasyonunda Uzun-Kodlama Yapmayan RNA NEAT1'in Rolü, Burslu, Sonuçlandı, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, 1001 - Araştırma, ARDEB, Proje Katılma/Ayrıma Tarihleri: 03.03.2021 - 01.12.2022, Proje Başlangıç/Bitiş Tarihleri: 01.07.2020 - 01.12.2022.

218S527, Polarize Mikrogliya Kökenli Ekzozomal mikroRNA'ların Nöral Kök Hücre Farklılaşması Üzerine Etkilerinin Değerlendirilmesi, Burslu, Sonuçlandı, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, 1001 - Araştırma, ARDEB, Proje Katılma/Ayrıma Tarihleri: 17.01.2020 - 19.02.2021, Proje Başlangıç/Bitiş Tarihleri: 15.06.2019 - 15.12.2022.

214S171, Santral Sinir Sistemi Hücrelerinde İnflamazom Aktivasyonuna ve İnflamazom Aktivasyonu İle İndüklenen Hücre Ölümüne Sülfurafan'ın Etkisi, Burslu, Sonuçlandı, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, 1001 - Araştırma, ARDEB, Proje Katılma/Ayrıma Tarihleri: 01.06.2016 - Devam ediyor, Proje Başlangıç/Bitiş Tarihleri: 15.04.2015 - 15.04.2017.

BİDEB Destekleri

BURAK İBRAHİM ARIÖZ, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2250-Lisansüstü Bursları Performans Programı, Destek Sona Erdi, 2022 - 1.

BURAK İBRAHİM ARIÖZ, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2250-Lisansüstü Bursları Performans Programı, Destek Sona Erdi, 2023 - 1.

BURAK İBRAHİM ARIÖZ, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2250-Lisansüstü Bursları Performans Programı, Destek Sona Erdi, 2023 - 2.

BURAK İBRAHİM ARIÖZ, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2250-Lisansüstü Bursları Performans Programı, Destek Sona Erdi, 2024 - 1.

Panelistlik/İzleyicilik/Raportörlük Sayısı

Görev	ARDEB	BİDEB	BİLİM TOPLUM	ÜİDB	TEYDEB	Toplam
Hakemlik/Panelistlik/Dış Danışmanlık Sayısı	0	0	0	0	0	0
Moderatorluk Sayısı	0	0	0	0	0	0
İzleyicilik/Danışmanlık Sayısı	0	0	0	0	0	0
Raportörlük Sayısı	0	0	0	0	0	0
Katılmadığı Panelistlik/Dış Danışmanlık Sayısı	0	0	0	0	0	0

8.2. PUBLICATION LIST



Dimethyl Fumarate Alleviates NLRP3 Inflammasome Activation in Microglia and Sickness Behavior in LPS-Challenged Mice

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NLRP3 inflammasome activation contributes to several pathogenic conditions, including lipopolysaccharide (LPS)-induced sickness behavior characterized by reduced mobility and depressive behaviors. Dimethyl fumarate (DMF) is an immunomodulatory and anti-oxidative molecule commonly used for the symptomatic treatment of multiple sclerosis and psoriasis. In this study, we investigated the potential use of DMF against microglial NLRP3 inflammasome activation both *in vitro* and *in vivo*. For *in vitro* studies, LPS- and ATP-stimulated N9 microglial cells were used to induce NLRP3 inflammasome activation. DMF's effects on inflammasome markers, pyroptotic cell death, ROS formation, and Nrf2/NF- κ B pathways were assessed. For *in vivo* studies, 12–14 weeks-old male BALB/c mice were treated with LPS, DMF + LPS and ML385 + DMF + LPS. Behavioral tests including open field, forced swim test, and tail suspension test were carried out to see changes in lipopolysaccharide-induced sickness behavior. Furthermore, NLRP3 and Caspase-1 expression in isolated microglia were determined by immunostaining. Here we demonstrated that DMF ameliorated LPS and ATP-induced NLRP3 inflammasome activation by reducing IL-1 β , IL-18, caspase-1, and NLRP3 levels, reactive oxygen species formation and damage, and inhibiting pyroptotic cell death in N9 murine microglia via Nrf2/NF- κ B pathways. DMF also improved LPS-induced sickness behavior in male mice and decreased caspase-1/NLRP3 levels via Nrf2 activation. Additionally, we showed that DMF pretreatment decreased miR-146a and miR-155 both *in vivo* and *in vitro*. Our results proved the effectiveness of DMF on the amelioration of microglial NLRP3 inflammasome activation. We anticipate that this study will provide the foundation consideration for further studies aiming to suppress NLRP3 inflammasome activation associated with in many diseases and a better understanding of its underlying mechanisms.

Keywords: dimethyl fumarate (DMF), microglia, NLRP3 inflammasome, lipopolysaccharide (LPS), sickness behaviors, pyroptosis



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Research article

Proteome profiling of neuron-derived exosomes in Alzheimer's disease reveals hemoglobin as a potential biomarker

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ABSTRACT

Alzheimer's disease is a chronic and progressive neurodegenerative disorder, which is the most common cause of dementia worldwide. Although amyloid plaques and neurofibrillary tangles are identified as the hallmarks of the disease, the only valid diagnostic method yet is post-mortem imaging of these molecules in brain sections. Exosome is a type of extracellular vesicles secreted into extracellular space and plays fundamental roles in healthy and pathological conditions, including cell-to-cell communication. In this study, we aimed to investigate the proteomic contents of neuron-derived exosomes (NDEs) from AD patients and healthy controls (HCs) to identify a possible marker for AD diagnosis. We identified alpha-globin, beta-globin, and delta-globin increase in neuron-derived exosomes of AD patients compared to HCs with LC-MS/MS proteomics analysis. Then, we confirmed the high hemoglobin (Hb) level in NDEs of AD patients with ELISA. We found the area under the curve of hemoglobin level as 0.6913 with ROC analysis. Cargo proteins of NDEs may be useful diagnostic biomarker for AD.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder with cognitive decline and dementia [1]. The most prominent pathological hallmark of AD is the deposition of amyloid plaque and tau hyperphosphorylation, which can be definitely analyzed by post mortem observations [2]. Pathological hallmarks of AD start decades before clinical symptoms arise; therefore, the clinical use of early biomarkers is urgently required [3]. Cerebrospinal fluid (CSF) and blood biomarkers are progressively analyzed for AD diagnosis. However, they may not reflect the progression during the pre-symptomatic initial phases of AD pathogenesis [1]. Exosomes are the member of the extracellular vesicle family, which are formed through the endosomal route by inward

budding of late endosomes to form multivesicular bodies (MVBs) and the fusion of MVBs with the cell plasma membrane [4,5]. They are released from almost all types of cells, including neurons, and found in bodily fluids, such as plasma, serum, urine, breast milk, and cerebrospinal fluid [6–11]. Exosomes mediate the cell-to-cell communication process by their cargo, which may contain proteins, nucleic acids, (microRNA, long non-coding RNA, DNA), lipids, and sugars [12]. The content of the cargo reflects the state of the donor cell and affect the phenotype and functions of the recipient cells [13]. Thereby, exosomal cargo characterization and identification of protein content are promising hot topics for disease diagnosis and drug research [14].

In the last decade, analysis of cell type's specific exosomes attracted biomarker researchers for AD. Neuronal exosomes, which may reflect

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Targeting NLRP3 Inflammasome With Nrf2 Inducers in Central Nervous System Disorders

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The NLRP3 inflammasome is an intracellular multiprotein complex that plays an essential role in the innate immune system by identifying and eliminating a plethora of endogenous and exogenous threats to the host. Upon activation of the NLRP3 complex, pro-inflammatory cytokines are processed and released. Furthermore, activation of the NLRP3 inflammasome complex can induce pyroptotic cell death, thereby propagating the inflammatory response. The aberrant activity and detrimental effects of NLRP3 inflammasome activation have been associated with cardiovascular, neurodegenerative, metabolic, and inflammatory diseases. Therefore, clinical strategies targeting the inhibition of the self-propelled NLRP3 inflammasome activation are required. The transcription factor Nrf2 regulates cellular stress response, controlling the redox equilibrium, metabolic programming, and inflammation. The Nrf2 pathway participates in anti-oxidative, cytoprotective, and anti-inflammatory activities. This prominent regulator, through pharmacologic activation, could provide a therapeutic strategy for the diseases to the etiology and pathogenesis of which NLRP3 inflammasome contributes. In this review, current knowledge on NLRP3 inflammasome activation and Nrf2 pathways is presented; the relationship between NLRP3 inflammasome signaling and Nrf2 pathway, as well as the pre/clinical use of Nrf2 activators against NLRP3 inflammasome activation in disorders of the central nervous system, are thoroughly described. Cumulative evidence points out therapeutic use of Nrf2 activators against NLRP3 inflammasome activation or diseases that NLRP3 inflammasome contributes to would be advantageous to prevent inflammatory conditions; however, the side effects of these molecules should be kept in mind before applying them to clinical practice.

Keywords: NLRP3 inflammasome, Nrf2, inflammation, central nervous system, dimethyl fumarate, sulforaphane

Abbreviations: AD, Alzheimer's Disease; ALS, Amyotrophic Lateral Sclerosis; ARE, Antioxidant Response Element; ATP, Adenosine Triphosphate; ASC, Apoptosis-associated Speck-like Protein containing a CARD; CBP, CREB-binding protein; CNS, Central Nervous System; DAMPs, Danger-Associated Molecular Patterns; DMF, Dimethyl Fumarate; GSDMD, Gasdermin D; HAMPs, Homeostasis-Altering Molecular Processes; IL, Interleukin; KEAP1, Kelch-like ECH associated protein; LncRNA, Long Non-coding RNA; LPS, Lipopolysaccharide; miRNA, MicroRNA; MS, Multiple Sclerosis; NF- κ B, Nuclear Factor Kappa B; NLRP3, NLR Family Pyrin Domain Containing Protein 3; Nrf2, Nuclear Factor Erythroid 2-related Factor 2; PAMPs, Pathogen-associated molecular patterns; PD, Parkinson's Disease; PPI, Protein-Protein Inhibitor; PRR, Pattern Recognition Receptors; ROS, Reactive Oxygen Species; RMS, Relapsing-Remitting Multiple Sclerosis; SFN, Sulforaphane; TLR, Toll-like Receptor; TXNIP, Thioredoxin-interacting protein.



Review

The Role of Melatonin on NLRP3 Inflammasome Activation in Diseases

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Abstract: NLRP3 inflammasome is a part of the innate immune system and responsible for the rapid identification and eradication of pathogenic microbes, metabolic stress products, reactive oxygen species, and other exogenous agents. NLRP3 inflammasome is overactivated in several neurodegenerative, cardiac, pulmonary, and metabolic diseases. Therefore, suppression of inflammasome activation is of utmost clinical importance. Melatonin is a ubiquitous hormone mainly produced in the pineal gland with circadian rhythm regulatory, antioxidant, and immunomodulatory functions. Melatonin is a natural product and safer than most chemicals to use for medicinal purposes. Many in vitro and in vivo studies have proved that melatonin alleviates NLRP3 inflammasome activity via various intracellular signaling pathways. In this review, the effect of melatonin on the NLRP3 inflammasome in the context of diseases will be discussed.

Keywords: melatonin; NLRP3 inflammasome; disease

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1. Introduction

The NLRP3 inflammasome is a crucial step in innate immune responses and contributes to immune pathogenesis of several diseases including neurodegenerative, cardiac, pulmonary, gastrointestinal, and metabolic diseases. Therefore, inhibition of NLRP3 inflammasome activation is a novel therapeutic target for inflammation-related disorders. In recent years, many natural or synthetic molecules targeting NLRP3 have been evaluated for this purpose. Melatonin is one of the best candidate agents due to it being a natural and endogenous molecule. Here, we reviewed the suppressive effect of melatonin on the NLRP3 inflammasome in various in vitro and in vivo models of diseases and injuries. We performed a literature search in the National Library of Medicine's PubMed and PubMed Central. The following queries were utilized: "melatonin AND NLRP3" in PubMed and "(melatonin[Body-All Words] AND NLRP3[Body-All Words])" in PubMed Central. We identified a total of 411 (48 from PubMed and 363 from PubMed Central) articles. After excluding duplicates and inaccessible articles, and applying our inclusion criteria, 38 articles were selected for our review. In our review, the following criteria were applied for article inclusion: (1) using melatonin treatment in vivo or in vitro; (2) reporting any protective effect of melatonin against NLRP3 inflammasome (we also included an article that investigates a synthetic derivative of melatonin); (3) full-text research articles in the English language. Others were excluded unless specified otherwise.

2. Melatonin

Melatonin (*N*-acetyl-5-methoxy tryptamine) is a hormone produced from L-tryptophan via a chain of enzymatic reactions, mostly in the pineal gland [1,2]. One of the well-recognized functions of melatonin is circadian rhythm regulation. Additionally, anti-inflammatory, cytoprotective, free radical scavenging, antioxidative, anti-cancer, anti-aging,



Melatonin Alters the miRNA Transcriptome of Inflammasome Activation in Murine Microglial Cells

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Abstract

Systemic inflammation can have devastating effects on the central nervous system via its resident immune cells, the microglia. One of the primary mediators of this inflammation is inflammasomes, multiprotein complexes that trigger a release of inflammatory proteins when activated. Melatonin, a hormone with anti-inflammatory effects, is an attractive candidate for suppressing such inflammation. In this study, we have investigated how melatonin alters the microRNA (miRNA) transcriptome of microglial cells. For that purpose, we have performed RNA sequencing on a lipopolysaccharide and adenosine triphosphate (LPS + ATP) induced NLR family pyrin domain containing 3 (NLRP3) inflammasome activation model in the N9 mouse microglial cell line, with and without melatonin pre-treatment. We have identified 136 differentially expressed miRNAs in cells exposed to LPS + ATP compared to controls and 10 differentially expressed miRNAs in melatonin pre-treated cells compared to the inflammasome group. We have identified miR-155-3p as a miRNA that is upregulated with inflammasome activation and downregulated with melatonin treatment. We further confirmed this pattern of miR-155-3p expression in the brains of mice injected intraperitoneally with LPS. Moreover, an overexpression study with miRNA-155-3p mimic supported the idea that the protective effects of melatonin in NLRP3 inflammasome activation are partly associated with miRNA-155-3p inhibition.

Keywords Melatonin · Microglia · NLRP3 inflammasome · microRNA · LPS · ATP

Abbreviations

ATP	Adenosine triphosphate
ASC	Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
CNS	Central nervous system
Ctrl	Control
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GSDMD	Gasdermin D
IL	Interleukin
IP	Intraperitoneal
KEGG	Kyoto encyclopedia of genes and genomes
LA	LPS and ATP
LPS	Lipopolysaccharide
miRNA	MicroRNA
MLA	Melatonin and LPS and ATP
MT	Melatonin
NLRP3	NLR family pyrin domain containing protein 3
PBS	Phosphate-buffered saline
qPCR	Quantitative real-time PCR
TLR	Toll-like receptor

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Introduction

Microglia are the primary immune cells in the central nervous system (CNS), responsible for or auxiliary to the inflammatory processes that protect, regulate, and occasionally damage the neuronal milieu [1]. Inflammation in the brain involves a significant degree of inflammasome



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Biomarkers of NRF2 signalling: Current status and future challenges

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ABSTRACT

The cytoprotective transcription factor NRF2 regulates the expression of several hundred genes in mammalian cells and is a promising therapeutic target in a number of diseases associated with oxidative stress and inflammation. Hence, an ability to monitor basal and inducible NRF2 signalling is vital for mechanistic understanding in translational studies. Due to some caveats related to the direct measurement of NRF2 levels, the modulation of NRF2 activity is typically determined by measuring changes in the expression of one or more of its target genes and/or the associated protein products. However, there is a lack of consensus regarding the most relevant set of these genes/proteins that best represents NRF2 activity across cell types and species. We present the findings of a comprehensive literature search that according to stringent criteria identifies GCLC, GCLM, HMOX1, NQO1, SRXN1 and TXNRD1 as a robust panel of markers that are directly regulated by NRF2 in multiple cell and tissue types. We assess the relevance of these markers in clinically accessible biofluids and highlight future challenges in the development and use of NRF2 biomarkers in humans.

1. Introduction

The basic region-leucine zipper (bZip) transcription factor nuclear

factor erythroid 2-like 2 (NRF2), encoded by the *NFE2L2* gene, was first described in connection with its ability to modulate xenobiotic metabolism in cells [1,2]. To date, several hundred genes have been shown to be modulated by this transcription factor. In addition to being an

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Proximity extension assay-based proteomics studies in neurodegenerative disorders and multiple sclerosis

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Abstract

Neurodegenerative diseases impact the structure and operation of the nervous system, causing progressive and irreparable harm. Efforts for distinguishing neurodegenerative diseases in their early stages are continuing. Despite several biomarkers being identified, there is always search for more accurate and abundant ones. Additionally, it can be difficult to pinpoint the precise neurodegenerative disorder affecting a patient as the symptoms of these conditions frequently overlap. Numerous studies have shown that pathological changes occur years before clinical signs appear. Therefore, it is crucial to discover blood-based biomarkers for neurodegenerative diseases for easier and earlier diagnosis. Proximity extension assay is a unique proteomics method that uses antibodies linked to oligonucleotides for quantifying proteins with real-time PCR. Proximity extension assay can identify even low-quantity proteins using a small volume of specimens with increased sensitivity compared to conventional methods. In this article, we reviewed the employment of proximity extension assay technology to detect biomarkers or protein profiles for several neurodegenerative diseases.

KEYWORDS

Alzheimer's disease, biomarker, neurodegenerative diseases, Parkinson's disease, proteomics

Abbreviations: AD, Alzheimer's disease; ADAD, Autosomal dominant Alzheimer's disease; AIDS, Acquired immunodeficiency syndrome; ALS, Amyotrophic lateral sclerosis; ANI, Asymptomatic neurocognitive impairment; APD, Atypical Parkinsonism; APOE, Apolipoprotein E; APP, Aβ, Amyloid β-protein; CBS, Corticobasal syndrome; CCL13, C-C motif chemokine 13 (monocyte chemoattractant protein 4); CCL19, C-C motif chemokine 19; CCL2, C-C motif chemokine 2 (monocyte chemoattractant protein 1); CCL28, C-C motif chemokine 28; CCL28, Mucosae-associated epithelial chemokine; CCL3, C-C motif chemokine 3 (macrophage inflammatory protein 1 alpha); CCL4, C-C motif chemokine 4 (macrophage inflammatory protein 1 beta); CCL8, C-C motif chemokine 8 (monocyte chemoattractant protein 2); CD27, Cluster of differentiation 27; CD49d, Cluster of differentiation 49d; CD69, Cluster differentiation 69; CDH3, Cadherin 3; CDH6, Cadherin 6; CHIT1, Chitinase 1; CIS, Clinically isolated syndrome; CNS, Central nervous system; CSF, Cerebrospinal fluid; CSF2RA, Colony stimulating factor 2 receptor alpha subunit; CX3CL1, C-X3-C Motif chemokine Ligand 1 (fractalkine); CXCL1, C-X-C Motif Chemokine Ligand 1 (growth-regulated alpha protein); CXCL10, C-X-C motif chemokine 10; CXCL5, C-X-C motif chemokine 5; CXCL5, C-X-C Motif Chemokine Ligand 5; CXCL6, C-X-C motif chemokine 6; CXCR3, C-X-C Motif Chemokine Receptor 3; DLB, Dementia with Lewy Bodies; DNER, Delta/Notch-like Epidermal Growth Factor-Related Receptor; ds-DNA, Double-stranded DNA; EV, Extracellular vesicle; EZR, Ezrin; FCRL2, Fc Receptor-Like 2; FGF-19, Fibroblast growth factor 19; FGF-5, Fibroblast growth factor 5; FTD, Frontotemporal dementia; FUS, Fused in sarcoma; HAD, HIV-associated dementia; HAGH, Hydroxyacylglutathione hydrolase;

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8.3. Ethics Approval



İZMİR BİYOTIP VE GENOM MERKEZİ
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KARARI

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Bilgilerinizi ve gereğini rica ederiz.

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