University of Nebraska Medical Center
BREAKTHROUGHS FOR LIFE

University of Nebraska Medical Center
DigitalCommons@UNMC

Theses & Dissertations

Graduate Studies

Fall 12-14-2018

Sequence-Specific Extracellular MicroRNAs Activate TLR7

Niming Wu
University of Nebraska Medical Center

Follow this and additional works at: https://digitalcommons.unmc.edu/etd

 Part of the Medical Immunology Commons, and the Medical Neurobiology Commons

Recommended Citation

Wu, Niming, "Sequence-Specific Extracellular MicroRNAs Activate TLR7" (2018). *Theses & Dissertations*. 327.
https://digitalcommons.unmc.edu/etd/327

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@UNMC. It has been accepted for inclusion in Theses & Dissertations by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.

SEQUENCE-SPECIFIC EXTRACELLULAR MICRORNAS ACTIVATE TLR7

By

NIMING WU

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy

Medical Sciences Interdepartmental Area Graduate Program
(Pharmacology & Experimental Neuroscience)

Under the Supervision of Professor Howard S. Fox

University of Nebraska Medical Center
Omaha, Nebraska

November, 2018

Supervisory Committee:
Howard S. Fox, M.D., Ph.D. Stephen I. Rennard, M.D.
John S. Davis, Ph.D. Eleanor G. Rogan, Ph.D. Kristina L. Bailey, M.D.

Sequence-Specific Extracellular MicroRNAs Activate TLR7

Niming Wu, Ph.D.

University of Nebraska, 2018

Advisor: Dr. Howard S. Fox, M.D., Ph.D.

Toll-like receptors (TLRs) play an important role in the innate immune system. Emerging evidence shows that TLRs, especially endosomal TLRs, can participate in CNS diseases by increasing the production of proinflammatory cytokines via recognition of microRNAs (miRNAs), however which of the miRNAs are able to activate signaling and whether specific sequence motifs are involved remains incompletely defined. Here we found that numerous miRNAs induced TNF-α production across multiple myeloid cell types, including microglia, and that this effect was abolished in cells deficient of TLR7. In particular, miR-20a-5p and miR-148b-3p preferentially stimulate cytokine secretion compared to miR-20b-5p and miR-148a-3p, respectively, despite sharing similar sequences. Further examination of closely related miRNAs that differed in their ability to activate TLR7 resulting in the identification of a motif (UGCUUAU) as well as specific nucleotides (all of the uridines but surprisingly including the cytosine as well), that were vital for secretion of cytokines through TLR7 stimulation. A minimal 10-nucleotide sequence including this motif was found to be the shortest ssRNA that was identified to signal through TLR7. A miRNA containing this motif induced multiple pro-inflammatory molecules, which required the PI3K, MAPK and NF-κB signaling pathways. Wild-

type mice that were administered miR-20a-5p (containing this motif) demonstrated increased leukocyte migration. This effect was significantly ameliorated in TLR7 knockout mice, and mice injected with miR-20b-5p (in which the motif is mutated) failed to exert this effect. Our results provide a detailed analysis of miRNAs that activate endosomal TLR7, and identify key nucleotide features of sequence motif recognized by TLR7.

LIST OF ABBREVIATIONS

miRNA micro-ribonucleic acid

TLR toll-like receptor

CNS central nervous system

TNF-α tumor necrosis factor alpha

PI3K phosphoinositide 3-kinase

MAPK mitogen-activated protein kinase

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

PAMP pathogen-associated molecule pattern

MyD88 myeloid differentiation factor 88

TRIF TIR domain-containing adaptor inducing interferon-β

IL-1β interleukin 1 beta

ssRNA single strand ribonucleic acid

EV extracellular vesicle; also known as exosome

AD alzheimer's disease

DOTAP N-[1-(2,3-Dioleoyloxy) propyl]-N, N, N-trimethylammonium chloride

IL-6 interleukin 6

ELISA enzyme-linked immunosorbent assay

WT wild-type

KO knockout

MLK-3 mixed-lineage protein kinase 3

URMC-099 3-(1H-indol-5-yl)-5-[4-[(4-methyl-1-piperazinyl) methyl] phenyl]-1H-pyrrolo[2,3-b] pyridine

BMS-345541 4(2'-aminoethyl) amino-1,8-dimethylimidazo(1,2-a) quinoxaline

PCR polymerase chain reaction

CXCL10 c-x-c motif chemokine 10

PTSG-2 prostaglandin-endoperoxide synthase 2

CCL5 chemokine (c-c motif) ligand 5

GAPDH glyceraldehyde 3-phosphate dehydrogenase

Ly-6C lymphocyte antigen 6C

Ly-6G lymphocyte antigen 6G

CD11b cluster of differentiation molecule 11b

MHC class II major histocompatibility complex class II

CD170 cluster of differentiation molecule 170

LPM large peritoneal macrophage

SPM small peritoneal macrophage

Acknowledgement

I sincerely express my deep sense of gratitude to a number of people who have been constantly supporting, encouraging and motivating me during my graduate studies. First and foremost, I am deeply indebted to Dr. Fox for accepting me to be a part of his lab. Dr. Fox was always supportive and guided me throughout this project with many scientific discussions. I have been inspired and encouraged by his strong enthusiasm in research and knowledge. I would also like to thank Dr. Rennard for his mentorship during my early graduate training. He has been a great influence in my personal and professional life, and taught me numerous qualities that are required for becoming a good physician-scientist. I am also enormously grateful to my supervisory committee, Dr. John Davis, Dr. Eleanor Rogan and Dr. Kristina Bailey, for their valuable inputs, suggestions and guidance during my graduate training here at UNMC. I would like to thank the past and present members of the Fox lab. I would like to thank Brenda Morsey who has made so many constructs in this project. I would like to thank Katy Emanuel who has done a lot of previous work for this study. Ben Lamberty has always been there to help with anything and I truly appreciate his Lebron jokes. None of the achievements would be possible without the support from other members of the Fox lab. I am indebted to Dr. Fumihiko Makino, Dr. Martin Liu and Dr. Hesham Basma for nurturing my research skills and for all the valuable suggestions and inputs during my previous studies. I would like to thank the UNMC Core Facility, Li Wu and all of the PEN department administrative staff. Finally, I would like to thank my mother and my fiancé. They are my greatest spiritual support throughout my graduate training.

Table of Contents

LIST OF FIGURES AND TABLES

Figures

Tables

Chapter 1. Introduction

1.1 Toll-like receptors, miRNAs and Extracellular Vesicles

1.1.1 Toll-like receptors

Toll-like receptors (TLRs), an important family of pattern recognition receptors (PRRs), classically recognize conserved pathogen-associated molecular patterns (PAMPs) from infectious pathogens. This recognition triggers the production of large amounts of proinflammatory cytokines (such as IL-1β and TNF-α), chemokines, and antiviral proteins through the activation of interferon regulatory factor (IRF) 3, myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor inducing interferon- β (TRIF), activator protein-1 (AP-1), and nuclear factor-kappa B (NF-κB) (1-5). This family of receptors is named after the *Toll* gene, which was first described in *Drosophila melanogaster* as being important in development, and then found to be essential in immunity (6). Since then, 13 members of the TLR family have been identified in mice and 11 in human (7). Studies on *Toll* and TLRs have led to Nobel prizes in Medicine in 1995 and 2011. TLRs are located on the cell surface, in endosomes, lysosome compartments as wells as in the endoplasmic reticulum, where they bind to their ligands. Several mechanisms are responsible for the regulation of the TLR-signaling pathways. These include conformational changes, phosphorylation, ubiquitylation, and proteasome-mediated degradation involving various regulatory molecules (2,8,9). TLRs recognize distinct PAMPs from pathogenic organisms, such PAMPs include lipids, lipopeptides, proteins, and nucleic acids. While such molecules in general are specific to non-self organisms, it has been found that self-mitochondrial DNA (which evolutionarily likely originated from a symbiotic organism) can be recognized by TLR9, which recognizes certain unmethylated CpG DNA sequences, and that certain microRNAs (miRNAs) can be recognized by TLR7 and TLR8, which recognize certain single strand RNA (ssRNA) sequences.

Since then, miRNAs have received considerable attention as a newly identified family of regulators involved in fine-tuning of the TLR-signaling pathways. While these TLRs are present in throughout mammals, in mice TLR8 is non-functional for signaling in response to ssRNA (10,11).

1.1.2 MiRNAs – biogenesis and maturation

MiRNAs are small noncoding ssRNAs (about 20 - 22 nucleotides in length) that are largely studied for their binding to the 3-untranslated regions (UTRs) of target messenger RNAs (mRNAs). This binding regulates their expression post-transcriptionally, causing mRNA degradation or protein translation repression (12,13). Canonical miRNAs are derived from endogenous precursors called primary miRNAs (pri-miRNAs), which are transcribed by RNA Polymerase II and cleaved by RNase III Drosha/DGCR8 complex. The pri-miRNAs constitute miRNA precursor (pre-miRNA) and is transported into the cellular cytoplasm. In the cytoplasm, cleavage of the pre-miRNAs takes place once they have been loaded onto the Dicer-TRBP (TAR-RNA binding protein) complex, which removes the loop from the pre-miRNAs to produce dsRNA complex that contains both the mature miRNA and the so-called passenger strand. Within this complex, the passenger strand is removed by DICER-TRBP complex resulting in a single stranded mature miRNA, which functions by being partially complementary to 3'-UTRs of target mRNA (14,15). Aberrant expression or function of miRNAs have been identified under various physiological and pathological conditions, e.g., in neurodegeneration and in neuroinflammation conditions (16,17).

1.1.3 Extracellular Vesicles as miRNA carriers in brain

While miRNA activities have been well characterized within their producing cells, and thus affecting the eventual translation of gene products, miRNAs can be transported out of the cell type of origin and carry their cargo to affect other cells in a paracrine or endocrine fashion.

This is best documented via Extracellular Vesicles (EVs), small vesicles (between 30 and 100 nm) released by cells with an endosome-derived membrane. EVs contain cell type-specific loads of proteins, mRNAs and miRNAs (18). EVs can be taken up by cells and release their loads, such as miRNAs, into cellular compartments (19,20). Transportation of miRNAs are thus secured in EVs while the biological function of miRNAs can be retained.

In the central nervous system (CNS), neurons, astrocytes, oligodendrocytes, microglia can release EVs into the extracellular environment (21-24); outside of the CNS a wide variety of cells can also release EVs. One of the best studied cargoes of EVs are miRNAs (18). Interestingly a novel role for EV miRNA was found in a study on cancer, where certain miRNAs released from cancer cells were found to bind to, and activate, mouse TLR7 as well as human TLR8 (25). This has been similarly found in a number of systems and our lab has found EVs from brains of monkeys with SIVE to carry miRNAs that can activate TLR7 (26). Although one other study reported that free extracellular miRNA let-7b can activate mouse TLR7 signaling in microglial cells, the mechanism by which free miRNAs can enter brain cells and reach the endosomes to induce signaling however, remains unclear (27). It is suggested that internalization of EVs by target cells must precede before binding of EVs to the cell surface. The components of EVs can thus be released into the extracellular environment. By complexing miRNAs with cationic liposomes (such as DOTAP, lipofectamine or LyoVec) and exposing the conjugates to cells, miRNAs have been found to enter cells and locate in the endosomes in close proximity to TLR7/8, thus mimicking the effects of natural EVs. This effect has been best documented in cells of the myeloid lineage. For example in macrophages, miR-21 and miR-29a (complexed with DOTAP) have been shown to signal through mouse TLR7 and human TLR8 to induce TNF-α and IL-6 production (25), whereas miR-142 and miR-146a (complexed with lipofectamine) were found to signal through mouse TLR7, leading in turn, to MIP-2, TNF-α and IL-6 production (28). Intriguingly, in

human macrophages non-miRNAs such as HIV-1 encoded ssRNA (complexed with LyoVec) have been shown to signal through TLR8, with subsequent release of TNF-α (29,30), thus likely representing the traditional function of TLRs as pathogen receptors, which may have been co-opted by miRNAs in EVs.

1.2 TLR7 signaling pathways mediated by miRNAs

TLR7 is type I glycoprotein expressed within endosomal compartments in a variety of innate immune cells. Its extracellular domain consists of a leader signal sequence, which directly interact with TLR7 ligands. Endosomal and lysosomal TLRs (TLR3, 7, 8 and 9) recognize nucleic acids and are endoplasmic reticulum (ER)-resident, and move to endosomes/lysosomes upon ligand stimulation (31). Many signaling proteins, molecules and transcription factors are also recruited during TLR7 activation. Endosomal/lysosomal TLRs respond to DNA/RNA derived from pathogens and dead cells. For example, ssRNA and dsDNA directly bind to endosomal TLR3, 7 and 9. Furthermore, TLR7 binds to miRNAs. In innate immune cells, miRNA binding to TLR7, cellular colocalization/trafficking of TLR7, and TLR7-associated downstream signaling cascade can together play critical roles and trigger the activation of TLR7. The precise mechanism of action of TLR7 is still unclear.

1.2.1 Direct binding of endosomal TLRs by miRNAs

Previous studies have shown that endosomal and lysosomal ssRNAs are ligand of TLR7 (10,11). Structural studies of TLR7 indicates that TLR7 interacts with guanosine and the triribonucleotide UUU (32). It is also hypothesized that the dimerization of the extracellular domain of TLRs is the triggering mechanisms of TLRs activation by ssRNA. TLR7 and TLR8 are located in the endosomal components of cells and recognize ssRNA, including miRNAs of certain sequences (29,30,33-35). Lehmann et al. found that let-7b can act as a potent activator of TLR7

signaling in neurons and that this activation can induce neurodegeneration (27). Previous works from our lab and others show that miR-21 can bind to TLR7 and is neurotoxic (13,25,26,36). Specific sequences, such as GU-rich elements or the number of U ribonucletoi-des, were previously described to be important for innate immune activation via ssRNAs (10,11). Fabbri et al. suggested that GU-content as well the tridimensional structure of the miRNAs may affect miRNA ability to functionally activate TLR7 (36). Lehmann et al. postulated that let-7b can act as a signaling activator of TLR7 because the let-7b sequence contains a core GU-rich motif that is also present in the HIV ssRNA40, which is a known ligand of TLR7 (27). GUUG for miR-21 and GGUU for miR-29a is essential for the miRNA-TLR7 recognition (13,25,26,36). Feng et al. found that miRNA mimics (miR-133a, -146a, and -208a) induced cy-tokine production and the effects of these miRNAs were diminished by Uridine to Adenosine mutation (28). However, it is still unknown about consensus sequences needed to activate TLR7. It is also important to note that miRNA binding to TLR7 alone cannot trigger the activa-tion of TLR7. A previous study performed by Iavarone et al. indicates that although wild-type TLR7 and TLR7rsq (mutated TLR7) bind similarly to ssRNA, mutated TLR7 is not functional and fails to activate JNK, p38 and NF-κb. Other than the ability of TLR7 to bind to its ligands such as ssRNA, features that are critically linked to downstream signal transduction and TLR7 traffick-ing are also crucial to TLR7 activation (37).

1.2.2 Cellular localization and trafficking of TLR7

Endosomal TLRs, such as TLR3, 7, 8 and 9, recognize nucleic acid and are reported to be lo-calized in the ER. Subcellular compartmentalization and cellular trafficking of these endosomal TLRs seem to be also crucial for ligand recognition and downstream signaling cascades (Fig 1.1). Upon cellular stimulation or infection, TLR7 transcription is induced in the nucleus via NF-κb and synthesized in the ER. TLR7 then exits the ER and translocate to the Golgi by uncoordinat-

ed 93 homolog B1 (UNC93B1). In the Golgi, UNC93B1 ubiquitinates TLR7 and HRS/ESCRT machinery sorts ubiquitinated TLR7 for endosomal-lysosomal- transport. Adaptor proteins such as AP-4 and AP-3 deliver TLR7 from the Golgi to endosomes and lysosomes. Once TLR7 reaches the endosomes, endosomal acidification occurs for proteolytic cleavage by proteases, including furin proprotein convertases (PC). TLR7 cleavage separates the N-terminal ectodomain from the C-terminal ectodomain, transmembrane domain, and cytosolic region. The N terminal region forms disulfide bonds with the C-terminal region and remains in the endosome for optimal signaling (31). Kensuke et al. also suggests that TLR7 in steady-state plasmacytoid Dendritic cells is localized in perinuclear lysosomes. Upon activation by ssRNA, TLR7 activates two signaling pathways. One induces pro-inflammatory cytokines. The other signal activates a small GTPase Arl8b to link TLR7-containing lysosomes with microtubules and moves them to the peripheral region (38) for secretion of IFNs. Overall, TLR trafficking as a potential mechanism linking TLR7 activation and inflammation cascade remains to be investigated.

1.2.3 MiRNA-dependent regulation of TLR7-associated signaling proteins/molecules and transcription factors

Although miRNAs can directly bind to TLR7, TLR7-associated downstream signaling cascade is also critical for activation of TLR7. Lavarone et al. reported that although wild-type TLR7 and TLR7rsq (mutated TLR7) bind similarly to ssRNA, downstream JNK, p38 and NF-kb cannot be activated by ssRNA in mutated TLR7 . Activation of TLR7 causes recruitment of many types of signaling proteins and molecules. These proteins and molecules include adaptor molecules MyD88, TRIF, TRIF-related adaptor molecule (TRAM); kinases and transcription factors such as MAP kinases, IKK1/2, NF-κB and STAT3 (Table 1.1). However, in addition to directly stimulating TLR7, many miRNAs, by their more traditional roles of interacting with specific mRNAs, have

been shown to affect TLR7 signaling. These miRNAs include several (miR-155, miR-21 and miR-29a) that are known to be ligands for TLR7 activation and downstream effects (26,27,39).

One such miRNA is miR-155, which can target several signaling molecules as shown in Table 1.1. MiR-155 can regulate TAB2 in human monocyte-derived dendrocytes (40) as well as MyD88 (41). MyD88 is an important adaptor molecule used by almost all TLRs except TLR3 to activate the transcription factor NF-kB. Similarly, miR-21 can also target MyD88 as well as IRAK1 (42). In addition, it was demonstrated that miR-155 can directly control the level of TAB2, an important signal transduction molecule and down-regulates inflammatory cytokine production in response to stimuli (40). Mi-155 also targets the Fas-associated death domain protein (FADD), IkappaB kinase epsilon (IKKepsilon), and the receptor (TNFR superfamily)-interacting serine-threonine kinase 1 (Ripk1) while enhancing TNF-alpha translation (40).

The tumor necrosis factor receptor (TNFR)-associated factor 4 (TRAF4) is a member of TRAF family proteins that act as major signal transducers of the TNF receptor and the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) superfamily. TRAF4 has been reported to be over-expressed in various human cancers. TRAF4 was upregulated when cells were transfected with inhibitor of miR-29a, implying miR-29a regulation of the TRAF4 mRNA (43).

Transcription factors, such as NF-κB, MAPK and STAT, are also activated following TLR7 activation. NF-κB is one of the most important transcription factors in TLR-dependent inflammation signaling and causes production of cytokines such as IL-6 and TNF-α. It has been shown that miR-21, which we and others have found to be an activating ligand for TLR7, can directly target mRNAs resulting in enhancement of the NF-κB pathway (44). Interestingly, it is also shown that let-7 represses the activation of NF-κB (44). MiRNAs can also target other TLR-activated transcription factors. For example, miR-20a-5p and miR-17-5p can target STAT3 in

myeloid-derived suppressor cells and alleviate the suppressive function of myeloid-derived suppressor cells (45).

In addition to miR-155 targeting the signaling molecules such as TAB2 and MyD88 as described above, it also targets transcription factors including NF-κB, MAPK and FOXP3 (40,46). Among these, FOXP3 is a transcription factor that is essential for the normal development of regulatory T cells. The relationship to miR-155 was definitely shown in knockout mice in which, in the absence of miR-155, Foxp3(+) Tregs develop but failed to maintain immune homeostasis, leading to a scurfy-like disease (46). In summary, miRNAs can directly target expression of various signaling molecules as well as transcription factors. However, it remains to be investigated whether and what signaling molecules and transcription factors are involved in TLR7-dependent signaling by miRNAs.

1.2.4 MiRNA-dependent regulation of TLR7-associated cytokines and chemokines

Activation of TLR7 signaling leads to the transcriptional activation of genes encoding for proinflammatory cytokines and chemokines. These cytokines and chemokines play important roles in eradicating pathogens and recruiting inflammatory cells to the infection site for effective host defense. Several key TLR7-associated cytokines and chemokines such as IL-1β, IFN-β, TNF-α, IL-6, CCL-5, PTSG-2 and CXCL-10 have been demonstrated to be regulated by miRNAs (Table 1.1). For example, previous studies in our lab shows that the expression of IFN-b as well as IFN-induced chemokine CXCL10 are decreased in miR-155 knockout mice after brain injury (47). Transfection experiments also show marked increases of TNF-a, IL-1b and IL-6 in macrophages transfected with miR-125b or let-7b inhibitor (48). A variety of effects have been found in vivo and in vitro regarding miRNAs and cytokines.

Cytokines or chemokines are regulated by miRNAs by several mechanisms. First, miRNAs can directly bind to the target 3'-UTR regions with subsequent regulation of mRNA. It has long been known that miRNAs play important biological roles in cells through classic post-transcriptional regulation of target mRNAs. For example, let-7b can directly regulate the expression of IFN-β by targeting the IFN-β 3'-UTR (47,48). In addition, bioinformatics analysis indicates that the 3'-UTR of the IL-6 mRNA contains a let-7-binding site (49). Let-7b inhibitor can increase the IL-6 production in macrophages (50), however, it remains to be experimentally determined whether let-7b can suppress IL-6 directly. It has been demonstrated that miR-146a is involved in negatively regulating PTGS2 expression in human gastric epithelial cells, and miR-146a can decrease PTGS2 expression by degradation of its mRNA (47). TNF mRNA contains a binding site that can be targeted by miR-125b in mouse RAW 264.7 macrophages (51).

Next, production of cytokines and chemokines can also be indirectly controlled by miRNAs. Stability of proteins is crucial for their biological functions. Increasing evidences indicate that miRNAs, together with RNA-binding proteins, regulate cytokine mRNA stability and (or) translation through the AU-rich elements (AREs) of their 3'-UTR regions. ARE-binding proteins such as tristetraprolin (TTP), AU-rich binding factor1 (AUF1) and members of the Hu protein R (HUR) family are regulated by miRNAs such as miR-146a. In addition, TNF mRNAs contain long AREs targeted by TTP, a key factor in mRNA destabilization downstream of the TLR-signaling pathway (52). These finding indicate that AREs can indirectly regulate the production of cytokines by miRNAs.

Last but not least, miRNA binding to endosomal TLR7 can trigger the inflammation cascade and thus indirectly regulate the production of cytokines and chemokines. Lehmann et al. found that let-7b can act as a potent activator of TLR7 signaling in microglial cells and triggers production of TNF-α (27). Fabbri et al. reported that miR-21, miR-29a and miR-147 induce TLR7

activation and secretion of TNF-α as well as IL-6 (25,36). The excessive production of type I IFNs is a hallmark and a main mechanism of many autoimmune diseases such as systemic lupus erythematosus. The sustained production of type I IFNs is dependent on the improper activation of plasmacytoid DCs. Savli et al. showed that synthetic miR-574 activates human plasmacytoid DCs to secrete IFN-α via TLR7 (53).

While cytokine or chemokine expression can be regulated directly by miRNAs binding to the target mRNA or indirectly through AREs/TLRs, a number of cytokines can also regulate miRNA synthesis. For instance, IL-1β and TNF-α are potent stimulators for miR-146a and miR-155 induction in a variety of cell types (54). Interestingly, it has also been shown that miR-155 is required for TNF mRNA stabilization and miR-155-deficient B cells and (or) miR-155-deficient mice fail to produce TNF-α after LPS treatment (51,55). In addition to the insights discussed above, further studies are needed to elucidate whether other TLR7-associated cytokines and (or) chemokines are subject to regulation by miRNAs.

In summary, miRNAs can have dual activities in terms of regulation of cytokines and chemokines: On the one hand, they bind to proteins and guide the silencing of target genes, and on the other hand, they act independently of regulation of expression of genes and proteins by interacting directly with endosomal TLRs such as TLR7. The interplay between the two pathways require further investigations to assess the biological and pathological significance of the dual roles of these miRNAs.

Figure 1.1

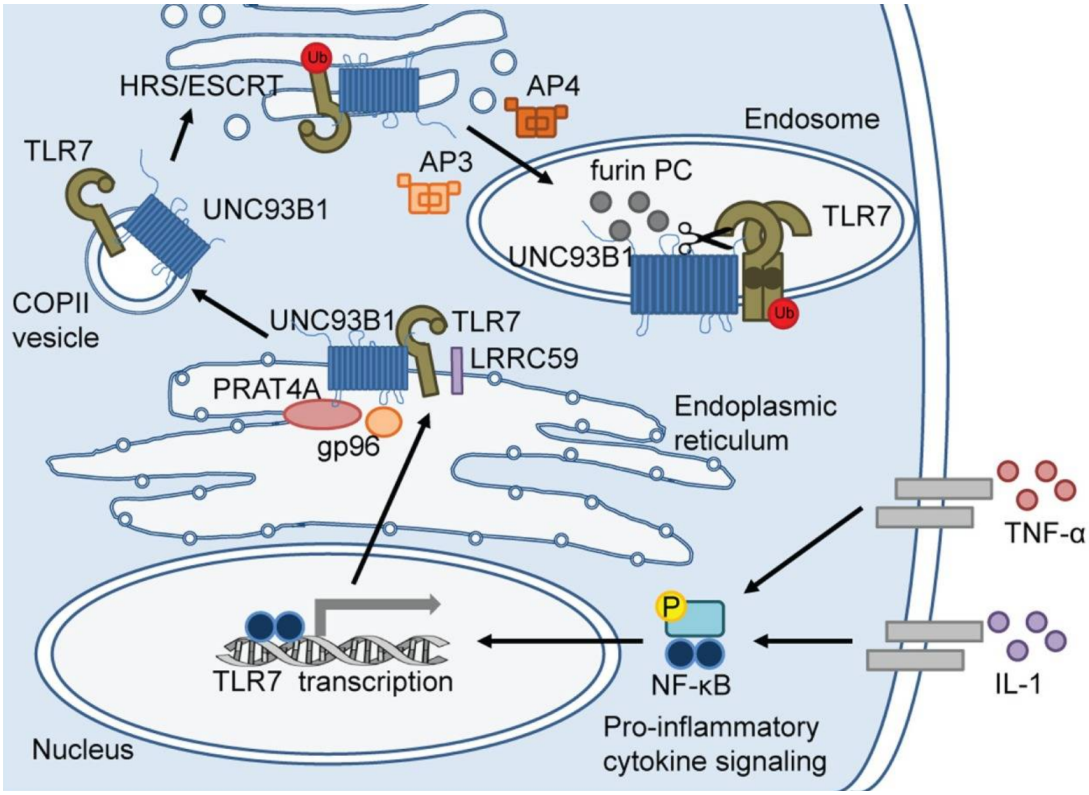

Ub
HRS/ESCRT
AP4
Endosome
TLR7
AP3
furin PC
UNC93B1
TLR7
UNC93B1
Ub
COPII
vesicle
UNC93B1
TLR7
LRRC59
PRAT4A
gp96
Endoplasmic
reticulum
TNF-α
P
TLR7 transcription
NF-κB
IL-1
Nucleus
Pro-inflammatory
cytokine signaling

Figure 1.1. TLR7 trafficking from the endoplasmic reticulum (ER) to the endosome.

In response to pro-inflammatory cytokine signaling, TLR7 transcription is induced via nuclear factor (NF)-κB. TLR7 protein is synthesized in the ER where ER-resident chaperone proteins, gp96 and protein-associated with TLR4 (PRAT4A), are required to facilitate proper folding of TLR7 before exiting the ER. Upon cellular stimulation (e.g. TLR7 ligand Imiquimod) or infection, leucine-rich repeat (LRR) containing protein 59 (LRRC59) promotes uncoordinated 93 homolog B1 (Caenorhabditis elegans) (UNC93B1)-mediated packaging of TLR7 into coat protein complex II (COPII)-coated vesicles to exit the ER and translocate to the Golgi. In the Golgi, UNC93B1 ubiquitinates TLR7 and HRS/ESCRT machinery sorts ubiquitinated TLR7 for endolysosomal transport. Adaptor protein AP-4 and AP-3 deliver TLR7 from the Golgi to endosomes. Once TLR7 reaches the endosome, endosomal acidification occurs for proteolytic cleavage by proteases, including furin proprotein convertases (PC). TLR7 cleavage separates the N-terminal ectodomain from the C-terminal ectodomain, transmembrane domain, and cytosolic region. The N terminal region forms disulfide bonds with the C-terminal region and remains in the endosome for optimal signaling (31).

Table 1.1

Targets of miRNAs in TLR7-associated signaling pathway

Signaling molecule	miRNA	Reference(s)
MyD88	miR-21, miR-155, let-7b	(41,42,56)
IRAK1	miR-21	(42)
TRAF4	miR-29a	(43)
IKK	miR-155	(40,51)
FADD	miR-155	(40,51)
TAB2	miR-155	(40,51)
RIPK1	miR-155	(40,51)

Transcription Factor	miRNA	Reference(s)
NF-κB	miR-21, let-7	(44)
STAT3	miR-20a, miR-17	(45)
MAPK	miR-155	(40)
FOXP3	miR-155	(46)

Cytokines, Chemokines	miRNA	Reference(s)
IL-1β	miR-125b, let-7b	(50)
IFN-β	let-7b	(48,57)
TNF-α	miR-125b, miR-146a, let-7b	(27,50,51)
IL-6	miR-125b, let-7b	(50)
CCL-5	miR-21, miR-200c	(58,59)
PTSG-2	miR-146a, miR-21	(47,60)
CXCL-10	miR-155	(61)

1.3 Interplay Between EVs, miRNAs and TLR7 in neurodegenerative diseases

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) are characterized by neuronal cell losses. Neurodegenerative diseases cause progressive degeneration and death of nerve cells, which further leads to movement disorders such as ataxias, or dementias. Dementias are responsible for the greatest burden of neurodegenerative diseases, with Alzheimer's representing approximately 70% of dementia cases. Despite significant research efforts, the primary causes of neurodegenerative diseases remain mostly unknown.

Most of the neurodegenerative diseases and their pathological alterations, including AD, PD, ALS and Huntington's disease, have been associated with miRNAs. This suggests that miRNAs may be one of the contributing factors in neurodegenerative diseases and maybe used as diagnostic biomarkers or for therapeutic purposes. It has been previously reported that miRNA expression levels are altered in the blood of AD, PD and ALS patients. Studies have been conducted in order to utilize miRNAs for early diagnosis and treatments (62), but these have yet to be definitive.

In Alzheimer's disease research, Liu et al. reported that overexpression of miR-193b could suppress the expression of amyloid precursor protein (APP). APP is the precursor molecule whose proteolysis generates beta amyloid (Aβ), a polypeptide containing 37 to 49 amino acid residues, whose amyloid fibrillar form is the primary component of amyloid plaques found in the brains of Alzheimer's disease patients. This shows that miR-193b may get involved in the neurodegenerative process, and therefore miR-193b has the potential to be a unique biomarker for AD (63). In Parkinson's disease (PD) research by Gui et al., microRNA profiling strategy for exosomal miRNAs isolated from CSF was suggested to serve for differential diag-

nosis of PD with AD. The purpose of exosomal miRNA biomarker researches is to be able predict future cognitive decline in asymptomatic individuals and the progression of the disease in patients with early dementia.

Neuroinflammation can be observed during progression of neurodegenerative diseases and involves brain cells such as microglia, oligodendrocytes, astrocytes, neuronal cells, as well as macrophages crossing the brain-blood barrier, which can be damaged in the inflamed brain (64). Much of this appears to involve the innate immune system, within which TLRs are important components. Whereas brief neuroinflammatory responses are considered to be neuroprotective and may even contribute neuronal development; when such responses persist they result in neurodegeneration (65-69). Among the known TLRs so far, TLR7 can be activated by miRNAs and result in neuro-inflammation (27). It is still unknown whether neuro-inflammation in the CNS leads to the progress of neurodegenerative diseases. However, increasing evidence indicate the participation of TLR7-dependent pathways in neurodegenerative diseases. Lehmann et al have reported that TLR7 recognize GU-rich miRNAs as ligands in the CNS (27). For example, miRNA let-7b, which is highly expressed in both microglia and neuronal cells, interacts with TLR7 to regulate transcription factors, such as MyD88, induced by TLR7. Therefore, it is inferred that let-7b binds to miRNA-sensing TLR7 and consequently induces neurodegeneration through neuronal TLR7 (27). Our lab has shown that EVs isolated from the brains of monkeys with SIV induced CNS disease activates TLR7 and were neurotoxic when compared to EVs from control animals (26). Coleman et. al found that ethanol induced TLR7 and let-7b expression, caused TLR7-associated neuroimmune gene induction and initiated the release of let-7b in EVs, enhancing TLR7-mediated neurotoxicity (70).

In summary, endogenous miRNAs may serve important functions as ligands of TLR7-promotion of neuroinflammation. In addition to miRNAs, it is important to note that roles for

EVs in transporting pathogenic proteins such as alpha-synuclein (α-syn) and amyloid precursor protein (APP) in PD and AD, respectively, have been proposed (20,71,72). Additional information on the roles of miRNA and other pathogenic aspects of neurodegenerative disorders is presented next.

1.3.1 Alzheimer's Disease

AD is a chronic neurodegenerative disease that usually starts slowly and worsens over time. It is the most common cause of dementia in the world (73). AD has been identified as a protein misfolding disease, caused by plaque accumulation of abnormally folded amyloid beta protein (Aβ), and tau protein in the brain (74). Plaques are made up of small peptides, 39-43 amino acids in length, called amyloid beta (Aβ). Aβ is a fragment from the larger amyloid precursor protein (APP). APP is a transmembrane protein that penetrates through the neuron's membrane and is critical to neuron growth, survival, and post-injury repair (75,76). In AD, the APP is proteolytically processed by the beta- and gamma-secretases to release the Aβ peptide that is neurotoxic and aggregates in the brain (77). AD is also considered a tauopathy due to abnormal aggregation of the tau protein. Every neuron has a cytoskeleton, an internal support structure partly made up of structures called microtubules. These microtubules act like tracks, guiding nutrients from the body of the cell to the ends of the axon and back. The tau protein stabilizes the microtubules via phosphorylation and is therefore called a microtubule-associated protein. In AD, the tau protein is hyperphosphorylated and pairs with other threads, creating neurofibrillary tangles and disintegrating the neuron's transport system (78).

Aβ clearance is crucial to progress of AD and microglial cells are activated during removal of these amyloid deposits (79). Activated microglia can clear the Aβ deposits by phagocytic activity pro-inflammatory cytokines However, microglia activation is often insufficient to re-

move amyloid deposits from the aged brain and may even causes damages to neighboring cells (80,81). In AD, activation of microglia involves endosomal TLRs such as TLR9, and boost the clearance of Aβ deposits in the brain (82). Tahara et al. reported that activation of BV-2 cells with TLR9 ligands markedly increased ingestion of Aβ . Li et al. on the other side, reported that activation of TLR3, TLR4, TLR7, but not TLR9, reduced primary microglial CD36 transcription and cell surface CD36 protein and reduced Aβ phagocytosis as well (83). The role of TLRs, especially endosomal TLRs, is still debated and further studies need to be conducted.

Recent studies have revealed that the catabolism of APP occurs in endosome. Aβ peptides can be secreted from the cells in association with exosomes. Exosomal proteins were found to accumulate in the plaques of AD patient brains, suggesting a role in the pathogenesis of AD (59). The EVs associated with Aβ can be internalized into microglia and transported to microglial lysosomes, where it is degraded. Thus, neuronal EVs, which can trap Aβ, act as couriers of Aβ for Aβ clearance. In co-cultures of human APP-transfected N2a cells and BV2 cells, promotion of EV generation in N2a by knockdown of sphingomyelin synthase increases engulfment of extracellular Aβ by BV2 and decreases the levels of Aβ in the culture medium. Infusion of neuronal EVs into APP transgenic mice decreases Aβ levels and attenuates Aβ toxicity in the hippocampus (84). These studies indicate that Aβ clearance is EV-dependent and improvement of Aβ clearance by EV infusion may provide a novel therapeutic approach for AD.

It is also likely that miRNAs, especially exosomal miRNAs, play vital roles in the progression of AD. Previously studies using blood samples from AD patients have identified more than 60 miRNAs up- or down-regulated in these patients in comparison with healthy individuals (85,86). The miRNAs bind to complementary sites within the 3'-UTR region of target genes that determine the expression of APP and beta-site APP-cleaving enzyme (BACE) (87,88). Extracellular

Aβ deposits, which ultimately lead to progressive loss of neurons, are derived from processing of APP by BACE. Significantly dysregulated miRNAs targeting APP like miR-193b (63), miR-101 (89), miR-155 (90) or BACE1 like miR-29c (87,88), miR-9 (91), miR-124 (92), miR-15b (93), miR-124 (94) influence Aβ ingestion in AD brain. Furthermore, miR-193b has been shown to be present in the hippocampus of AD mice and overexpression of miR-193b could suppress the expression of APP (63). The levels of miRNA let-7 are also enhanced in AD patients (27). Lehmann et al. reported that let-7 activates endosomal TLR7, and thus, induces neurodegeneration in these patients.

1.3.2 Parkinson's Disease

Parkinson's Disease (PD) is a progressive nervous system disorder that affects movements and is characterized by degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Symptoms start gradually, sometimes starting with a barely noticeable tremor in one hand. The disorder also commonly causes stiffness, bradykinesia and postural instability (95). The main pathological characteristics of PD are the presence of intracellular inclusion termed Lewy bodies (accumulations of the fibrillar alpha-synuclein) in the neurons and cell death in the basal ganglia. The loss of neurons is accompanied by the death of astrocytes as well as increase of microglial cells in the substantia nigra (96).

In PD and other Lewy body disorders, the main pathology has been accredited to the accumulation of alpha-synuclein. There are a number of proposed mechanisms by which alpha-synuclein results in neurodegeneration, some of which involve the innate immune system. For example the accumulation of alpha-synuclein activates microglia, increasing dopaminergic neurotoxicity (97,98).

Although initially alpha-synuclein was considered a purely intracellular protein, accumulating evidences from recent studies suggests that alpha-synuclein can spread readily between cells (99,100). It has also been shown that such cell-to-cell transfer can occur as neuron-to-neuron, glial cell-to-glial cell as well as neuron-to-microglia (101,102). Although the precise molecular mechanisms are not fully understood, cell-to-cell transfer of alpha synuclein via EVs has been reported. Alpha-synuclein is present in EVs from cultured cells and secreted via a calcium-dependent mechanism (103). When inhibiting lysosomal action in alpha-synuclein overexpressing neural cell lines, EV secretion of alpha-synuclein increased and promoted cell-to-cell transfer of alpha-synuclein (103,104). Furthermore, cell-derived EVs containing alpha-synuclein were found to induce cell death in neuronal cell lines (103).

The human studies have identified a large number of dysregulated microRNAs in PD brain tissue samples (72). The profile of miRNA dysregulation differs among various regions of the PD brains: Included among those having upregulated expression are: miR-199b, -544a, -488, -221, -144 (cingulate gyri); miR-221-3p, -95 (putamen); miR-21, -224, -373, -26b, -106a, -301b (substantia nigra); miR-224, -373 (amygdala); miR-200b, -200a, -195, -424 (frontal cortex); miR-16-5p (prefrontal cortex). Those with downregulated expression are: miR-7, -145 (cingulate gyri); miR-155-5p, -34b (putamen); miR-198, -485-5p, -339-5p, -208b, -135b, -299-5p, -330-5p, -542-3p, -379, -337-5p, -34b, -34c (substantia nigra); miR-34b, -34c (amygdala); miR- 200a, -199a-3p, -148a, -451, -144, -429, -190, -34b, -34c -205 (frontal cortex); let-7i-3p, miR-184, -127-5p (prefrontal cortex).

Downstream target gens of several miRNAs have also been identified in the previous PD studies, and alterations of these miRNAs can greatly affect the pathology and progression of PD. By and large, PD has not been considered to be a genetic disease and the majority of cases are idiopathic. However, leucine-rich repeat kinase2 (LRRK2) is one of the greatest known ge-

netic contributors to PD, and mutations of LRRK2 account for 15 to 20 percent of PD cases in the Ashkenazi Jewish, and about 40 percent of cases in the North African Arab Berbers. It has been demonstrated that the levels of let-7 are correlated with the expression of alpha-synuclein and LRRK2, coded by the two main genes associated with PD (105). A recent report has also indicated that let-7 represses the expression of alpha-synuclein and is downregulated in PD models (106). LRRK2 is also an experimentally validated target of miR-1224, which is downregulated in PD (71). The miRNAs also target the 3'-UTR regions of targeted genes and proteins. For example, upregulated miR-21 in PD can directly target the 3'-UTR of lysosome-associated membrane protein 2A (LAMP2A) (107); Downregulated miR-135a-5p targets the 3'-UTR of rho-associated protein kinase2 (ROCK2) and plays a role in the protective effects of hydrogen sulfide against PD (108).

Increasing evidences suggest a close relationship between PD and TLRs. It has been recently shown that extracellular alpha-synuclein induces BV-2 microglial cells activation and secrete proinflammatory mediators such as TNF-α and IL-1β (109). In addition, the expression of TLR1, TLR7, MyD88, NF-κB, TNF-α and IL-1β are increased while the expression of TLR4, TLR6, TLR9 and CD36 are decreased or unchanged (109). These findings indicate that alpha-synuclein can activate microglia through TLR7. However, further studies need to be conducted in order to show that TLR7 can mediate cell death in mouse model of Parkinson disease (such as MPTP model).

Chapter 2. MATERIAL AND METHODS

Oligonucleotides

MiRNAs with phosphorothioate or phosphodiester linkages were synthesized, desalted, and purified by reverse-phase cartridge by Sigma-Aldrich (St. Louis, MO, USA). MiRNAs were used by complexing with (or where specified without) DOTAP reagent (Sigma-Aldrich).

Cells

RAW 264.7 macrophages (American Type Culture Collection, Manassus, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and penicillin-streptomycin. N1E-115 neuroblast cells (American Type Culture Collection) were cultured in DMEM with 4.5 g/L glucose without sodium pyruvate (Invitrogen) supplemented with 10% FBS. SH-SY5Y neuroblastoma cells were obtained from American Type Culture Collection and grown in Dulbecco's modified eagle's medium/Nutrient mixture F-12 (DMEM/F12) (Invitrogen) with 10% FBS supplemented with L-glutamine. BV-2 immortalized microglial cells (American Type Culture Collection) were grown and routinely maintained in DMEM with 10% FBS and incubated at 37° C and 5% CO_2. The NR-9460 microglial cell line, derived from wild-type mice (denoted WT microglia), and the NR-19980 microglial cell line, derived from TLR7 knockout mice (denoted TLR7ko microglia) (BEI Resources, Manassus, VA, USA), were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 10 μg/ml ciprofloxacin. Cells were grown to confluence at 37° C in humidified air with 5% CO_2.

AlamarBlue Assay for cellular survival/viability

Responsiveness of N1E-115 cell to miRNAs was determined by alamarBlue assay. The active ingredient of alamarBlue (resazurin) is a nontoxic, cell permeable compound that is blue in

color and virtually nonfluorescent. Upon entering cells, resazurin is reduced and produces red fluorescence. Nonviable cells have no metabolic capacity and will not reduce the dye. 1.25 x 10^4 cells were plated in 96-well plates and were incubated for the indicated times with let-7b-3p (Sigma-Aldrich) complexed (or not) to the transfection agent DOTAP (Sigma-Aldrich), using various ratios of miRNAs to DOTAP. After incubation with alamarBlue (Invitrogen) for 4 h at room temperature, the absorbance was read at 570 nm using a microplate reader according to the manufacturer's recommendations.

Isolation of SH-SY5Y-derived Extracellular Vesicles

EVs were isolated from SH-SY5Y neuroblastoma cells as previously described, with minor modifications (110,111). Confluent SH-SY5Y cells were washed with 1X phosphor-buffered saline (PBS) (Invitrogen) and then grown in DMEM/F12 without phenol red supplemented with exosome-depleted FBS (System Biosciences, Palo Alto, CA, USA) to avoid potential disturbance of the following assay with Nanosight (112).Culture supernatants were centrifuged at 300 g for 20 minutes at room temperature to remove cell debris and large particles. The resultant supernatants were ultracentrifuged at 150,000 at 4° C for 1 hour. The resulting pellets were washed and resuspended with 1X PBS.

TNF-α and IL-6 ELISA

Cells were incubated for the indicated times with the indicated concentrations of miRNAs complexed to the transfection agent DOTAP (Sigma-Aldrich), using a ratio of miRNAs to DOTAP of 1:6. Conditioned medium was collected and analyzed by mouse TNF-α or IL-6 ELISA kit from eBioscience (San Diego, CA, USA), with lower limit of detection of 8 pg/ml and upper limit of linearity of 1000 pg/ml (8pg/ml, 500 pg/ml for IL-6 assay). For each determination technical duplicates were averaged. The number of biological replicates is given in each figure legend.

Final cytokine concentrations were calculated based on a standard curve constructed in each independent experiment. Values <8 and >1000 pg/ml were recorded as 8 and 1000 pg/ml, respectively (8 and 500 pg/ml for IL-6 assay).

Inhibitors of signal transduction pathways

Microglial cells were pre-treated with 100 nM MLK-3 inhibitor URMC-099 (Cayman Chemical, Ann Arbor MI, USA), 1 μM PI3K inhibitor Wortmannin (Sigma-Aldrich) or 10 μM IKK inhibitor BMS-345541 (Sigma-Aldrich) for 30 minutes prior to miRNA-DOTAP stimulation.

RNA extraction and analysis

To prepare total RNA from cultured microglial cell lines, cells were plated at the density of 1.5 x 10^6 cells/well in 6-well plates. Cells were then subjected to RNA extraction using Trizol reagent according to the manufacturer's instructions (Invitrogen). Two micrograms of total RNA from cultured cells was used for subsequent cDNA synthesis. A real-time PCR assay was performed using the AppliedBiosystems StepOnePlus Real-Time PCR system. Primers and probes were purchased premade stocks from AppliedBioSysystems (Pleasanton, CA, USA), for the following genes: TNF- α (Mm00443258_m1), IL-6 (Mm00446190_m1), CXCL10 (Mm00445235_m1), PTSG-2 (Mm00478374_m1), IL-1 β (Mm00433228_m1) and CCL5(Mm01302427_m1). Relative levels were determined by the ddCt method, using GAPDH (Mm99999915_g1) as the housekeeping control. Each PCR was run as a technical duplicate and averaged, the number of biological replicates is given in the figure legend.

Animals

Wild-type (WT, C57BL/6) and TLR7ko mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and bred in the University of Nebraska Medical Center animal facility. The

animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center, and followed National Institutes of Health guidelines.

Culture and proliferation of primary bone marrow-derived macrophages

Immortalized primary bone marrow-derived macrophages (BMDMs) were generated from mice femurs as previously described (113) and cultured in RPMI-1649 supplemented with 10% FBS, penicillin-streptomycin and M-CSF. Cells were plated in 96-wells culture plates at 250,000 cells per well for future transfections.

Primary mouse microglial cell isolation

Primary microglia cells were obtained from 1- to 3-day-old C57BL/6 newborn pups and isolated as previously described. After removal of brains, the cortices were dissected and digested in Hanks buffered salt solution (Invitrogen) containing 0.25% trypsin (Invitrogen). Mixed glial cultures were then prepared by re-suspending the cell suspension in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were plated at a density of 5×10^6 cells/flask in 75 cm^2 cell culture flasks. Cell medium was replaced every 5 days, and after the first medium change, 0.25 ng/ml macrophage colony stimulating factor (Invitrogen) was added to the flasks to promote microglial proliferation. Upon reaching confluence (7 to 10 days), cultures were shaken at 37° C at 220 rpm for 2 h to detach microglia from the flask surface. The cell medium, containing the released microglia cells, was collected and centrifuged at 1000xg for 5 min to collect the pelleted cells, which were then plated on 96-well tissue culture plates at 25,000 cells/well for all subsequent experiments.

In vivo miRNA treatment

WT and TLR7 knockout mice (8-12 weeks of age) were treated with 20 μg miRNA-mimics complexed with DOTAP, negative control (DOTAP only) or saline. Animals were injected intraperitoneally and 20 hours post-injection, under light isoflurane anesthesia, mice were sacrificed by cervical dislocation and their abdomen opened for the collection of intraperitoneal fluid. The peritoneal cavity was flushed with 5 ml PBS with 3% FBS, and gently massaged before removing the fluid containing intraperitoneal leukocytes. The fluid was centrifuged to pellet cells, resuspended in sterile PBS, counted and used for flow cytometric analysis. Each experiment consisted of one mouse of each genotype, and five biological replicates were performed.

Flow Cytometry

Isolated intraperitoneal cells were washed with PBS and stained for live/dead assay (L23105, Invitrogen). Live/dead reagent was removed by centrifugation and cells were blocked for 5 min with rat CD16/CD32 Fc Block (BD Biosciences) and then stained with fluorescently labeled antibodies: anti-mouse Ly-6C (HK1.4), Ly-6G (1A8), CD11b (M1/70), MHC class II (M5/114.15.2), CD170 (IRNM44N), F4/80 (bm8) (eBioscience), for 45 minutes on ice. After washing and fixing, data were acquired on an LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA) in the University of Nebraska Medical Center Flow Cytometry core facility, and analyzed using BD FACSDiva software version 6.2, Firmware version 1.12 (Becton Dickinson) or Flowjo 6.0 (Flowjo, LLC). Due to inconsistent staining the CD170 (used in order to identify eosinophils) results were not used in the analysis.

Statistics

Statistics were performed using Prism 7.0 (GraphPad Software, La Jolla, CA, USA). For the *in vitro* studies one-way ANOVA was followed by Dunnett's multiple comparison test to the con-

trol condition, for the *in vivo* studies two-way ANOVA was followed by Sidak's post-hoc test with the p value adjusted for multiple comparisons. Alpha was set to 0.05 for significance.

Chapter 3. Activation of TLR7 by miRNAs in brain cells

3.1 Rationale

In the present study we hypothesized that many miRNAs can activate endosomal TLR7 of brain innate immune cells, such as macrophages and microglia. We investigated the toxicity of our transfection complex and utilized various brain cells. Based on a comprehensive screen of of cytokine production via activation of microglialTLR7 by miRNAs, the study was conducted to explore the role of miRNAs in the brain and to identify potential pathogenic pathways in neurodegenerative diseases.

3.2 Results

3.2.1 MiRNA-DOTAP complex does not affect the viability of N1E-115 cells

In order to test the effect of the miRNA-DOTAP complex on cell viability and to determine the optimal ratio of miRNA to DOTAP to be used, we chose to assess the absorbance of alamarBlue after incubation of N1E-115 cells with various ratios of miRNA to DOTAP for indicated time (Fig. 3.1). MiRNA-DOTAP complex does not affect the viability of cells and appears to have little cytotoxicity.

Figure 3.1

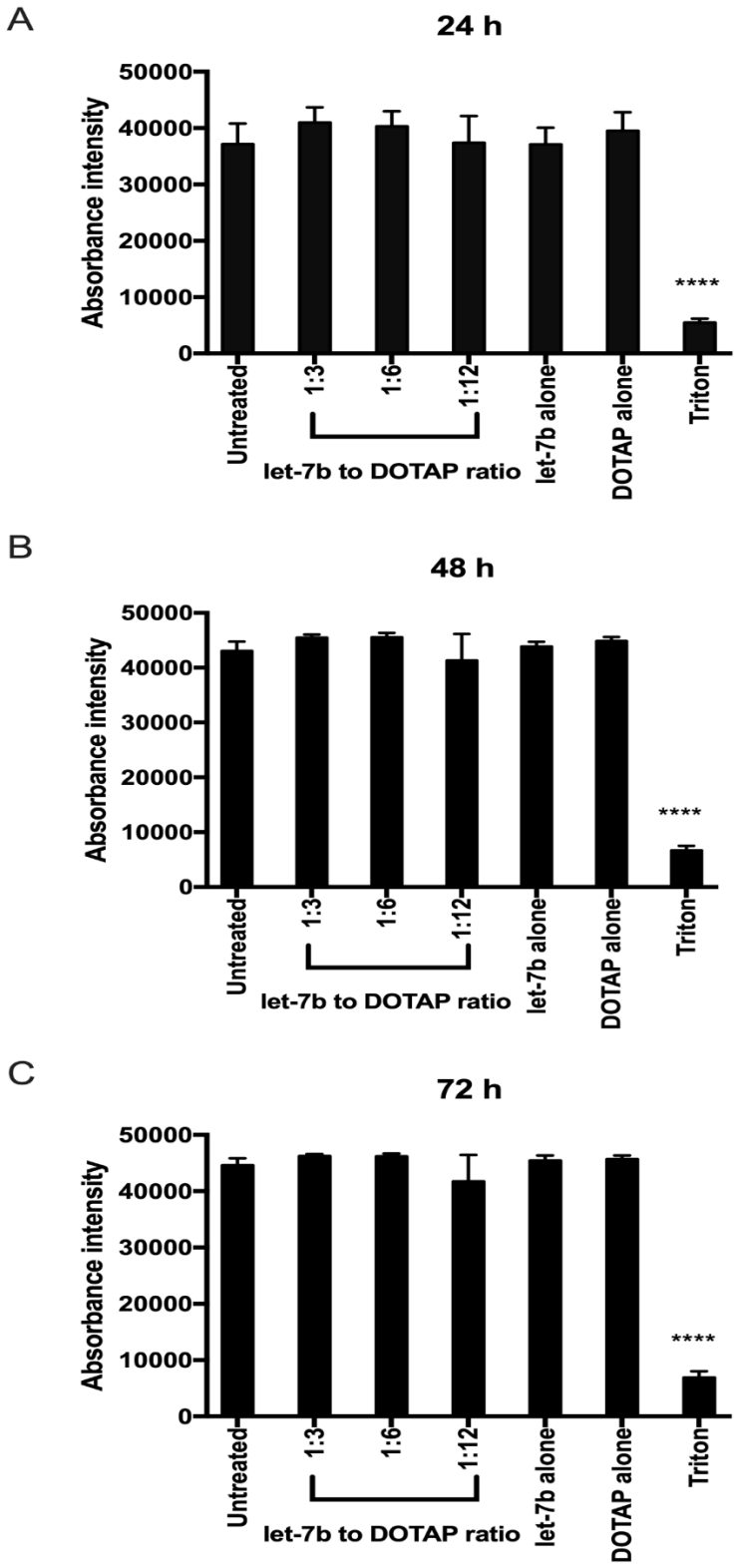

A
24 h
Absorbance intensity
50000
40000
30000
20000
10000
0
****
Untreated
1:3
1:6
1:12
let-7b to DOTAP ratio
let-7b alone
DOTAP alone
Triton
B
48 h
Absorbance intensity
50000
40000
30000
20000
10000
0
****
Untreated
1:3
1:6
1:12
let-7b to DOTAP ratio
let-7b alone
DOTAP alone
Triton
C
72 h
Absorbance intensity
50000
40000
30000
20000
10000
0
****
Untreated
1:3
1:6
1:12
let-7b to DOTAP ratio
let-7b alone
DOTAP alone
Triton

Figure 3.1. miRNA-DOTAP complex does not affect the viability of N1E-115 neuroblast cells.

N1E-115 neuroblast cells were treated with or without let-7b complexed to DOTAP at indicated ratios, let-7b-3p alone, DOTAP alone or Triton for A) 24 hours, B) 48 hours or C) 72 hours followed by alamarBlue assays. The results are presented as mean ± SEM. ***p < 0001 when compared to the untreated. n=3 for all experiments. MiRNA-DOTAP complex is not toxic to cells.

3.2.2 MiRNAs activate RAW 264.7 cells in a dose-dependent manner

The macrophage population of the CNS include the microglia, perivascular macrophages, meningeal macrophages, macrophage of the circumventricular organs and the macrophages of the choroid plexus. We first investigated the response of TLR7 expressing RAW 264.7 macrophages to indicated miRNAs based on previously published literatures (26,27,114,115). It should be noted that RAW 264.7 macrophages also contain may other PAMPs including TLRs such as TLR4, which is activated by pathogens like LPS (116), thus this is not specific for TLR7 but enabled a screen of whether a large number of miRNAs could activate PAMPs. Following incubation with various doses of indicated miRNAs complexed to transfection agent DOTAP for 16 hours, TNF-α levels in the culture supernatants were measured by ELISA. At 2 μg/ml, miR-21, miR-9 and let-7i caused release of more than 800 pg/ml TNF-α release. Let-7b only caused release of less than 200 pg/ml TNF-α (Fig 3.2).

Figure 3.2

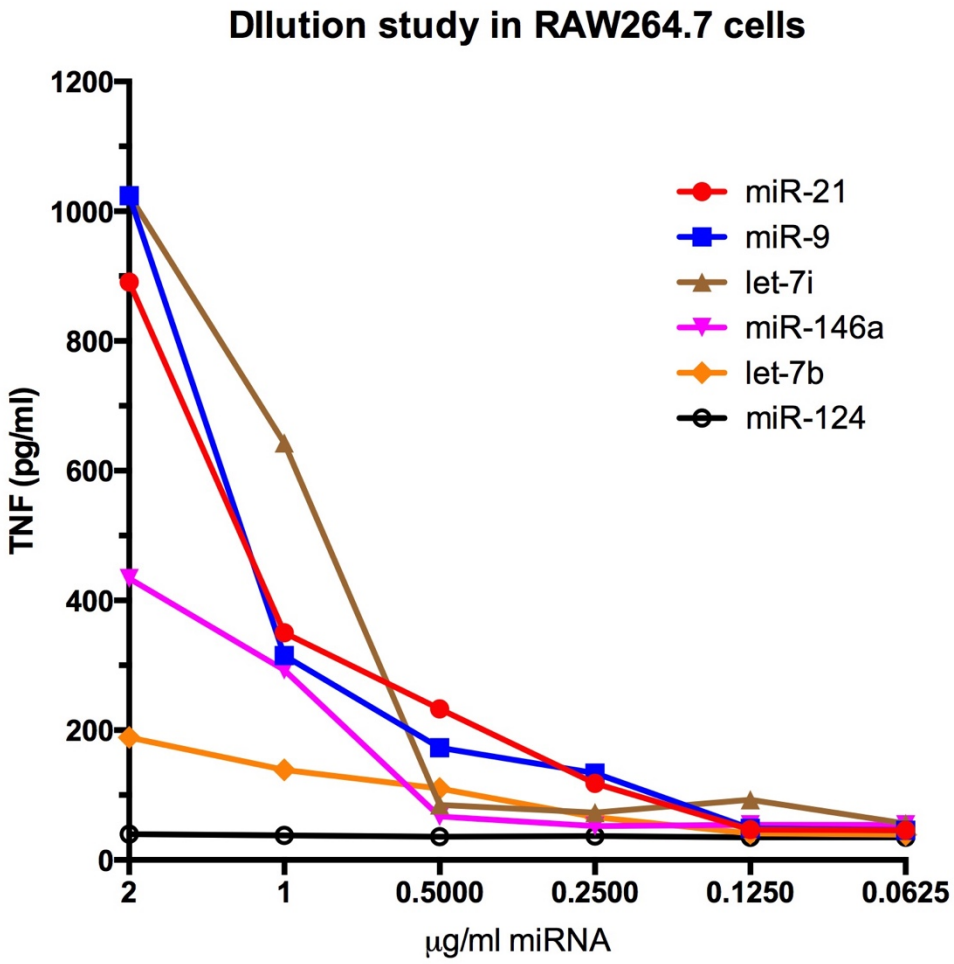

Dllution study in RAW264.7 cells
TNF (pg/ml)
1200
1000
800
600
400
200
0
2
1
0.5000
0.2500
0.1250
0.0625
µg/ml miRNA
miR-21
miR-9
let-7i
miR-146a
let-7b
miR-124

Figure 3.2. Synthetic miRNAs stimulate RAW 264.7 macrophages to release TNF-alpha in a dose-dependent manner.

RAW 264.7 cells were incubated for 16 hours with various doses of miRNAs. Subsequently, TNF-α amounts in the culture supernatants were determined by ELISA. Imiquimod (5 μg/ml) was used as a positive control and caused more than 1000 pg/ml TNF-α release (data not shown).

Table 3.1

miRNA	Sequence	RAW 264.7 cells TNF-α (mean ± SEM)
let-7a-5p	5'-UGAGGUAGUAGGUUGUAUAGUU-3'	38.3 ± 0.4
let-7b-5p	5'-UGAGGUAGUAGGUUGUGUGGUU-3'	224.3 ± 10.5
let-7c-5p	5'-UGAGGUAGUAGGUUGUAUGGUU-3'	143.1 ± 7.9
let-7d-5p	5'-AGAGGUAGUAGGUUGCAUAGUU-3'	28.9 ± 0.8
let-7e-5p	5'-UGAGGUAGGAGGUUGUAUAGUU-3'	392.4 ± 29
let-7f-5p	5'-UGAGGUAGUAGAUUGUAUAGUU-3'	76.2 ± 3
let-7g-5p	5'-UGAGGUAGUAGUUUGUACAGUU-3'	313.5 ± 21.8
let-7i-5p	5'-UGAGGUAGUAGUUUGUGCUGUU-3'	high
miR-100-5p	5'-AACCCGUAGAUCCGAACUUGUG-3'	high
miR-101a-3p	5'-UACAGUACUGUGAUAACUGAA-3'	28.9 ± 1.7
miR-103-3p	5'-AGCAGCAUUGUACAGGGCUAUGA-3'	290.2 ± 30.9
miR-106b-5p	5'-UAAAGUGCUGACAGUGCAGAU-3'	38.1 ± 1
miR-107-3p	5'-AGCAGCAUUGUACAGGGCUAUCA-3'	high
miR-10a-5p	5'-UACCCUGUAGAUCCGAAUUUGUG-3'	high
miR-10b-5p	5'-UACCCUGUAGAACCGAAUUUGUG-3'	high
miR-122-5p	5'-UGGAGUGUGACAAUGGUGUUUG-3'	high
miR-124-3p	5'-UAAGGCACGCGGUGAAUGCC-3'	25.9 ± 0.1
miR-125a-5p	5'-UCCCUGAGACCCUUUAACCUGUGA-3'	high
miR-125b-5p	5'-UCCCUGAGACCCUAACUUGUGA-3'	high
miR-126a-3p	5'-UCGUACCGUGAGUAAUAAUGCG-3'	30.7 ± 1.3
miR-126a-5p	5'-CAUUAUUACUUUUGGUACGCG-3'	960.6 ± 58.3
miR-128-3p	5'-UCACAGUGAACCGGUCUCUUU-3'	347.1 ± 19.3
miR-129-5p	5'-CUUUUUGCGGUCUGGGCUUGC-3'	653.8 ± 12.6
miR-130a-3p	5'-CAGUGCAAUGUUAAAAGGGCAU-3'	122.3 ± 2.7
miR-130b-3p	5'-CAGUGCAAUGAUGAAAGGGCAU-3'	31 ± 1.2
miR-132-3p	5'-UAACAGUCUACAGCCAUGGUCG-3'	147.4 ± 8.7
miR-133a-3p	5'-UUUGGUCCCCUUCAACCAGCUG-3'	509.1 ± 21.4
miR-133b-3p	5'-UUUGGUCCCCUUCAACCAGCUA-3'	489.3 ± 48.7
miR-134-5p	5'-UGUGACUGGUUGACCAGAGGGG-3'	33.2 ± 1.4
miR-135a-5p	5'-UAUGGCUUUUUAUUCCUAUGUGA-3'	high
miR-135b-5p	5'-UAUGGCUUUUCAUUCCUAUGUGA-3'	high

miR-137-3p	5'-UUAUUGCUUAAGAAUACGCGUAG-3'	212.5 ± 6
miR-138-5p	5'-AGCUGGUGUUGUGAAUCAGGCCG-3'	30.6 ± 1.3
miR-140-3p	5'-UACCACAGGGUAGAACCACGG-3'	287 ± 14.9
miR-140-5p	5'-CAGUGGUUUUACCCUAUGGUAG-3'	high
miR-141-3p	5'-UAACACUGUCUGGUAAAGAUGG-3'	182.1 ± 2.3
miR-142a-3p	5'-UGUAGUGUUUCCUACUUUAUGGA-3'	755.9 ± 43.4
miR-142a-5p	5'-CAUAAAGUAGAAAGCACUACU-3'	32.4 ± 1.3
miR-143-3p	5'-UGAGAUGAAGCACUGUAGCUC-3'	404.2 ± 14.2
miR-144-3p	5'-UACAGUAUAGAUGAUGUACU-3'	88.7 ± 1.8
miR-145a-5p	5'-GUCCAGUUUUCCCAGGAAUCCCU-3'	133.9 ± 3.1
miR-146a-5p	5'-UGAGAACUGAAUUCCAUGGGUU-3'	high
miR-146b-5p	5'-UGAGAACUGAAUUCCAUAGGCU-3'	high
miR-147-3p	5'-GUGUGCGGAAAUGCUUCUGCUA-3'	high
miR-148a-3p	5'-UCAGUGCACUACAGAACUUUGU-3'	763.5 ± 57.2
miR-148b-3p	5'-UCAGUGCAUCACAGAACUUUGU-3'	high
miR-149-5p	5'-UCUGGCUCCGUGUCUUCACUCCC-3'	high
miR-150-5p	5'-UCUCCCAACCCUUGUACCAGUG-3'	high
miR-152-3p	5'-UCAGUGCAUGACAGAACUUGG-3'	913.6 ± 48.1
miR-15a-5p	5'-UAGCAGCACAUAAUGGUUUGUG-3'	high
miR-15b-5p	5'-UAGCAGCACAUCAUGGUUUACA-3'	662.5 ± 3.8
miR-16-5p	5'-UAGCAGCACGUAAAUAUUGGCG-3'	28.7 ± 0.5
miR-17-5p	5'-CAAAGUGCUUACAGUGCAGGUAG-3'	86.7 ± 1.5
miR-181a-1-3p	5'-ACCAUCGACCGUUGAUUGUACC-3'	766.2 ± 30.9
miR-181a-5p	5'-AACAUUCAACGCUGUCGGUGAGU-3'	345.2 ± 2.9
miR-181b-5p	5'-AACAUUCAUUGCUGUCGGUGGGU-3'	158.2 ± 10
miR-181c-5p	5'-AACAUUCAACCUGUCGGUGAGU-3'	high
miR-181d-5p	5'-AACAUUCAUUGUUGUCGGUGGGU-3'	861.1 ± 28.8
miR-183-5p	5'-UAUGGCACUGGUAGAAUUCACU-3'	833.6 ± 6.9
miR-184-3p	5'-UGGACGGAGAACUGAUAAGGGU-3'	24.7 ± 0.5
miR-185-5p	5'-UGGAGAGAAAGGCAGUUCCUGA-3'	380 ± 5.3
miR-186-5p	5'-CAAAGAAUUCUCCUUUUGGGCU-3'	101.2 ± 0.6
miR-187-3p	5'-UCGUGUCUUGUGUUGCAGCCGG-3'	high
miR-18a-5p	5'-UAAGGUGCAUCUAGUGCAGAUAG-3'	40.1 ± 0.2
miR-191-5p	5'-CAACGGAAUCCCAAAAGCAGCUG-3'	49.9 ± 0.2

miR-192-5p	5'-CUGACCUAUGAAUUGACAGCC-3'	150.4 ± 3.3
miR-193a-3p	5'-AACUGGCCUACAAAGUCCCAGU-3'	25.2 ± 0.3
miR-194-5p	5'-UGUAACAGCAACUCCAUGUGGA-3'	74.5 ± 0.9
miR-195a-5p	5'-UAGCAGCACAGAAAUAUUGGC-3'	34.4 ± 0.5
miR-196a-5p	5'-UAGGUAGUUUCAUGUUGUUGGG-3'	high
miR-196b-5p	5'-UAGGUAGUUUCCUGUUGUUGGG-3'	741.2 ± 22.9
miR-199a-5p	5'-CCCAGUGUUCAGACUACCUGUUC-3'	high
miR-19a-3p	5'-UGUGCAAAUCUAUGCAAAACUGA-3'	58.2 ± 3.4
miR-19b-3p	5'-UGUGCAAAUCCAUGCAAAACUGA-3'	49.3 ± 1.8
miR-1a-3p	5'-UGGAAUGUAAAGAAGUAUGUAU-3'	96.6 ± 5.8
miR-200a-3p	5'-UAACACUGUCUGGUAACGAUGU-3'	59.9 ± 2.6
miR-200b-3p	5'-UAAUACUGCCUGGUAAUGAUGA-3'	57 ± 3.3
miR-200c-3p	5'-UAAUACUGCCGGGUAAUGAUGGA-3'	22.9 ± 0.2
miR-203-3p	5'-GUGAAAUGUUUAGGACCACUAG-3'	368.6 ± 43.3
miR-204-5p	5'-UUCCCUUUGUCAUCCUAUGCCU-3'	high
miR-205-5p	5'-UCCUUCAUUCCACCGGAGUCUG-3'	369.2 ± 4
miR-206-3p	5'-UGGAAUGUAAGGAAGUGUGUGG-3'	26.5 ± 0.3
miR-20a-5p	5'-UAAAGUGCUUAUAGUGCAGGUAG-3'	high
miR-20b-5p	5'-CAAAGUGCUCAUAGUGCAGGUAG-3'	415.5 ± 12.9
miR-210-3p	5'-CUGUGCGUGUGACAGCGGCUGA-3'	43.3 ± 1.2
miR-214-3p	5'-ACAGCAGGCACAGACAGGCAGU-3'	25.8 ± 0.6
miR-218-5p	5'-UUGUGCUUGAUCUAACCAUGU-3'	997.7 ± 14.2
miR-21a-5p	5'-UAGCUUAUCAGACUGAUGUUGA-3'	high
miR-22-3p	5'-AAGCUGCCAGUUGAAGAACUGU-3'	111.8 ± 11.8
miR-221-3p	5'-AGCUACAUUGUCUGCUGGGUUUC-3'	99.5 ± 9.2
miR-222-3p	5'-AGCUACAUCUGGCUACUGGGU-3'	29.1 ± 0.7
miR-223-3p	5'-UGUCAGUUUGUCAAAUACCCCA-3'	high
miR-23a-3p	5'-AUCACAUUGCCAGGGAUUUCC-3'	high
miR-23b-3p	5'-AUCACAUUGCCAGGGAUUACC-3'	403.8 ± 41.8
miR-24-3p	5'-UGGCUCAGUUCAGCAGGAACAG-3'	928.3 ± 19.3
miR-25-3p	5'-CAUUGCACUUGUCUCGGUCUGA-3'	high
miR-26a-5p	5'-UUCAAGUAAUCCAGGAUAGGCU-3'	385.5 ± 34.5
miR-26b-5p	5'-UUCAAGUAAUUCAGGAUAGGU-3'	191.5 ± 13.7
miR-27a-3p	5'-UUCACAGUGGCUAAGUUCCGC-3'	high

miR-27b-3p	5'-UUCACAGUGGCUAAGUUCUGC-3'	high
miR-28a-5p	5'-AAGGAGCUCACAGUCUAUUGAG-3'	43.3 ± 0.7
miR-296-5p	5'-AGGGCCCCCCCUCAAUCCUGU-3'	23.3 ± 0.1
miR-29a-3p	5'-UAGCACCAUCUGAAAUCGGUUA-3'	high
miR-29b-3p	5'-UAGCACCAUUUGAAAUCAGUGUU-3'	261.9 ± 7
miR-29c-3p	5'-UAGCACCAUUUGAAAUCGGUUA-3'	827.1 ± 0.2
miR-301a-3p	5'-CAGUGCAAUAGUAUUGUCAAAGC-3'	high
miR-302d-3p	5'-UAAGUGCUUCCAUGUUUGAGUGU-3'	high
miR-30a-5p	5'-UGUAAACAUCCUCGACUGGAAG-3'	43.6 ± 0.3
miR-30b-5p	5'-UGUAAACAUCCUACACUCAGCU-3'	49.3 ± 1.2
miR-30c-5p	5'-UGUAAACAUCCUACACUCUCAGC-3'	54 ± 2.4
miR-30d-5p	5'-UGUAAACAUCCCCGACUGGAAG-3'	64.9 ± 4.1
miR-30e-5p	5'-UGUAAACAUCCUUGACUGGAAG-3'	152.3 ± 14.3
miR-32-5p	5'-UAUUGCACAUUACUAAGUUGCA-3'	163.2 ± 0.3
miR-320-3p	5'-AAAAGCUGGGUUGAGAGGGCGA-3'	66.4 ± 3.6
miR-328-3p	5'-CUGGCCCUCUCUGCCCUUCCGU-3'	328.2 ± 88.9
miR-33-5p	5'-GUGCAUUGUAGUUGCAUUGCA-3'	511.3 ± 12.1
miR-335-5p	5'-UCAAGAGCAAUAACGAAAAAUGU-3'	63.8 ± 0.6
miR-338-3p	5'-UCCAGCAUCAGUGAUUUUGUUG-3'	high
miR-339-5p	5'-UCCCUGUCCUCCAGGAGCUCACG-3'	114.5 ± 21
miR-342-3p	5'-UCUCACACAGAAAUCGCACCCGU-3'	82.1 ± 5.3
miR-34a-5p	5'-UGGCAGUGUCUUAGCUGGUUGU-3'	high
miR-34c-5p	5'-AGGCAGUGUAGUUAGCUGAUUGC-3'	967.5 ± 13
miR-375-3p	5'-UUUGUUCGUUCGGCUCGCGUGA-3'	high
miR-381-3p	5'-UAUACAAGGGCAAGCUCUCUGU-3'	672.8 ± 42.9
miR-409-3p	5'-GAAUGUUGCUCGGUGAACCCCU-3'	298.3 ± 70.7
miR-411-5p	5'-UAGUAGACCGUAUAGCGUACG-3'	90 ± 3.5
miR-423-5p	5'-UGAGGGGCAGAGAGCGAGACUUU-3'	82.3 ± 8.9
miR-425-5p	5'-AAUGACACGAUCACUCCCGUUGA-3'	86.9 ± 1.8
miR-431-5p	5'-UGUCUUGCAGGCCGUCAUGCA-3'	169.2 ± 9.9
miR-433-3p	5'-AUCAUGAUGGGCUCCUCGGUGU-3'	646.8 ± 19.2
miR-451a	5'-AAACCGUUACCAUUACUGAGUU-3'	118.2 ± 8.4
miR-455-5p	5'-UAUGUGCCUUUGGACUACAUCG-3'	430 ± 0.6
miR-484	5'-UCAGGCUCAGUCCCCUCCCGAU-3'	85.6 ± 1.2

miR-485-5p	5'-AGAGGCUGGCCGUGAUGAAUUC-3'	157.3 ± 2.7
miR-499-5p	5'-UUAAGACUUGCAGUGAUGUUU-3'	high
miR-574-3p	5'-CACGCUCAUGCACACACCCACA-3'	69.2 ± 0.9
miR-652-3p	5'-AAUGGCGCCACUAGGGUUGUG-3'	71.1 ± 2.4
miR-744-5p	5'-UGCGGGGCUAGGGCUAACAGCA-3'	66.4 ± 0.7
miR-7a-5p	5'-UGGAAGACUAGUGAUUUUGUUGU-3'	61.4 ± 0.9
miR-9-3p	5'-AUAAAGCUAGAUAACCGAAAGU-3'	117.4 ± 4.1
miR-9-5p	5'-UCUUUGGUUAUCUAGCUGUAUGA-3'	high
miR-93-5p	5'-CAAAGUGCUGUUCGUGCAGGUAG-3'	high
miR-96-5p	5'-UUUGGCACUAGCACAUUUUUGCU-3'	966.6 ± 26.3
miR-98-5p	5'-UGAGGUAGUAAGUUGUAUUGUU-3'	458.9 ± 25.3
miR-99a-5p	5'-AACCCGUAGAUCCGAUCUUGUG-3'	high

Table 3.1 Names, sequences, and TNF-α release when miRNA:DOTAP complex was used to stimulate RAW 264.7 cells.

Values indicate TNF-α levels (pg/ml) in the culture supernatant 16 hours after addition of miRNA:DOTAP complex, average of technical duplicates and standard error of the mean are reported. High: values above the linear range of detection of the assay 1000 pg/ml.

3.2.3 Numerous miRNAs cause TNF-α release in RAW 264.7 cells

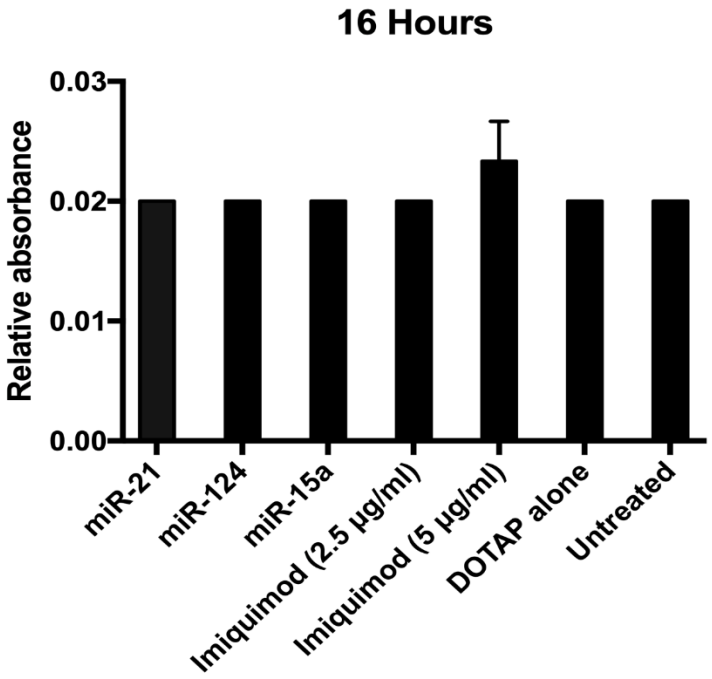
We extended the study and investigated the response of RAW 264.7 monocyte-macrophage cell line to a series of commonly studied miRNAs (Table 3.1). Sixteen hours after stimulation with indicated miRNAs complexed to DOTAP (1 to 6 ratio), conditioned medium was collected and analysed for TNF-α release. A wide range of miRNAs were found capable of producing TNF-α inRAW 264.7 cells. For values below or above the linear range of detection of the assay (8 pg/ml and 1000 pg/ml, respectively) values of 8 or 1000 are reported. Many of the miRNAs elicited high levels of TNF-α after transfection. 40 of the tested miRNAs led to production of TNF-α at or above the detection level of the assay (1000 pg/ml). Another 57 of the miRNAs elicited the release of 100 to 1000 pg/ml of TNF-α. Again it must be pointed out that RAW macrophages express not only TLR7, but also other PAMPs. Thus the effect of activation may be due to PAMP including other TLRs instead of TLR7 alone (116). This study provides initial sight of the ability of miRNAs activating the TLRs.

3.2.4 Validation of miRNA-induced TNF-α release in N1E-115 and SH-SY5Y cells

In order to further validate the miRNA induced TNF-α in RAW 264.7 cells, indicated miRNAs were combined with DOTAP and incubated with N1E-115 cells and SH-SY5Y cells. Although miR-21, miR-15a and let-7b have been previously reported to be biological relevant and induced significant amount of TNF-α in RAW 264.7 cells, they did not cause increased production of TNF-α in N1E-115 or SH-SY5Y cells (Fig 3.3 & 3.4).

Figure 3.3

A


16 Hours
Relative absorbance
0.03
0.02
0.01
0.00
miR-21
miR-124
miR-15a
Imiquimod (2.5 µg/ml)
Imiquimod (5 µg/ml)
DOTAP alone
Untreated

B

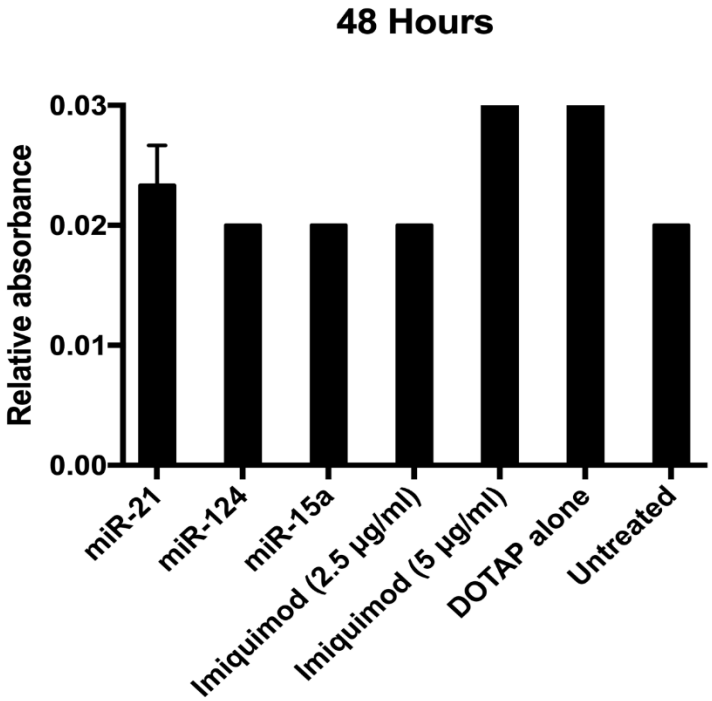

48 Hours
Relative absorbance
0.03
0.02
0.01
0.00
miR-21
miR-124
miR-15a
Imiquimod (2.5 µg/ml)
Imiquimod (5 µg/ml)
DOTAP alone
Untreated

Figure 3.3. Validation of miRNA-induced TNF-α release in N1E-115 cells.

N1E-115 cells were incubated for 16 hours or 48 hours with 6 μg/ml of indicated miRNAs. Subsequently, relative absorbance of TNF-α in the culture supernatants were determined by ELISA. Results are presented as mean ± SEM, n=3. Tested miRNAs did not induce release TNF-α release in N1E-115 cells.

Figure 3.4

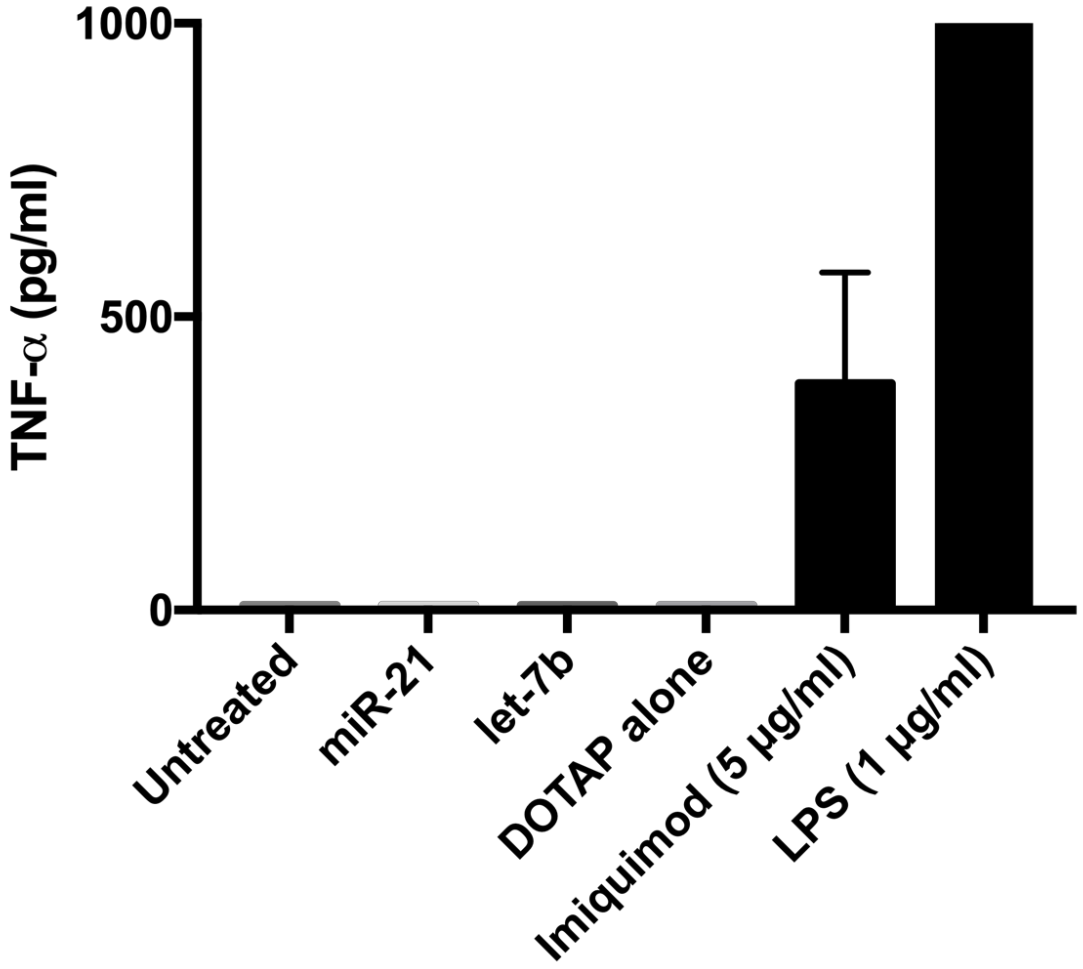

1000
500
0
TNF-α (pg/ml)
Untreated
miR-21
let-7b
DOTAP alone
Imiquimod (5 µg/ml)
LPS (1 µg/ml)

Figure 3.4. Validation of miRNA-induced TNF-α release in SH-SY5Y cells.

SH-SY5Y cells were incubated for 16 hours with 6 μg/ml of indicated miRNAs. Subsequently, the amount of TNF-α in the culture supernatants were determined by ELISA. Results are presented as mean ± SEM, n=2. Tested miRNAs did not induce release TNF-α release in SH-SY5Y cells.

3.2.5 MiRNAs induce the release of TNF-α from immortalized wild-type or TLR7 knock out bone marrow derived macrophages (BMDM).

To confirm the specificity of TLR7-activation via miRNAs, BMDMs from wild type and TLR7 deficient mice were then tested with miRNAs that induced significant amount of TNF-α in RAW 264.7 cells (Table 3.1). BMDMs were isolated and then incubated with indicated miRNAs. Subsequently, TNF-α release in the culture supernatants was determined by ELISA. As shown in Table 3.2, incubation with miR-21 and miR-15a stimulated TNF-α release (34.4 pg/ml and 15.1 pg/ml). All miRNAs did not cause release of TNF-α in TLR7 knock out BMDMs. However, let-7 family miRNAs still did not show any stimulation of TNF-α release while miR-138 produced TNF-α of over 120 pg/ml.

Table 3.2

miRNA	WT BMDM	TLR7ko BMDM
miR-149	147.7	8.0
miR-138	121.3	8.0
miR-93	112.5	8.0
miR-146b	80.6	8.0
miR-135b	65.4	8.0
miR-146a	58.4	8.0
miR-302d	45.3	8.0
miR-233	45.0	8.0
miR-21	34.4	8.0
miR-375	34.4	8.0
miR-27a	22.7	8.0
miR-10a	21.0	8.0
miR-23a	19.1	8.0
miR-196b	17.8	8.0
miR-15a	15.1	8.0
miR-196a	11.3	8.0
miR-24	11.0	8.0
miR-199a	10.8	8.0
let-7i	9.5	8.0
miR-147b	9.5	8.0
miR-20a	9.2	8.0
miR-181d	8.6	8.0
miR-133b	8.0	8.0
miR-204	8.0	8.0
miR-15b	8.0	8.0
miR-125b	8.0	8.0
miR-218	8.0	8.0
miR-99a	8.0	8.0
miR-135a	8.0	8.0

miR-142a	8.0	8.0
miR-181a-13	8.0	8.0
miR-20b	8.0	8.0
miR-133a	8.0	8.0
miR-107	8.0	8.0
miR-98	8.0	8.0
miR-122	8.0	8.0
miR-124	8.0	8.0
miR-143	8.0	8.0
miR-499	8.0	8.0
miR-187	8.0	8.0
miR-150	8.0	8.0
miR-338	8.0	8.0
miR-100	8.0	8.0
miR-301a	8.0	8.0
miR-9	8.0	8.0
let-7b	8.0	8.0
miR-25	8.0	8.0
miR-27b	8.0	8.0
miR-29a	8.0	8.0
miR-34a	8.0	8.0
miR-124	8.0	8.0
miR-10b	8.0	8.0
miR-148	8.0	8.0
miR-181c	8.0	8.0
miR-125a	8.0	8.0
miR-34c	8.0	8.0
miR-96	8.0	8.0
miR-126a	8.0	8.0
miR-152	8.0	8.0
miR-183	8.0	8.0

miR-29c	8.0	8.0
miR-148a	8.0	8.0
miR-381	8.0	8.0
miR-129	8.0	8.0
miR-433	8.0	8.0
miR-33	8.0	8.0
miR-455	8.0	8.0

Table 3.2 Names and TNF-α release when 10 μg/ml of miRNA:DOTAP complex was used to stimulate the WT or TLR7ko BMDMs.

Primary BMDMs from WT and TLR7ko mice were cultured in 96-wells culture plates. Average value of 3 duplicates indicating TNF-α amounts (pg/ml) in the culture supernatant 16 hours after addition of 10 μg/ml of miRNA:DOTAP complex is reported. For values below the linear range of detection of the assay (8 pg/ml) values of 8 are reported.

3.2.6 MiRNAs induce the release of TNF-α and IL-6 in wild-type but not in TLR7 knock out microglial cells.

Microglial cells are the resident macrophages-like cells of the CNS with a broad role in the brain's innate immunity and inflammatory processes. In order to ensure that the effects seen by miRNAs are not just in macrophages and are TLR7-dependent, we utilized microglial cell lines derived from wild-type or TLR7 knock out mice. In the wild-type microglia, tested miRNAs (miR-93 and miR-25) were capable of inducing TNF-α production at various doses (Fig 3.5). In order to further assess the role microglial TLR7 in this stimulation induced by miRNAs, we extended our study via testing 145 miRNAs. In the wild-type microglia, many of the tested miRNAs were found to elicit TNF-α or IL-6 production (Table 3.3 & 3.4). In the TLR7ko microglia on the other hand, the stimulation effects were almost completed abolished (Table 3.3 & 3.4).

3.2.7 let-7b-5p does not induce TNF-α production in BV-2 microglia or primary mouse microglia.

Let-7 family miRNAs contains a GU-rich core motif in proximal to 3' end. Previous studies have shown the stimulation effects of let-7 family miRNAs, in particular let-7b (27). We were puzzled by our results with the let-7 family miRNAs in macrophages and microglial cells (Table 3.1, 3.2, 3.3). Only let-7i-tp induced TNF-α in RAW 264.7 macrophages and wild-type microglia in our experiments. We thus tested let-7b-5p in both the BV-2 microglial cells as well as the primary mouse microglia. Based on our previous data from RAW 264.7 cells and wild-type microglia, a potent and poor miRNA inducer of TNF-α (miR-20a-5p and miR-17-5p, respectively) were also tested. Let-7b-5p failed to induce TNF-α in BV-2 microglia or primary mouse microglia (Fig 3.6).

3.2.8 Modification of let-7 family miRNAs does not induce TNF-α production in wild-type mouse microglia.

While we utilized oligonucleotides with phosphodiester linkages, it has been reported that free (not complexed with DOTAP or other transfection molecules) let-7b could signal through TLR7. In addition, many studies use phosphorothioate linkages to make the oligonucleotides resistant to RNase. We thus treated the wild-type microglia with newer preparations of let-7b-5p (as well as two other strong miRNA inducer of TNF-α) containing phosphodiester or phosphorothioate linkages, as well as with phosphodiester linkages but in the absence of DOTAP. Under these treatments, let-7b-5p was still incapable of inducing TNF-α (Fig 3.7).

Figure 3.5

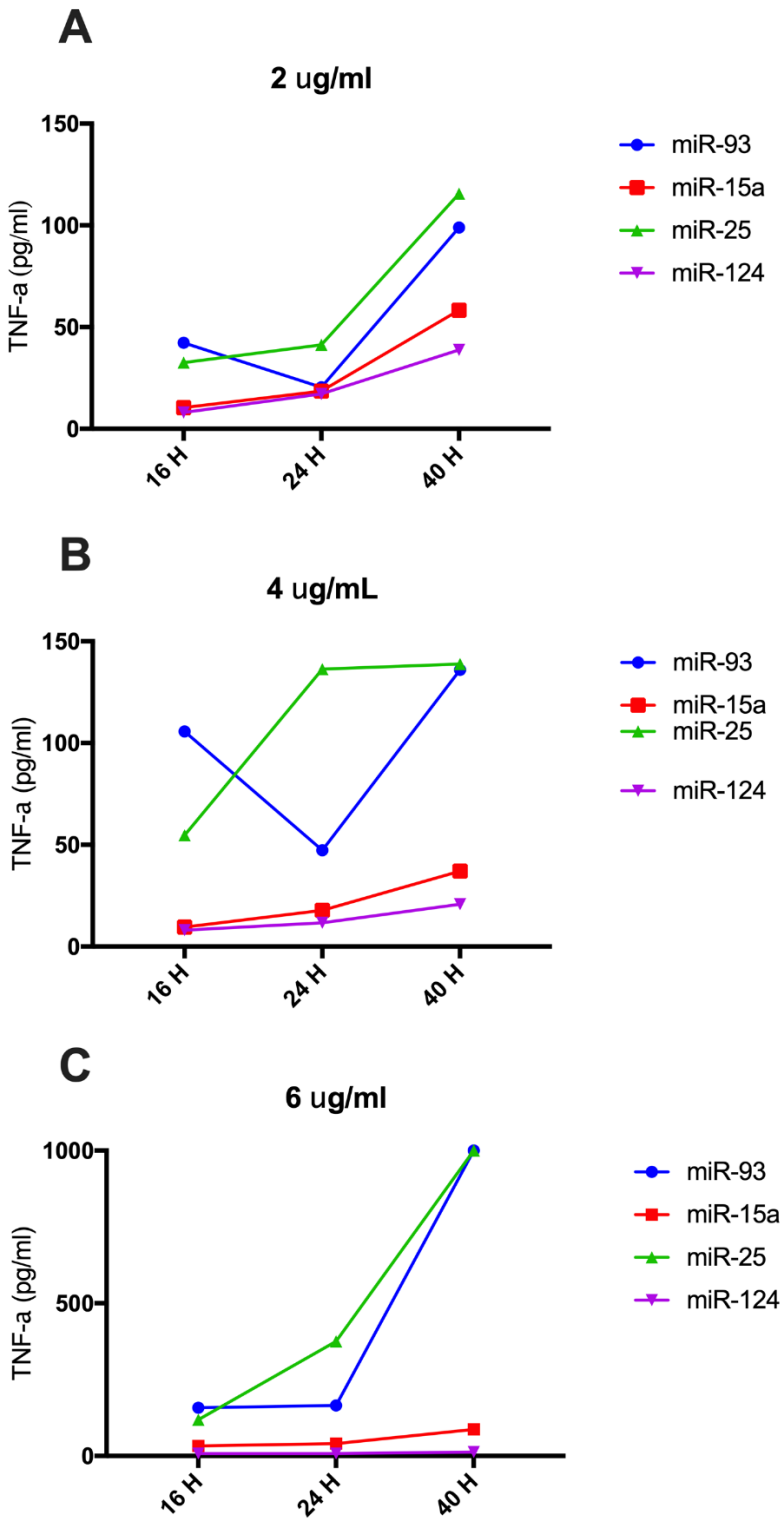

A
2 ug/ml
TNF-a (pg/ml)
150
100
50
0
16 H
24 H
40 H
miR-93
miR-15a
miR-25
miR-124
B
4 ug/mL
TNF-a (pg/ml)
150
100
50
0
16 H
24 H
40 H
miR-93
miR-15a
miR-25
miR-124
C
6 ug/ml
TNF-a (pg/ml)
1000
500
0
16 H
24 H
40 H
miR-93
miR-15a
miR-25
miR-124

Figure 3.5 Various doses of miRNAs induce the release of TNF-α in wild-type microglial cells.

Wild-type mice microglial cells were incubated with indicated doses of miRNAs for 16, 24 or 40 hours. Culture supernatants were collected and assesses by TNF-α ELISA. Data represent results of technical duplicates.

Table 3.3

miRNA	Sequence	WT	TLR ko
let-7a-5p	5'-UGAGGUAGUAGGUUGUAUAGUU-3'	33.08	n.d.
let-7b-5p	5'-UGAGGUAGUAGGUUGUGUGGUU-3'	n.d.	n.d.
let-7c-5p	5'-UGAGGUAGUAGGUUGUAUGGUU-3'	n.d.	n.d.
let-7d-5p	5'-AGAGGUAGUAGGUUGCAUAGUU-3'	n.d.	n.d.
let-7e-5p	5'-UGAGGUAGGAGGUUGUAUAGUU-3'	n.d.	n.d.
let-7f-5p	5'-UGAGGUAGUAGAUUGUAUAGUU-3'	0.61	n.d.
let-7g-5p	5'-UGAGGUAGUAGUUUGUACAGUU-3'	2.75	n.d.
let-7i-5p	5'-UGAGGUAGUAGUUUGUGCUGUU-3'	320.74	n.d.
miR-100-5p	5'-AACCCGUAGAUCCGAACUUGUG-3'	459.51	n.d.
miR-101a-3p	5'-UACAGUACUGUGAUAACUGAA-3'	n.d.	n.d.
miR-103-3p	5'-AGCAGCAUUGUACAGGGCUAUGA-3'	12.31	n.d.
miR-106b-5p	5'-UAAAGUGCUGACAGUGCAGAU-3'	n.d.	n.d.
miR-107-3p	5'-AGCAGCAUUGUACAGGGCUAUCA-3'	48.94	n.d.
miR-10a-5p	5'-UACCCUGUAGAUCCGAAUUUGUG-3'	225.40	n.d.
miR-10b-5p	5'-UACCCUGUAGAACCGAAUUUGUG-3'	129.36	n.d.
miR-122-5p	5'-UGGAGUGUGACAAUGGUGUUUG-3'	155.80	n.d.
miR-124-3p	5'-UAAGGCACGCGGUGAAUGCC-3'	n.d.	n.d.
miR-125a-5p	5'-UCCCUGAGACCCUUUAACCUGUGA-3'	40.14	n.d.
miR-125b-5p	5'-UCCCUGAGACCCUAACUUGUGA-3'	156.70	n.d.
miR-126a-3p	5'-UCGUACCGUGAGUAAUAAUGCG-3'	n.d.	n.d.
miR-126a-5p	5'-CAUUAUUACUUUUGGUACGCG-3'	33.02	n.d.
miR-128-3p	5'-UCACAGUGAACCGGUCUCUUU-3'	n.d.	n.d.
miR-129-5p	5'-CUUUUUGCGGUCUGGGCUUGC-3'	36.85	n.d.
miR-130a-3p	5'-CAGUGCAAUGUUAAAAGGGCAU-3'	n.d.	n.d.
miR-130b-3p	5'-CAGUGCAAUGAUGAAAGGGCAU-3'	n.d.	n.d.
miR-132-3p	5'-UAACAGUCUACAGCCAUGGUCG-3'	n.d.	n.d.
miR-133a-3p	5'-UUUGGUCCCCUUCAACCAGCUG-3'	72.28	n.d.
miR-133b-3p	5'-UUUGGUCCCCUUCAACCAGCUA-3'	72.51	n.d.
miR-134-5p	5'-UGUGACUGGUUGACCAGAGGGG-3'	n.d.	n.d.
miR-135a-5p	5'-UAUGGCUUUUUAUUCCUAUGUGA-3'	206.44	n.d.

miR-135b-5p	5'-UAUGGCUUUUCAUUCCUAUGUGA-3'	429.45	n.d.
miR-137-3p	5'-UUAUUGCUUAAGAAUACGCGUAG-3'	n.d.	n.d.
miR-138-5p	5'-AGCUGGUGUUGUGAAUCAGGCCG-3'	87.47	n.d.
miR-140-3p	5'-UACCACAGGGUAGAACCACGG-3'	n.d.	n.d.
miR-140-5p	5'-CAGUGGUUUUACCCUAUGGUAG-3'	8.92	n.d.
miR-141-3p	5'-UAACACUGUCUGGUAAAGAUGG-3'	7.65	n.d.
miR-142a-3p	5'-UGUAGUGUUUCCUACUUUAUGGA-3'	112.65	n.d.
miR-142a-5p	5'-CAUAAAGUAGAAAGCACUACU-3'	n.d.	n.d.
miR-143-3p	5'-UGAGAUGAAGCACUGUAGCUC-3'	n.d.	n.d.
miR-144-3p	5'-UACAGUAUAGAUGAUGUACU-3'	n.d.	n.d.
miR-145a-5p	5'-GUCCAGUUUUCCCAGGAAUCCCU-3'	n.d.	n.d.
miR-146a-5p	5'-UGAGAACUGAAUUCCAUGGGUU-3'	18.00	n.d.
miR-146b-5p	5'-UGAGAACUGAAUUCCAUAGGCU-3'	22.82	n.d.
miR-147-3p	5'-GUGUGCGGAAAUGCUUCUGCUA-3'	25.59	n.d.
miR-148a-3p	5'-UCAGUGCACUACAGAACUUUGU-3'	130.63	n.d.
miR-148b-3p	5'-UCAGUGCAUCACAGAACUUUGU-3'	453.95	n.d.
miR-149-5p	5'-UCUGGCUCCGUGUCUUCACUCCC-3'	156.73	n.d.
miR-150-5p	5'-UCUCCCAACCCUUGUACCAGUG-3'	110.40	n.d.
miR-152-3p	5'-UCAGUGCAUGACAGAACUUGG-3'	294.92	n.d.
miR-15a-5p	5'-UAGCAGCACAUAAUGGUUUGUG-3'	89.11	n.d.
miR-15b-5p	5'-UAGCAGCACAUCAUGGUUUACA-3'	n.d.	n.d.
miR-16-5p	5'-UAGCAGCACGUAAAUAUUGGCG-3'	n.d.	n.d.
miR-17-5p	5'-CAAAGUGCUUACAGUGCAGGUAG-3'	30.32	n.d.
miR-181a-1-3p	5'-ACCAUCGACCGUUGAUUGUACC-3'	2.23	n.d.
miR-181a-5p	5'-AACAUUCAACGCUGUCGGUGAGU-3'	n.d.	n.d.
miR-181b-5p	5'-AACAUUCAUUGCUGUCGGUGGGU-3'	n.d.	n.d.
miR-181c-5p	5'-AACAUUCAACCUGUCGGUGAGU-3'	6.28	n.d.
miR-181d-5p	5'-AACAUUCAUUGUUGUCGGUGGGU-3'	n.d.	n.d.
miR-183-5p	5'-UAUGGCACUGGUAGAAUUCACU-3'	19.24	n.d.
miR-184-3p	5'-UGGACGGAGAACUGAUAAGGGU-3'	n.d.	n.d.
miR-185-5p	5'-UGGAGAGAAAGGCAGUUCCUGA-3'	2.33	n.d.
miR-186-5p	5'-CAAAGAAUUCUCCUUUUGGGCU-3'	n.d.	n.d.
miR-187-3p	5'-UCGUGUCUUGUGUUGCAGCCGG-3'	112.44	n.d.

miR-18a-5p	5'-UAAGGUGCAUCUAGUGCAGAUAG-3'	n.d.	n.d.
miR-191-5p	5'-CAACGGAAUCCCAAAAGCAGCUG-3'	n.d.	n.d.
miR-192-5p	5'-CUGACCUAUGAAUUGACAGCC-3'	n.d.	n.d.
miR-193a-3p	5'-AACUGGCCUACAAAGUCCCAGU-3'	n.d.	n.d.
miR-194-5p	5'-UGUAACAGCAACUCCAUGUGGA-3'	n.d.	n.d.
miR-195a-5p	5'-UAGCAGCACAGAAAUAUUGGC-3'	n.d.	n.d.
miR-196a-5p	5'-UAGGUAGUUUCAUGUUGUUGGG-3'	85.89	n.d.
miR-196b-5p	5'-UAGGUAGUUUCCUGUUGUUGGG-3'	17.69	n.d.
miR-199a-5p	5'-CCCAGUGUUCAGACUACCUGUUC-3'	146.60	n.d.
miR-19a-3p	5'-UGUGCAAAUCUAUGCAAAACUGA-3'	n.d.	n.d.
miR-19b-3p	5'-UGUGCAAAUCCAUGCAAAACUGA-3'	n.d.	n.d.
miR-1a-3p	5'-UGGAAUGUAAAGAAGUAUGUAU-3'	5.44	n.d.
miR-200a-3p	5'-UAACACUGUCUGGUAACGAUGU-3'	n.d.	n.d.
miR-200b-3p	5'-UAAUACUGCCUGGUAAUGAUGA-3'	7.81	n.d.
miR-200c-3p	5'-UAAUACUGCCGGGUAAUGAUGGA-3'	6.91	n.d.
miR-203-3p	5'-GUGAAAUGUUUAGGACCACUAG-3'	24.83	n.d.
miR-204-5p	5'-UUCCCUUUGUCAUCCUAUGCCU-3'	78.63	n.d.
miR-205-5p	5'-UCCUUCAUUCCACCGGAGUCUG-3'	20.69	n.d.
miR-206-3p	5'-UGGAAUGUAAGGAAGUGUGUGG-3'	n.d.	n.d.
miR-20a-5p	5'-UAAAGUGCUUAUAGUGCAGGUAG-3'	438.41	n.d.
miR-20b-5p	5'-CAAAGUGCUCAUAGUGCAGGUAG-3'	75.49	n.d.
miR-210-3p	5'-CUGUGCGUGUGACAGCGGCUGA-3'	n.d.	n.d.
miR-214-3p	5'-ACAGCAGGCACAGACAGGCAGU-3'	14.91	n.d.
miR-218-5p	5'-UUGUGCUUGAUCUAACCAUGU-3'	25.59	n.d.
miR-21a-5p	5'-UAGCUUAUCAGACUGAUGUUGA-3'	102.39	n.d.
miR-22-3p	5'-AAGCUGCCAGUUGAAGAACUGU-3'	11.99	n.d.
miR-221-3p	5'-AGCUACAUUGUCUGCUGGGUUUC-3'	140.03	n.d.
miR-222-3p	5'-AGCUACAUCUGGCUACUGGGU-3'	n.d.	n.d.
miR-223-3p	5'-UGUCAGUUUGUCAAAUACCCCA-3'	118.81	n.d.
miR-23a-3p	5'-AUCACAUUGCCAGGGAUUUCC-3'	176.53	n.d.
miR-23b-3p	5'-AUCACAUUGCCAGGGAUUACC-3'	11.21	n.d.
miR-24-3p	5'-UGGCUCAGUUCAGCAGGAACAG-3'	49.22	n.d.
miR-25-3p	5'-CAUUGCACUUGUCUCGGUCUGA-3'	202.69	n.d.

miR-26a-5p	5'-UUCAAGUAAUCCAGGAUAGGCU-3'	6.49	n.d.
miR-26b-5p	5'-UUCAAGUAAUUCAGGAUAGGU-3'	27.05	n.d.
miR-27a-3p	5'-UUCACAGUGGCUAAGUUCCGC-3'	42.58	n.d.
miR-27b-3p	5'-UUCACAGUGGCUAAGUUCUGC-3'	134.92	n.d.
miR-28a-5p	5'-AAGGAGCUCACAGUCUAUUGAG-3'	n.d.	n.d.
miR-296-5p	5'-AGGGCCCCCCCUCAAUCCUGU-3'	4.49	n.d.
miR-29a-3p	5'-UAGCACCAUCUGAAAUCGGUUA-3'	88.01	n.d.
miR-29b-3p	5'-UAGCACCAUUUGAAAUCAGUGUU-3'	14.62	n.d.
miR-29c-3p	5'-UAGCACCAUUUGAAAUCGGUUA-3'	60.64	n.d.
miR-301a-3p	5'-CAGUGCAAUAGUAUUGUCAAAGC-3'	47.58	n.d.
miR-302d-3p	5'-UAAGUGCUUCCAUGUUUGAGUGU-3'	39.26	n.d.
miR-30a-5p	5'-UGUAAACAUCCUCGACUGGAAG-3'	n.d.	n.d.
miR-30b-5p	5'-UGUAAACAUCCUACACUCAGCU-3'	11.43	n.d.
miR-30c-5p	5'-UGUAAACAUCCUACACUCUCAGC-3'	17.98	n.d.
miR-30d-5p	5'-UGUAAACAUCCCCGACUGGAAG-3'	13.14	n.d.
miR-30e-5p	5'-UGUAAACAUCCUUGACUGGAAG-3'	11.31	n.d.
miR-32-5p	5'-UAUUGCACAUUACUAAGUUGCA-3'	14.99	n.d.
miR-320-3p	5'-AAAAGCUGGGUUGAGAGGGCGA-3'	11.81	n.d.
miR-328-3p	5'-CUGGCCCUCUCUGCCCUUCCGU-3'	92.96	n.d.
miR-33-5p	5'-GUGCAUUGUAGUUGCAUUGCA-3'	31.28	n.d.
miR-335-5p	5'-UCAAGAGCAAUAACGAAAAAUGU-3'	14.36	n.d.
miR-338-3p	5'-UCCAGCAUCAGUGAUUUUGUUG-3'	455.50	n.d.
miR-339-5p	5'-UCCCUGUCCUCCAGGAGCUCACG-3'	11.17	n.d.
miR-342-3p	5'-UCUCACACAGAAAUCGCACCCGU-3'	12.34	n.d.
miR-34a-5p	5'-UGGCAGUGUCUUAGCUGGUUGU-3'	259.98	n.d.
miR-34c-5p	5'-AGGCAGUGUAGUUAGCUGAUUGC-3'	31.39	n.d.
miR-375-3p	5'-UUUGUUCGUUCGGCUCGCGUGA-3'	111.60	n.d.
miR-381-3p	5'-UAUACAAGGGCAAGCUCUCUGU-3'	27.06	n.d.
miR-409-3p	5'-GAAUGUUGCUCGGUGAACCCCU-3'	14.27	n.d.
miR-411-5p	5'-UAGUAGACCGUAUAGCGUACG-3'	15.14	n.d.
miR-423-5p	5'-UGAGGGGCAGAGAGCGAGACUUU-3'	12.08	n.d.
miR-425-5p	5'-AAUGACACGAUCACUCCCGUUGA-3'	12.84	n.d.
miR-431-5p	5'-UGUCUUGCAGGCCGUCAUGCA-3'	13.32	n.d.

miR-433-3p	5'-AUCAUGAUGGGCUCCUCGGUGU-3'	16.37	n.d.
miR-451a	5'-AAACCGUUACCAUUACUGAGUU-3'	12.58	n.d.
miR-455-5p	5'-UAUGUGCCUUUGGACUACAUCG-3'	17.77	n.d.
miR-484	5'-UCAGGCUCAGUCCCCUCCCGAU-3'	12.61	n.d.
miR-485-5p	5'-AGAGGCUGGCCGUGAUGAAUUC-3'	24.73	n.d.
miR-499-5p	5'-UUAAGACUUGCAGUGAUGUUU-3'	52.46	n.d.
miR-574-3p	5'-CACGCUCAUGCACACACCCACA-3'	15.23	n.d.
miR-652-3p	5'-AAUGGCGCCACUAGGGUUGUG-3'	11.78	n.d.
miR-744-5p	5'-UGCGGGGCUAGGGCUAACAGCA-3'	10.81	n.d.
miR-7a-5p	5'-UGGAAGACUAGUGAUUUUGUUGU-3'	12.39	n.d.
miR-9-3p	5'-AUAAAGCUAGAUAACCGAAAGU-3'	67.42	n.d.
miR-9-5p	5'-UCUUUGGUUAUCUAGCUGUAUGA-3'	232.52	n.d.
miR-93-5p	5'-CAAAGUGCUGUUCGUGCAGGUAG-3'	438.93	n.d.
miR-96-5p	5'-UUUGGCACUAGCACAUUUUUGCU-3'	27.44	n.d.
miR-98-5p	5'-UGAGGUAGUAAGUUGUAUUGUU-3'	113.92	n.d.
miR-99a-5p	5'-AACCCGUAGAUCCGAUCUUGUG-3'	437.62	n.d.

Table 3.3 Names, sequences, and TNF-α release when 6 μg/ml miRNA:DOTAP complex was used to stimulate the wild-type (WT) or TLR7 knock out (TLR7ko) microglia.

Values indicate TNF-α levels (pg/ml) in the culture supernatant 16 hours after addition of 6 μg/ml miRNA:DOTAP complex, average of technical duplicates is reported.

n.d.: not detected.

Table 3.4

miRNA	Sequence	WT	TLR7 ko
miR-10a-5p	5'- UACCCUGUAGAUCCGAAUUUGUG -3'	n.d.	n.d.
miR-10b-5p	5'- UACCCUGUAGAACCGAAUUUGUG -3'	n.d.	n.d.
miR-135a-5p	5'- UAUGGCUUUUUAUUCCUAUGUGA -3'	17	n.d.
miR-135b-5p	5'- UAUGGCUUUUCAUUCCUAUGUGA -3'	57	n.d.
miR-146a-5p	5'- UGAGAACUGAAUUCCAUGGGUU -3'	n.d.	n.d.
miR-148a-3p	5'- UCAGUGCACUACAGAACUUUGU -3'	25	n.d.
miR-148b-3p	5'- UCAGUGCAUCACAGAACUUUGU -3'	170	n.d.
miR-149-5p	5'- UCUGGCUCCGUGUCUUCACUCCC -3'	n.d.	n.d.
miR-196a-5p	5'- UAGGUAGUUUCAUGUUGUUGGG -3'	10	n.d.
miR-196b-5p	5'- UAGGUAGUUUCCUGUUGUUGGG -3'	n.d.	n.d.
miR-20a-5p	5'- UAAAGUGCUUAUAGUGCAGGUAG -3'	192	n.d.
miR-20b-5p	5'- CAAAGUGCUCAUAGUGCAGGUAG -3'	n.d.	n.d.
miR-21-5p	5'- UAGCUUAUCAGACUGAUGUUGA -3'	n.d.	n.d.
miR-23a-3p	5'- AUCACAUUGCCAGGGAUUUCC -3'	29	n.d.
miR-23b-3p	5'- AUCACAUUGCCAGGGAUUACC -3'	n.d.	n.d.
miR-27b-3p	5'- UUCACAGUGGCUAAGUUCUGC -3'	n.d.	n.d.
miR-27a-3p	5'- UUCACAGUGGCUAAGUUCCGC -3'	n.d.	n.d.
miR-29a-3p	5'- UAGCACCAUCUGAAAUCGGUUA -3'	n.d.	n.d.
miR-29b-3p	5'- UAGCACCAUUUGAAAUCAGUGUU -3'	n.d.	n.d.
miR-29c-3p	5'- UAGCACCAUUUGAAAUCGGUUA -3'	n.d.	n.d.
miR-338-3p	5'- UCCAGCAUCAGUGAUUUUGUUG -3'	92	n.d.
miR-9-5p	5'- UCUUUGGUUAUCUAGCUGUAUGA -3'	17	n.d.
miR-93-5p	5'- CAAAGUGCUGUUCGUGCAGGUAG -3'	163	n.d.
let-7a-5p	5'- UGAGGUAGUAGGUUGUAUAGUU -3'	n.d.	n.d.
let-7b-5p	5'- UGAGGUAGUAGGUUGUGUGGUU -3'	n.d.	n.d.
let-7c-5p	5'- UGAGGUAGUAGGUUGUAUGGUU -3'	n.d.	n.d.
let-7d-5p	5'- AGAGGUAGUAGGUUGCAUAGUU -3'	n.d.	n.d.
let-7e-5p	5'- UGAGGUAGGAGGUUGUAUAGUU -3'	n.d.	n.d.
let-7f-5p	5'- UGAGGUAGUAGAUUGUAUAGUU -3'	n.d.	n.d.

let-7g-5p	5'- UGAGGUAGUAGUUUGUACAGUU -3'	n.d.	n.d.
let-7i-5p	5'- UGAGGUAGUAGUUUGUGCUGUU -3'	28	n.d.

Table 3.4 Names, sequences, and IL-6 release when 6 μg/ml miRNA:DOTAP complex was used to stimulate the wild-type (WT) or TLR7 knock out (TLR7ko) microglia.

Values indicate IL-6levels (pg/ml) in the culture supernatant 16 hours after addition of 6 μg/ml miRNA:DOTAP complex, average of technical duplicates is reported.

n.d.: not detected.

Figure 3.6

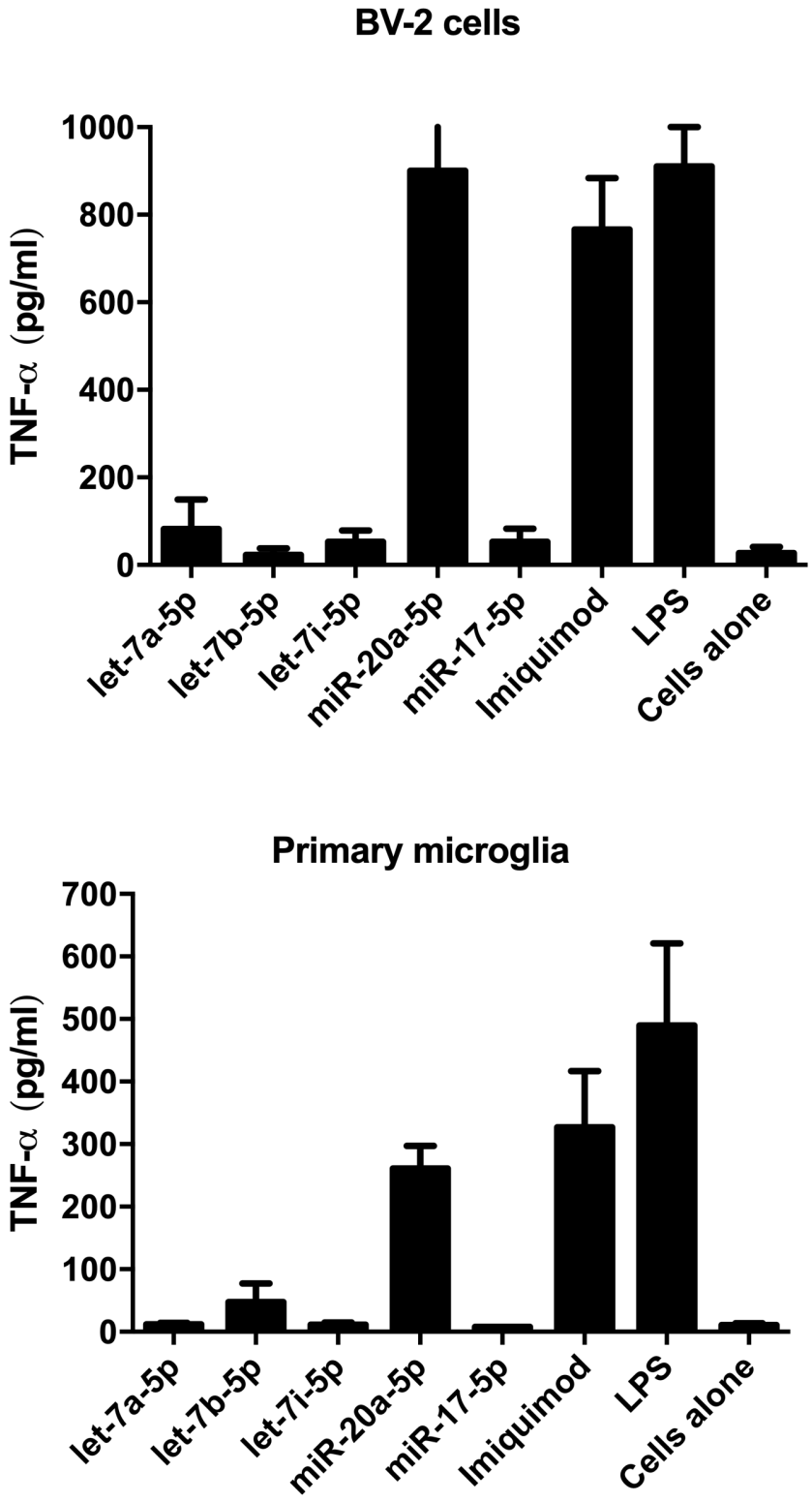

BV-2 cells
TNF-α (pg/ml)
1000
800
600
400
200
0
let-7a-5p
let-7b-5p
let-7i-5p
miR-20a-5p
miR-17-5p
Imiquimod
LPS
Cells alone
Primary microglia
TNF-α (pg/ml)
700
600
500
400
300
200
100
0
let-7a-5p
let-7b-5p
let-7i-5p
miR-20a-5p
miR-17-5p
Imiquimod
LPS
Cells alone

Figure 3.6. Let-7b-5p does not induce TNF-α in BV-2 microglia or primary mouse microglia.

A) BV-2 cells, treated with 6 μg/ml miRNAs as well as positive controls (5 μg/ml Imiquimod or 1 μg/ml LPS) for 16 hours or were left untreated (cells alone). B) Primary mouse microglia from C57BL/6 mice, treated with 10 μg/ml miRNA complexed to DOTAP, 10 μg/ml Imiquimod, 1 μg/ml LPS, or left untreated. Culture supernatants were collected and assesses by TNF-α ELISA. Data represent results mean ± SEM. n=3 independent experiments.

Figure 3.7

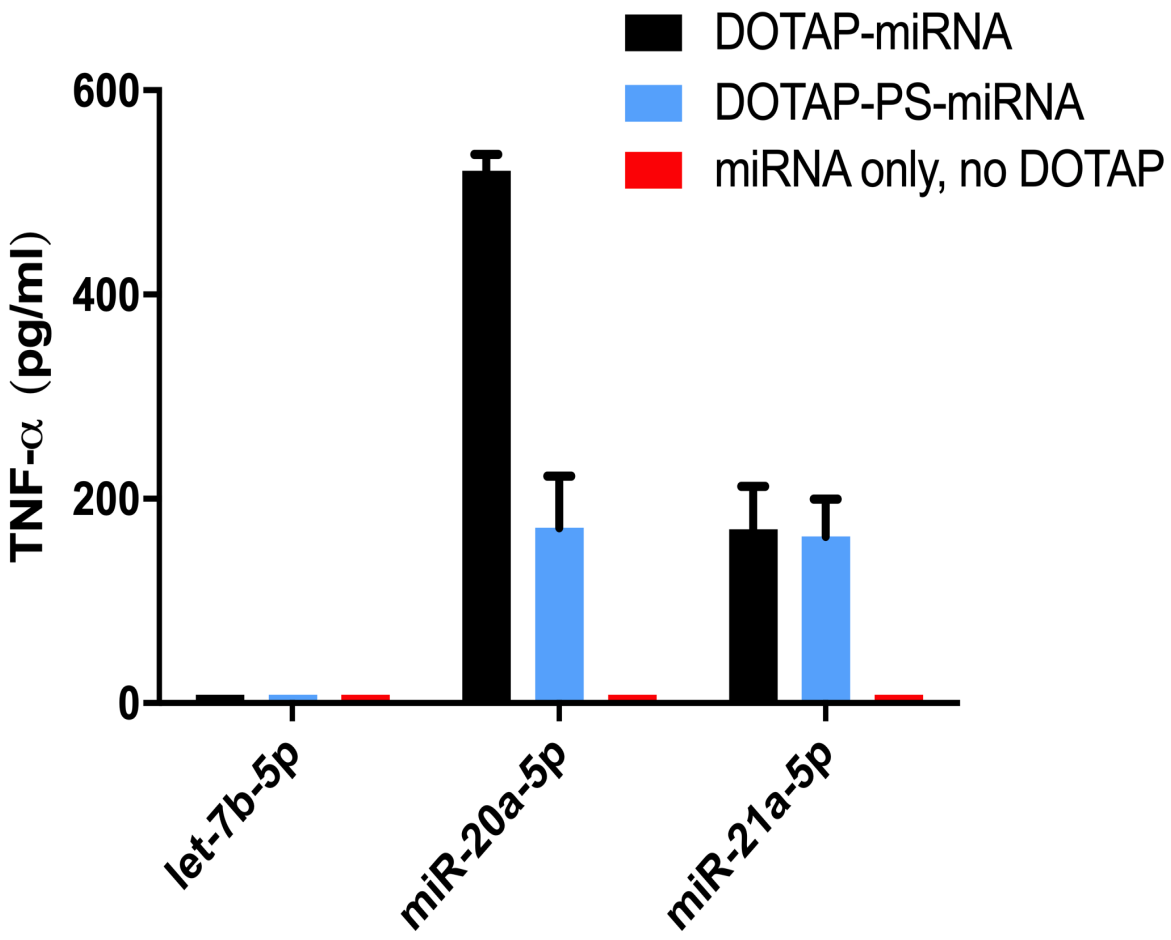

DOTAP-miRNA
DOTAP-PS-miRNA
miRNA only, no DOTAP
TNF-α (pg/ml)
600
400
200
0
let-7b-5p
miR-20a-5p
miR-21a-5p

Figure 3.7 Modification of let-7b-5p does not induce TNF-α in wild-type mouse microglia.

Wild-type microglial cells were treated with 6 μg/ml miRNAs containing phosphodiester or phosphorothioate (PS) linkage complexed (or not) to the transfection agent DOTAP for 16 hours. Culture supernatants were collected and assesses by TNF-α ELISA. Data represent results mean ± SEM. n=3 independent experiments.

3.3 Discussion

Our studies complement and expand upon knowledge of miRNA-TLR interactions, and provide significant new insights. 40 of the tested miRNAs led to production of TNF-α at or above the detection level of the assay (1000 pg/ml) in RAW 264.7 cells; Another 57 of the miRNAs elicited the release of 100 to 1000 pg/ml of TNF-α. Transfection with synthetic miRNAs complexed with DOTAP, especially with miR-21, also stimulated TNF-α release in wild-type BMDMs, whereas all miRNAs did not cause release of TNF-α in TLR7 knock out BMDMs. Previous work from our lab showed miR-21 was increased in EVs in traumatic brain injuries and can also lead to neuronal cell death (26,117,118), and others miR-21 as well as let-7c to suppress the dendritic growth of cortical neurons in a TLR7 dependent manner (34). Our works show that miRNAs can signal through TLR7 in macrophages.

We also found a broad range of miRNAs that can signal through TLR7 in WT microglia; 59 of the 145 miRNAs initially tested led to the production of >25 pg/ml of TNF-α, and 30 of those with levels of >100 pg/ml, whereas in TLR7ko microglia none reached the level of detection of the assay (8 pg/ml). Furthermore, miR-20a and miR-93 led to TNF-α and IL-6 productions while their closely related miRNAs (miR-20b and miR-17) caused no productions of TNF-α or IL-6. Infusion of miR-20a into the uninjured spinal cord caused inflammation and neuronal cell death similar to the pathology observed after trauma-induced Spinal Cord Injury in patients (119).

A number of miRNAs have been previously found to signal through TLR7 in different cell types (predominately in macrophages and microglia lineage): let-7a, -7b, -7c, -7g; and miR-21, -29a, -29b, -34a, -122, -133a, -142, -146a, -208a, -599 (25,27,28,35). Although miRNA:DOTAP complexes caused TLR7-dependent cytokine production in macrophages and microglial cells,

synthetic miRNAs did not induce TNF-α production in SH-SY5Y or N1E-115 cells in our experiments. We think that synthetic miRNAs may cause various effects depending on the morphology of cells. We were puzzled by the relative lack of TNF-α production following treatment with the let-7 family members (just low amounts with let-7a, higher levels only with let-7i) in our experiments, despite attempting to replication conditions (with and without DOTAP, and the use of phosphorothiolate as well as phosphodiesterase linkages. It is still not clear the reason for this difference. However the others that were tested in detail (miR-21, -29a, -34a, and -122) indeed activated microglia and macrophages through TLR7.

Current therapeutic activities for neurodegenerative disorders are restricted due to the limited understanding of their underlying mechanisms as well as the difficulties posed in accurately diagnosing. Pathological changes can initiate earlier than the appearance of clinical symptoms. Recently, miRNA microarray, miRNA qPCR assay and especially deep-sequencing technology has been applied for identification of differentially expresses miRNAs during the progress of neurodegenerative diseases(120,121). Exosomal miRNA profiles from human biological fluids such as CSF and plasma have prompted the potential application of miRNAs as diagnostic biomarkers for neurodegenerative diseases (63,122-124). However, current methods of isolation and purification of EVs via ultracentrifugation cannot achieve the goal of isolating the EVs with high-yield and may stand in the way of mass application of exosomal diagnostic biomarkers.

It is also worth noting that the free miRNAs without DOTAP complexation did not induce TNF-a release in vitro, indicating miRNA mimics need to be enveloped in some type of carriers, such as DOTAP, to protect them from degradation and/or enable them to efficiently reach the endosome. Circulating miRNAs are also enveloped in some type of carriers, such as EVs. Our

miRNA transfection complex is shown to be relatively intoxic, thus providing a physiological method of delivering synthetic miRNAs for potential therapeutic purposes.

In summary, we screened a wide profile of miRNAs inducing TNF-α and IL-6 responses in macrophages and microglial cells. Our studies provide further evidence that miRNAs can act as signals for TLR7 activation. These finding correlate with dysregulation of miRNAs in CNS diseases.

Chapter 4. Sequence-specific miRNA-induced TLR7 activation *in vitro* and *in vivo*

4.1 Introduction

In the current study, we screened a wide profile of miRNAs inducing TNF-α and IL-6 responses in macrophages and microglial cells. Our study provides further evidence that miRNAs can act as signals for TLR7 activation. However, little is known about consensus sequences needed to activate TLR7, although GU-content, GU-repeats, and poly-U are frequently implicated in this process (10,11,13,25,27,36). In the present study we hypothesized that several miRNAs could activate endosomal TLR7, leading in turn, to increased production of pro-inflammatory molecules, and induction of inflammatory responses in vivo. We thus chose miRNAs on the basis of their validated expression in tissues and diseases and focused on those with sequence homology in mice and human, as well as assessed these for TLR7-specific stimulation of TNF-α release. We also examined the signaling transduction pathways used by miRNAs for transcriptional stimulation of pro-inflammatory molecules. Additionally, we also analyzed the miRNA sequence motifs that could stimulate mouse TLR7, and that were active in vivo.

4.2.1 In-depth examination of closely related miRNAs

Our previous results have shown that miRNAs can induce TNF-α production in a TLR7-dependent manner. Despite the failure of let-7b family miRNAs to induce TNF-α production, we performed a more in-depth examination of a subset of miRNAs based on our data from wild-type microglia (Table 3.3). We focused on several families of miRNAs containing members sharing related sequences but divergent TNF-α responses. The TLR7 ligand Imiquimod and TLR4 ligand LPS were used as controls (Fig 4.1B). Parallel studies in TLR7 knock out microglia showed that none could stimulate TNF-α production (Fig 4.1B). Within the miR-20a-5p family

and miR-148b-3p family, a range of TNF-α was produced (Fig 4.1A). These findings suggested the existence of key motifs and/or nucleotides that could be responsible for TLR7 stimulation.

Figure 4.1

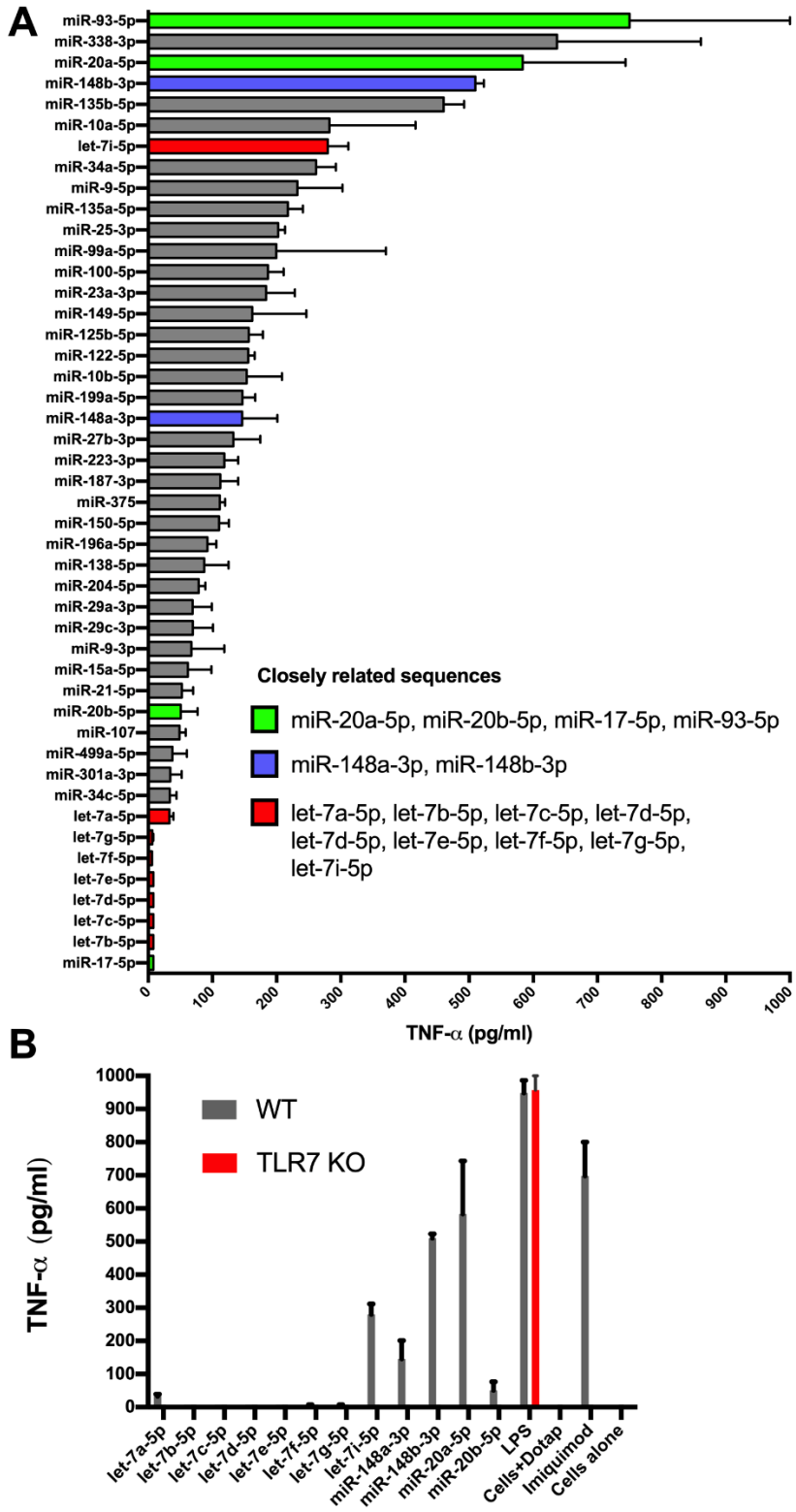

A
miR-93-5p
miR-338-3p
miR-20a-5p
miR-148b-3p
miR-135b-5p
miR-10a-5p
let-7i-5p
miR-34a-5p
miR-9-5p
miR-135a-5p
miR-25-3p
miR-99a-5p
miR-100-5p
miR-23a-3p
miR-149-5p
miR-125b-5p
miR-122-5p
miR-10b-5p
miR-199a-5p
miR-148a-3p
miR-27b-3p
miR-223-3p
miR-187-3p
miR-375
miR-150-5p
miR-196a-5p
miR-138-5p
miR-204-5p
miR-29a-3p
miR-29c-3p
miR-9-3p
miR-15a-5p
miR-21-5p
miR-20b-5p
miR-107
miR-499a-5p
miR-301a-3p
miR-34c-5p
let-7a-5p
let-7g-5p
let-7f-5p
let-7e-5p
let-7d-5p
let-7c-5p
let-7b-5p
miR-17-5p
Closely related sequences
miR-20a-5p, miR-20b-5p, miR-17-5p, miR-93-5p
miR-148a-3p, miR-148b-3p
let-7a-5p, let-7b-5p, let-7c-5p, let-7d-5p, let-7d-5p, let-7e-5p, let-7f-5p, let-7g-5p, let-7i-5p
0
100
200
300
400
500
600
700
800
900
1000
TNF-α (pg/ml)
B
TNF-α (pg/ml)
WT
TLR7 KO
let-7a-5p
let-7b-5p
let-7c-5p
let-7d-5p
let-7e-5p
let-7f-5p
let-7g-5p
let-7i-5p
miR-148a-3p
miR-148b-3p
miR-20a-5p
miR-20b-5p
LPS
Cells+Dotap
Imiquimod
Cells alone

Figure 4.1. Closely related miRNA sequences differ in TLR7 activation.

A) Wild-type microglia were treated with indicated miRNAs, conditions as in Table 3.3. Green, purple and red shading indicate miRNAs with closely related sequences. Results are shown as mean ± SEM. These experiments were performed with three independent experiments. B) The deficiency of TLR7 abolished imiquimod-induced TNF-α production in TLR7 knock out cells. The TLR7 ligand imiquimod was used at 5 µg/ml, the TLR4 ligand LPS was used at 1 µg/ml (as a positive control in WT and TLR7ko microglia), as a negative control DOTAP was added without any miRNA (cells+DOTAP). Results are presented as mean ± SEM. These experiments were performed with three independent experiments.

4.2.2 Specific U → C miRNA sequence mutations and "UGCUUAU" motif deletion diminishes miRNA-induced cytokine response

It was previously hypothesized that a GU-rich element contained in miRNAs can specifically stimulate TLR7 (27). Based on our data, miR-148b-3p caused significantly more TNF-α production in wild-type microglia than miR-148a-3p despite both having 18% G and 32% U elements (Table 4.1, Fig 4.1). These findings indicate that GU-rich may not be the sole requirement for TLR7 activation in microglia. Key nucleotides or sequence motifs of miRNAs may play a more important role in microglial TLR7 activation. In order to help reveal such key nucleotides or sequence motifs responsible for TLR7 stimulation, we used both natural occurring miRNAs members of the miR-148 family (miR-148a-3p, miR-148b-3p) and the miR-20 family (miR-20a-5p, miR-20b-5p, miR-17-5p, miR-93-5p), along with a series of their site-directed nucleotide substitution. In all these experiments tests were performed on both Wild-type and TLR7 knock out microglial cells in parallel. TLR7 knock out cells failed to produce TNF-α (data not shown).

We found that miR-148a-3p induced less than miR-148b-3p, yet the two miRNAs differed only by the presence of "CU" at bases 9-10 in miR-148a-3p vs "UC" in miR-148b-3p. Interestingly it is the "U" at base 9 (present in miR-148b-3p and 148mut1, but not miR-148a-3p or 148mut2) that was linked to higher TNF- α induction (Fig 4.2A). We then treated wild-type or TLR7 knock out macrophages with miR-148-a-3p, miR-148b-3p and their mutants (Fig 4.2A) and similar pattern of TNF-α production was seen in wild-type macrophages.

The miR-20 family is more complex. We first examined the "U" at the 5'-end of miR-20a-5p, since the other family members all contained a "C". The change (20a-mut1) made no difference in the induction of TNF- α.

Subsequently, using site-directed nucleotide substitutions based on 20a-mut1 as well as

using the natural family members, we found that the “U” at base 6 (20a-mut4), “C” at base 8 (20a-mut15), “U” at base 9 (20a-mut5), “U” at base 10 (miR-20b-5p), and “U” at base 12 (miR-17-5p) were all important in inducing TNF- α production (Fig 3.10B). These mutants mostly occurred as changes in the uridine residues within a “UGCUUAU” motif between bases 6 and 12, which has four “U”s out of the seven nucleotides, and, while the G was not essential, is 71% “GU.” We also noted that miR-93-5p that contains a “GU” at bases 10-11 instead of “UA” (as well as an A → C change at base 13) was still able to induce TNF-α. We saw similar pattern of TNF-α production in wild-type macrophages, which confirms our findings in wild-type micro-glial cells (Fig 4.2B). Interestingly 20a-mut15 changes the “C” at base 8 to an “A” and this change resulted in failure to induce cytokine production, without affecting GU content.

We also investigated the effects of GU-element for miR-29a-3p induced TNF-α production in wild-type microglia. GU-rich sequence does not “guarantee” the production of TNF-α pro-duction, as shown in Figure 4.2 and Table 4.2. However, mutations of G or U components in the “GGUUA” region of miR-29a-3p significantly diminished the TNF-α production.

Given the findings in this central region, we then undertook experiments using deletions from the 5’- and 3’- end of 20a-mut1 (Fig 4.2C). While the first 4 bases from the 5’-end are dis-pensable, as exhibited by (-4)miR20a, deleting 4 more bases, (-8)miR20a, entering into the mo-tif, eliminates TNF- α production. The last 8 bases at the 3’-end were also dispensable as seen in miR20a(-8), even when combined with deletion of 4 bases from the 5’-end, as in (-4)miR20a(-8). However, while removal of an additional base at from the 5’- or 3’-ends still re-sulted in TNF-α production in (-5)miR20a(-8) and (-4)miR20a(-9), removal of one addition base from the 3’-end in (-4)miR20a(-10) eliminated cytokine production.

Table 4.1.

miRNA	Sequence	G	U	A	C	G%	U%	A%	C%
miR-20a-5p	5'-UAAAGUGCUUAUAGUGCAGGUAG-3'	7	7	7	2	30%	30%	30%	9%
miR-20b-5p	5'-CAAAGUGCUCAUAGUGCAGGUAG-3'	7	5	7	4	30%	22%	30%	17%
miR-17-5p	5'-CAAAGUGCUUACAGUGCAGGUAG-3'	7	5	7	4	30%	22%	30%	17%
miR-93-5p	5'-CAAAGUGCUGUUCGUGCAGGUAG-3'	8	6	5	4	35%	26%	22%	17%
miR-148a-3p	5'-UCAGUGCACUACAGAACUUUGU-3'	4	7	6	5	18%	32%	27%	23%
miR-148b-3p	5'-UCAGUGCAUCACAGAACUUUGU-3'	4	7	6	5	18%	32%	27%	23%

Nucleotide composition analysis

Figure 4.2

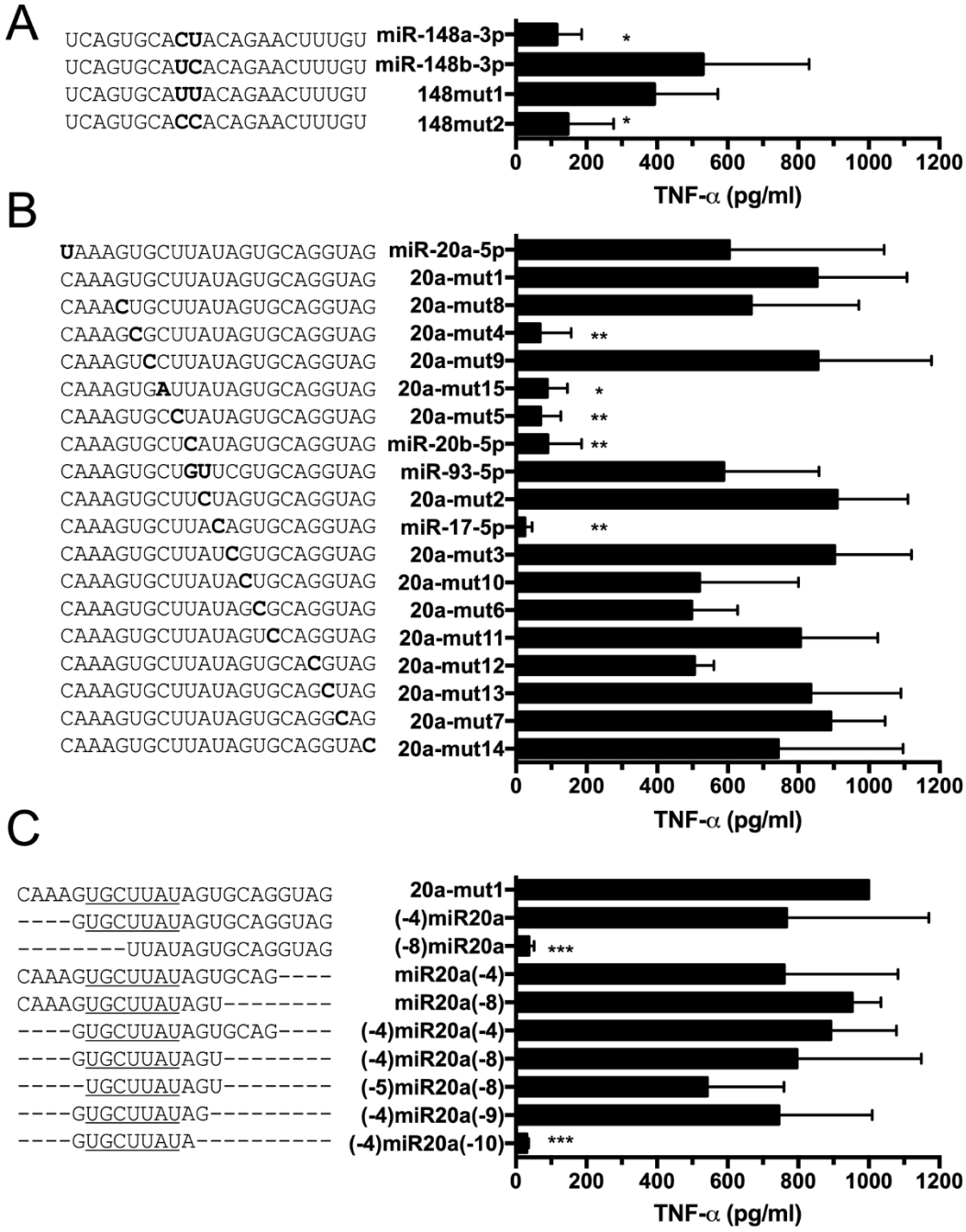

A
UCAGUGCACUACAGAACUUUGU miR-148a-3p
UCAGUGCAUCACAGAACUUUGU miR-148b-3p
UCAGUGCAUUACAGAACUUUGU 148mut1
UCAGUGCACCACAGAACUUUGU 148mut2
*
*
0 200 400 600 800 1000 1200
TNF-α (pg/ml)
B
UAAAGUGCUUAUAGUGCAGGUAG miR-20a-5p
CAAAGUGCUUAUAGUGCAGGUAG 20a-mut1
CAAACUGCUUAUAGUGCAGGUAG 20a-mut8
CAAAGCGCUUAUAGUGCAGGUAG 20a-mut4
CAAAGUCCUUAUAGUGCAGGUAG 20a-mut9
CAAAGUGAUUAUAGUGCAGGUAG 20a-mut15
CAAAGUGCCUAUAGUGCAGGUAG 20a-mut5
CAAAGUGCUCAUAGUGCAGGUAG miR-20b-5p
CAAAGUGCUGUUCGUGCAGGUAG miR-93-5p
CAAAGUGCUUCUAGUGCAGGUAG 20a-mut2
CAAAGUGCUUACAGUGCAGGUAG miR-17-5p
CAAAGUGCUUAUCGUGCAGGUAG 20a-mut3
CAAAGUGCUUAUACUGCAGGUAG 20a-mut10
CAAAGUGCUUAUAGCGCAGGUAG 20a-mut6
CAAAGUGCUUAUAGUCCAGGUAG 20a-mut11
CAAAGUGCUUAUAGUGCACGUAG 20a-mut12
CAAAGUGCUUAUAGUGCAGCUAG 20a-mut13
CAAAGUGCUUAUAGUGCAGGCAG 20a-mut7
CAAAGUGCUUAUAGUGCAGGUAC 20a-mut14
**
**
**
**
*
0 200 400 600 800 1000 1200
TNF-α (pg/ml)
C
CAAAGUGCUUAUAGUGCAGGUAG 20a-mut1
----GUGCUUAUAGUGCAGGUAG (-4)miR20a
--------UUAUAGUGCAGGUAG (-8)miR20a
CAAAGUGCUUAUAGUGCAG---- miR20a(-4)
CAAAGUGCUUAUAGU-------- miR20a(-8)
----GUGCUUAUAGUGCAG---- (-4)miR20a(-4)
----GUGCUUAUAGU-------- (-4)miR20a(-8)
-----UGCUUAUAGU-------- (-5)miR20a(-8)
----GUGCUUAUAG--------- (-4)miR20a(-9)
----GUGCUUAUA---------- (-4)miR20a(-10)
***
***
0 200 400 600 800 1000 1200
TNF-α (pg/ml)

Figure 4.2. Sequence-dependent stimulation of TLR7 in wild-type microglial cells.

The results are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0001 when compared to standard (top condition in each). Nucleotides tested by mutagenesis indicated in bold in A and B. Core motif underlined in C. (A) miR-148a-3p and miR-148b-3p and mutants; n=5 independent experiments for miR-148b-3p, n=3 for miR-148a-3p, n=4 for 148mut1 and 148mut2. Treatment conditions as in Table 3.3 legend. (B): miR-20a-5p, miR-20b-5p, miR-93-5p, miR-17-5p and mutants; n=5 independent experiments for all except miR-20a-5p (n=6), 20a-mut1 (n=3), and 20a-mut15 (n=4). Treatment conditions as in Figure 1 legend. (C) 20a-mut1 and deletion mutants, n=3 independent experiments for all. For 20a-mut1 6 μg of RNA complexed to DOTAP was used as in Figure 1 legend, for the deletion mutants the amount of RNA was adjusted to maintain equivalent molarity.

Figure 4.3

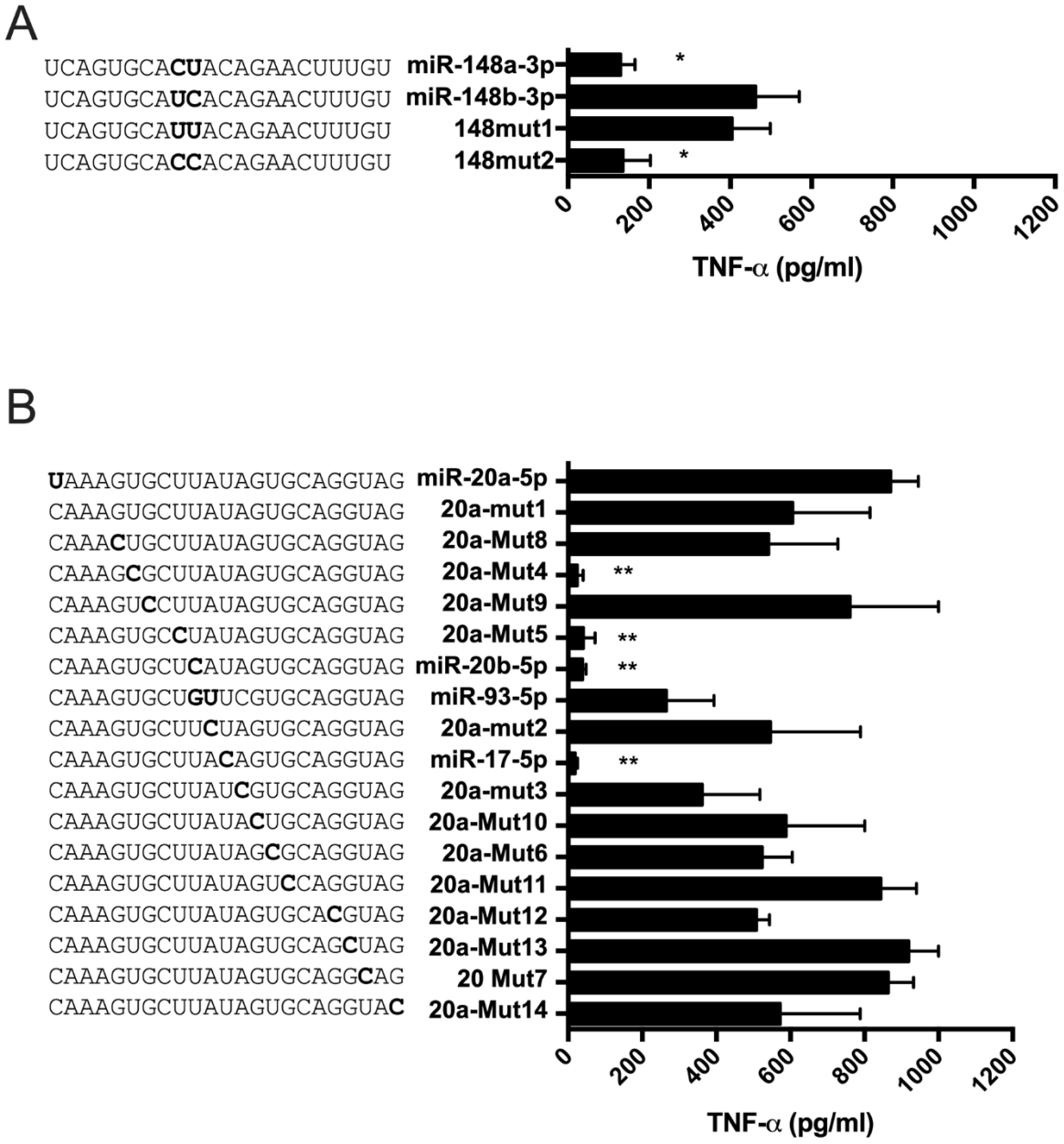

A
UCAGUGCACUACAGAACUUUGU miR-148a-3p
UCAGUGCAUCACAGAACUUUGU miR-148b-3p
UCAGUGCAUUACAGAACUUUGU 148mut1
UCAGUGCACCACAGAACUUUGU 148mut2
*
*
0
200
400
600
800
1000
1200
TNF-α (pg/ml)
B
UAAAGUGCUUAUAGUGCAGGUAG miR-20a-5p
CAAAGUGCUUAUAGUGCAGGUAG 20a-mut1
CAAACUGCUUAUAGUGCAGGUAG 20a-Mut8
CAAAGCGCUUAUAGUGCAGGUAG 20a-Mut4
CAAAGUCCUUAUAGUGCAGGUAG 20a-Mut9
CAAAGUGCCUAUAGUGCAGGUAG 20a-Mut5
CAAAGUGCUCAUAGUGCAGGUAG miR-20b-5p
CAAAGUGCUGUUCGUGCAGGUAG miR-93-5p
CAAAGUGCUUCUAGUGCAGGUAG 20a-mut2
CAAAGUGCUUACAGUGCAGGUAG miR-17-5p
CAAAGUGCUUAUCGUGCAGGUAG 20a-mut3
CAAAGUGCUUAUACUGCAGGUAG 20a-Mut10
CAAAGUGCUUAUAGCGCAGGUAG 20a-Mut6
CAAAGUGCUUAUAGUCCAGGUAG 20a-Mut11
CAAAGUGCUUAUAGUGCACGUAG 20a-Mut12
CAAAGUGCUUAUAGUGCAGCUAG 20a-Mut13
CAAAGUGCUUAUAGUGCAGGCAG 20 Mut7
CAAAGUGCUUAUAGUGCAGGUAC 20a-Mut14
**
**
**
0
200
400
600
800
1000
1200
TNF-α (pg/ml)

Figure 4.3. Sequence-dependent stimulation of TLR7 in wild-type macrophages.

The results are presented as mean ± SEM. *p < 0.05, **p < 0.01, when compared to standard (top condition in each). Nucleotides tested by mutagenesis indicated in bold in A and B. Treatment conditions as in Table 3.3 legend. (A) miR-148a-3p and miR-148b-3p and mutants; n=4 independent experiments for all. (B): miR-20a-5p, miR-20b-5p, miR-93-5p, miR-17-5p and mutants; n=3 independent experiments.

Figure 4.4

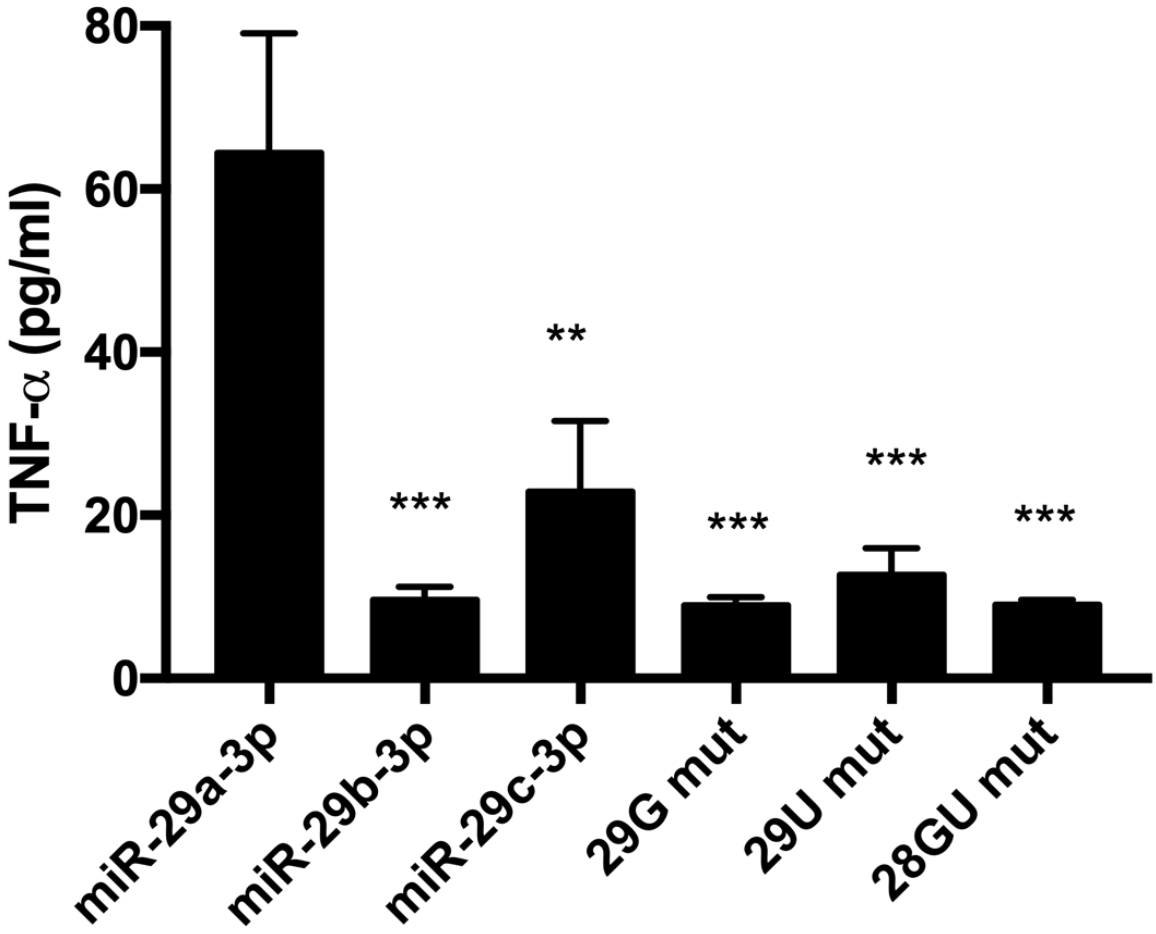

80
60
40
20
0
TNF-α (pg/ml)
***
**
***
***
***
miR-29a-3p
miR-29b-3p
miR-29c-3p
29G mut
29U mut
28GU mut

Figure 4.4. GU-dependent, miR-29a-3p induced stimulation of TLR7 in wild-type microglia.

The results are presented as mean ± SEM. **p < 0.01, ***p < 0001, when compared to standard (miR-29a-3p). Treatment conditions as in Table 3.3 legend. n=4 independent experiments for all. Note: Actual sequences of miR-29a-3p, miR-29b-3p and their mutants shown in Table 3.6.

Table 4.2.

Sequences of miR-29a-3p, miR-29b-3p and mutants

miRNA	Sequence	G %	U%
miR-29a-3p	5'-UAGCACCAUCUGAAAUCGGUUA-3'	27	18
miR-29b-3p	5'-UAGCACCAUUUGAAAUCAGUGUU-3'	35	17
29G mut	5'-UAGCACCAUCUGAAAUCAAUUA-3'	27	9
29Umut	5'-UAGCACCAUCUGAAAUCGGCCA-3'	18	18
29GU mut	5'-UAGCACCAUCUGAAAUCAACCA-3'	18	9

4.2.3 PI3K, MAPK and NF-κB promote miRNA-stimulated transcription of pro-inflammatory molecules

We used various signaling inhibitors to test what pathway is involved in miRNA-EV mimic-stimulated transcription of pro-inflammatory molecules. For this purpose, cells were incubated with inhibitors for 30 minutes prior to stimulation with miR-20a-5p for 16 hrs. Total RNA was isolated from the cells and cytokine mRNA levels were measured by quantitative RT-PCR. As shown in Figure 3.13, the MLK-3 inhibitor URMC-099, PI3K inhibitor Wortmannin and IKK1/2 inhibitor BMS-345541 treatment of WT microglial cells was effective at inhibiting the miR-20a-5p stimulated production of a number of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, CCL5, and CXCL10 as well as the inflammatory enzyme cyclooxygenase-2 (PTSG-2) (Fig 4.5). This observation indicates the essential requirements of PI3K, MAPK and NF-κB pathways in promoting the miR-20a-5p induced transcription of pro-inflammatory molecules.

Figure 4.5

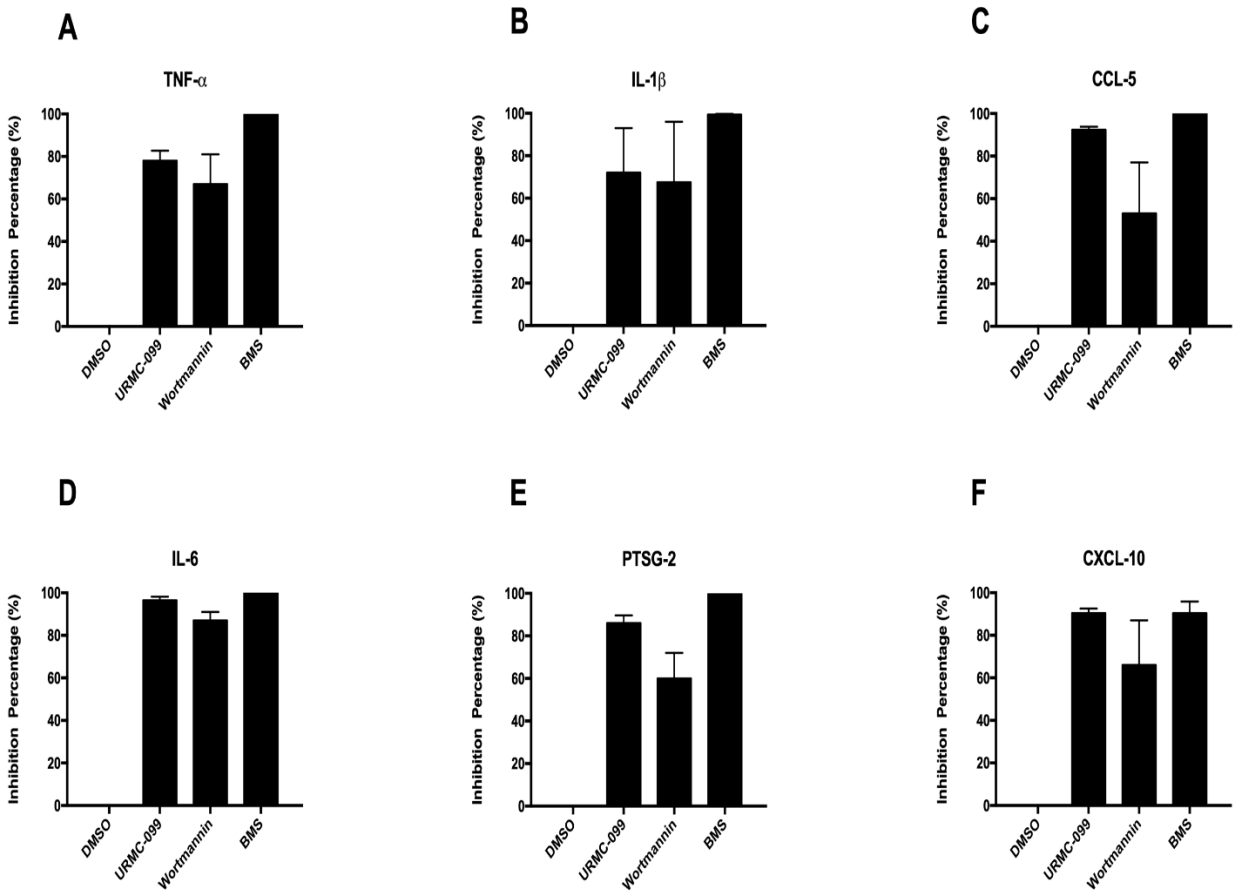

A
TNF-α
B
IL-1β
C
CCL-5
D
IL-6
E
PTSG-2
F
CXCL-10
Inhibition Percentage (%)
0
20
40
60
80
100
DMSO
URMC-099
Wortmannin
BMS

Figure 4.5. Effects of inhibitors of signal transduction pathways on miRNA-induced cytokine production in cells.

WT mice microglial cells were incubated for 30 minutes with DMSO (vehicle), URMC-099 (100 nM), Wortmannin (1 μM) or BMS-345541 (10 μM), followed by miR-20a-5p : DOTAP treatment as in the legend to Table 3.3. Cellular RNA was collected and assessed by qRT-PCR (using GAPDH as a control housekeeping gene) for relative levels of the indicated mRNA. Data represent mean ± SEM. n = 3 independent experiments for all except for wortmannin treatment where n=2.

4.2.4 miR-20a-5p, but not miR-20b-5p, causes peritoneal leukocyte migration *in vivo*

To examine the effectiveness of miRNAs with different sequences (based on prior results indicating TLR7-dependent stimulation) *in vivo*, we injected miRNAs complexed to DOTAP (miR-20a-5p and miR-20b-5p), as well as controls (DOTAP alone and saline) into the mouse peritoneal space and harvested the peritoneal lavage 20 h later. Cells were gated for identification of myeloid cells as shown in Supplementary Figure 1. MiR-20a-5p injection in WT mice, when compared with injection in TLR7ko mice, led to a significant increase in neutrophils ($2.96 \pm 1.01 \times 10^6$ vs. $0.68 \pm 0.54 \times 10^6$, $p < 0.01$) and monocytes ($5.42 \pm 1.74 \times 10^6$ vs. $1.37 \pm 1.18 \times 10^6$, $p < 0.05$) (Figure 4.7&4.8). We also examined the two types of peritoneal macrophages, large peritoneal macrophages (LPM), tissue resident macrophages that dominate by number in the steady-state, and small peritoneal macrophages (SPM) which infiltrate due to inflammatory stimuli (125,126). While no change was found with LPM, we found that miR-20a-5p caused a marked increase of SPM in WT mice compared with TLR7ko mice ($5.15 \pm 2.24 \times 10^5$ vs. $1.34 \pm 0.77 \times 10^5$, $p < 0.05$) (Figure 4.8). MiR-20b-5p did not alter the number of any of these cells between WT and TLR7ko mice. Therefore our data revealed that miR-20a-5p induced inflammatory leukocyte migration *in vivo* and further confirmed our *in vitro* finding that miR-20a-5p could activate TLR7 via a specific sequence motif.

Figure 4.6

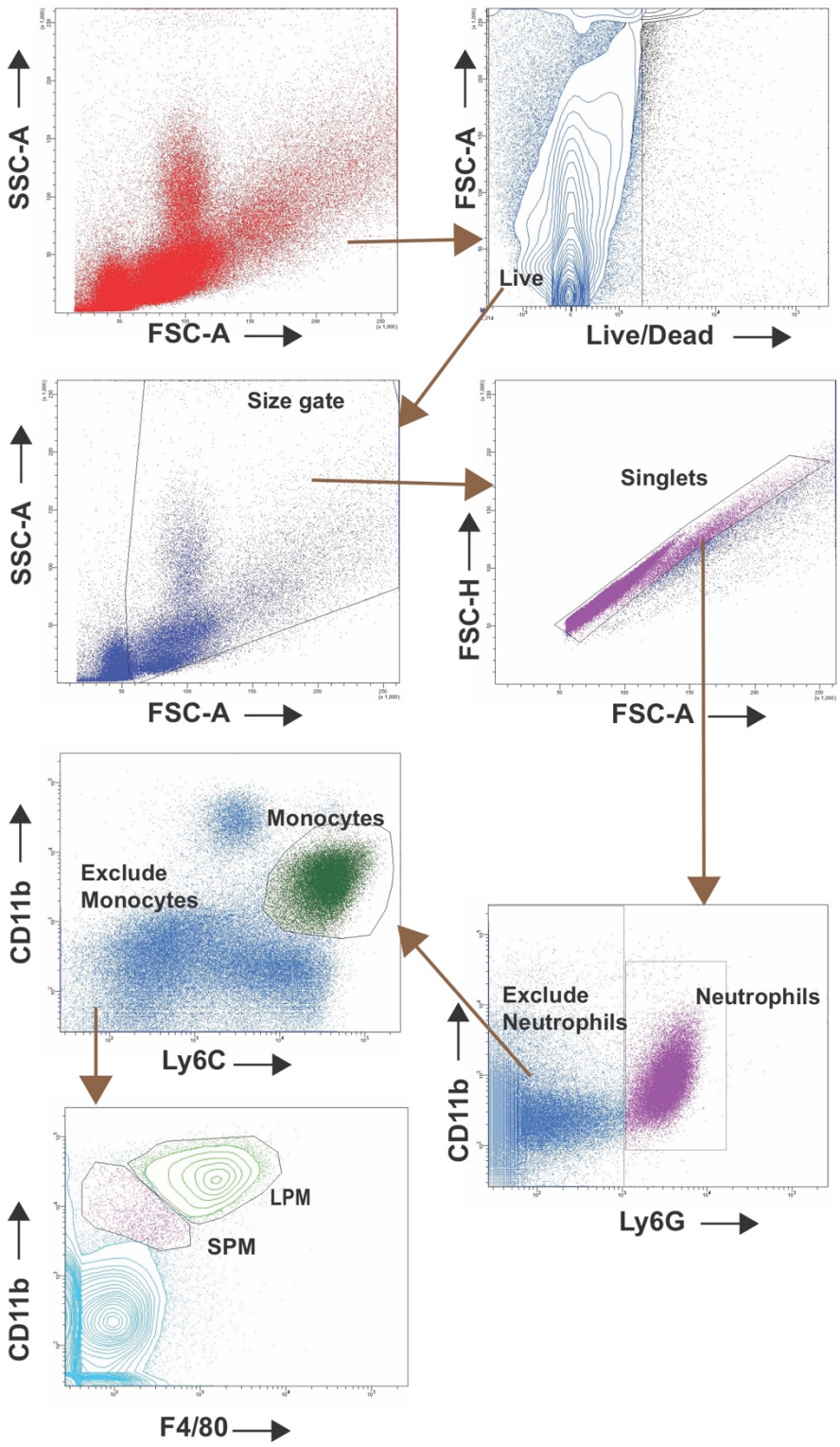

SSC-A
FSC-A
FSC-A
Live/Dead
Live
Size gate
SSC-A
FSC-A
FSC-H
FSC-A
Singlets
CD11b
Ly6C
Monocytes
Exclude
Monocytes
CD11b
Ly6G
Exclude
Neutrophils
Neutrophils
CD11b
F4/80
LPM
SPM

Figure 4.6. Gating strategy for peritoneal myeloid cells.

WT and TLR7ko mice were injected i.p. with 20 μg of miR-20a-5p, miR-20b-5p complexed with DOTAP or DOTAP alone. After 20 hr, the peritoneal cells were harvested, stained, and analyzed. Recovered cells (upper left) were first gated for live cells (live cells have low uptake of the dye, whereas dead cells have higher uptake). Live cells were then subjected to a size gate, based on forward (FSC) and side (SSC) scatter, and then doublets eliminated through examination of height and area of the forward scatter (FSC-H and FSC-A). From the singlets, Neutrophils were then identified as CD11b+Ly6G+, and from the non-neutrophils Monocytes identified as CD11b+Ly6C+. LPMs were then identified from the remaining cells as CD11b+F4/80+, whereas SPM had lower levels of both CD11b and F4/80.

Figure 4.7 (A)

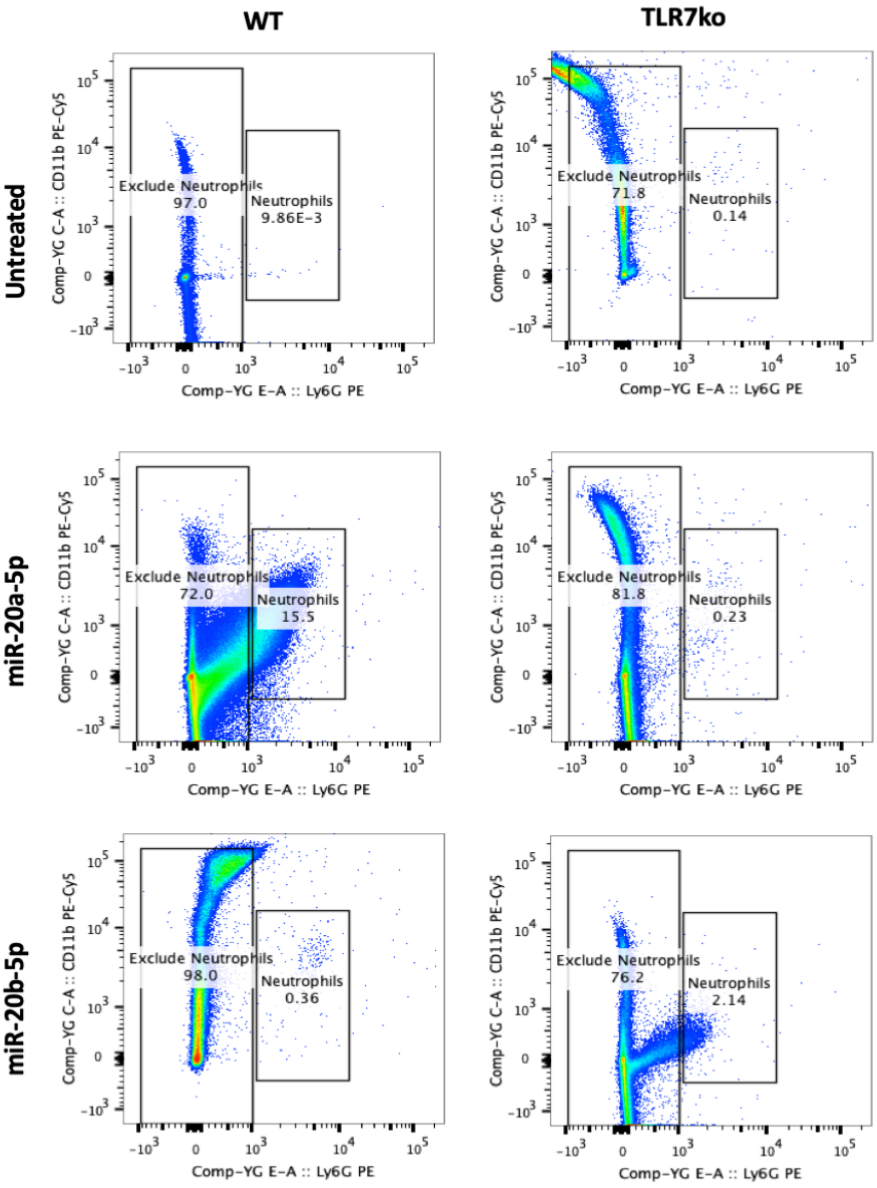

WT
TLR7ko
Untreated
miR-20a-5p
miR-20b-5p
Exclude Neutrophils
97.0
Neutrophils
9.86E-3
Exclude Neutrophils
71.8
Neutrophils
0.14
Exclude Neutrophils
72.0
Neutrophils
15.5
Exclude Neutrophils
81.8
Neutrophils
0.23
Exclude Neutrophils
98.0
Neutrophils
0.36
Exclude Neutrophils
76.2
Neutrophils
2.14
Comp-YG C-A :: CD11b PE-Cy5
Comp-YG E-A :: Ly6G PE

Figure 4.7 (B)

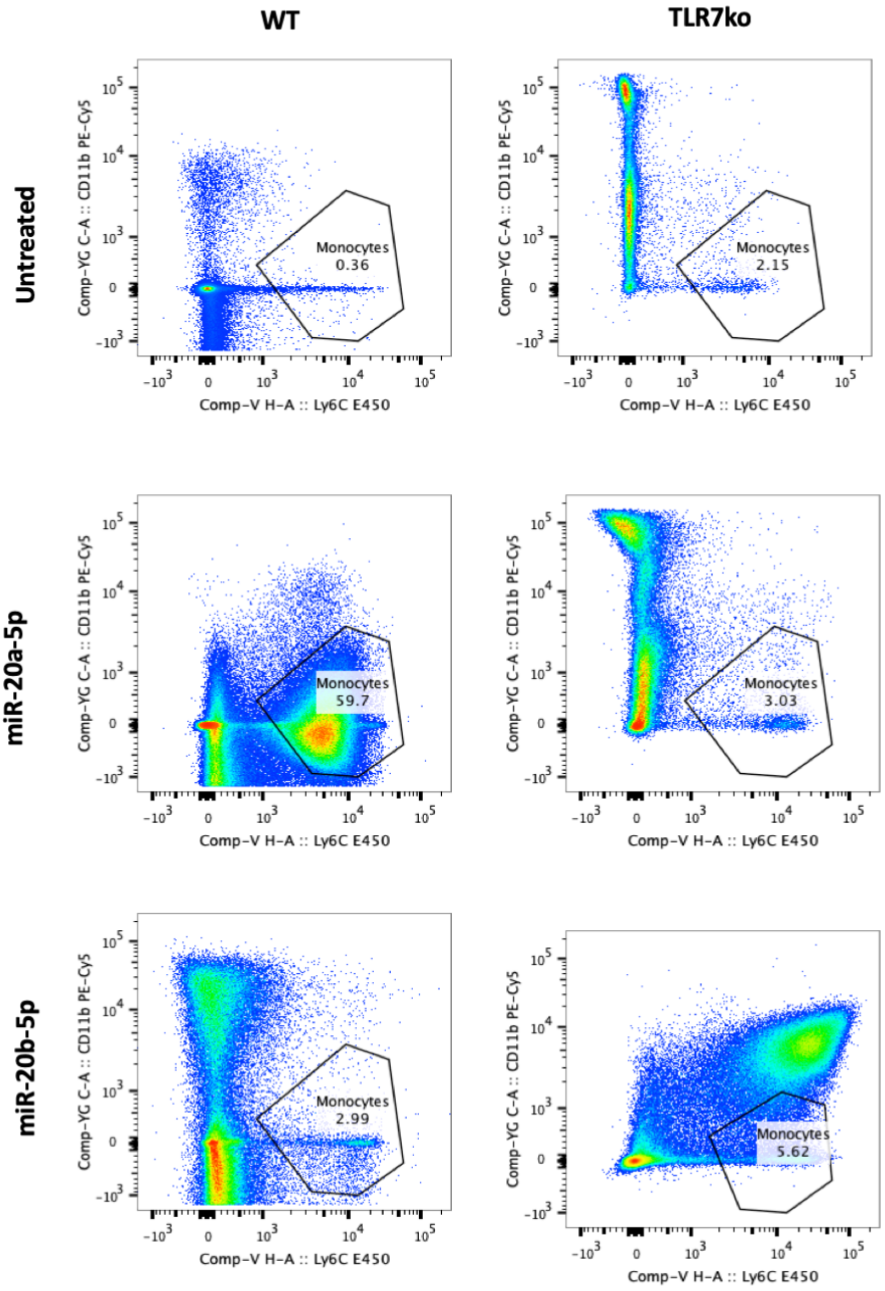

WT
TLR7ko
Untreated
miR-20a-5p
miR-20b-5p
Monocytes
0.36
Monocytes
2.15
Monocytes
59.7
Monocytes
3.03
Monocytes
2.99
Monocytes
5.62
Comp-YG C-A :: CD11b PE-Cy5
Comp-V H-A :: Ly6C E450

Figure 4.7. miR-20a-5p promotes peritoneal leukocyte recruitment in vivo.

The peritoneal lavage was harvested as described in Figure 4.7, and the peritoneal cells were analyzed with flow cytometry. miR-20a-5p, but not miR-20b-5p, promotes NE (A) and Monocytes (B) migration into the peritoneal cavity.

Figure 4.8

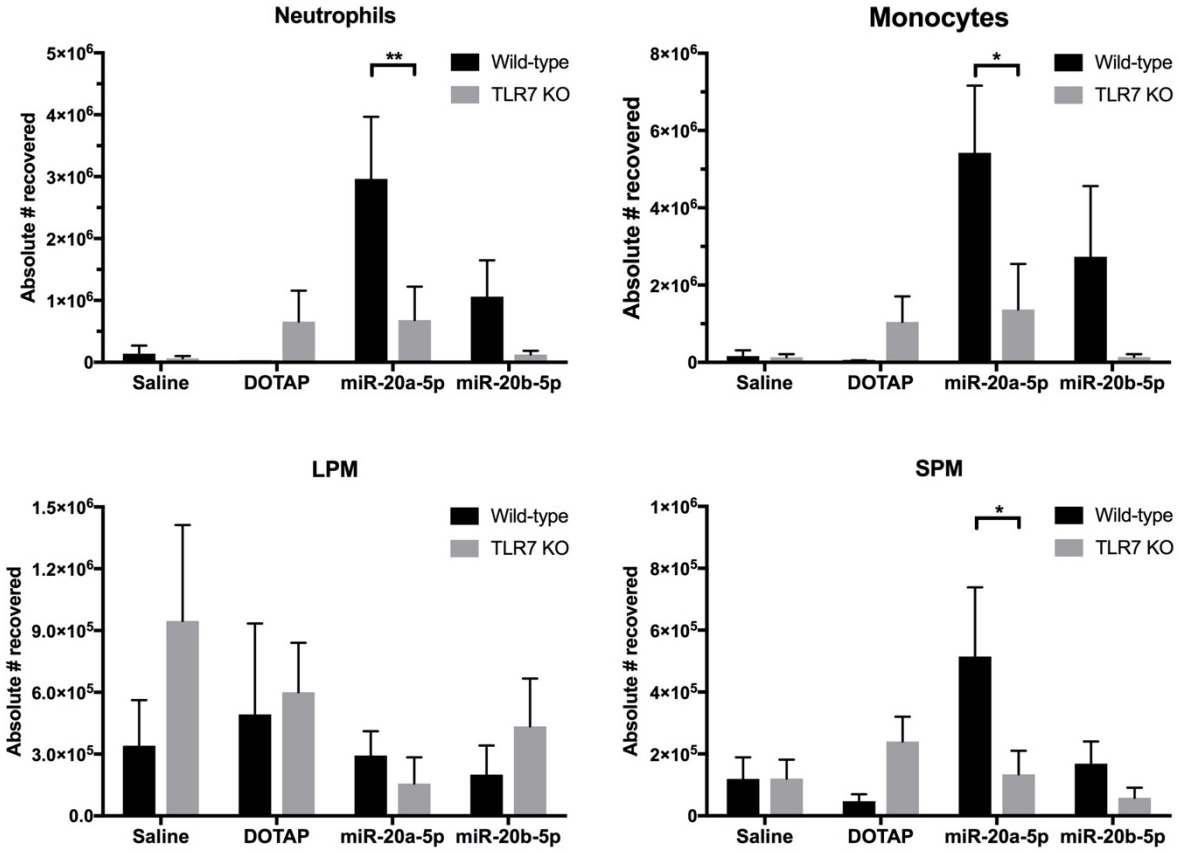

Neutrophils
Monocytes
LPM
SPM
Wild-type
TLR7 KO
Absolute # recovered
Saline
DOTAP
miR-20a-5p
miR-20b-5p
**
*
*
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
8×10^6
6×10^6
4×10^6
2×10^6
1.5×10^6
1.2×10^6
9.0×10^5
6.0×10^5
3.0×10^5
0.0
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5

Figure 4.8. MiR-20a-5p induced peritoneal leukocyte migration via TLR7 *in vivo*.

WT and TLR7ko mice were injected i.p. with 20 µg of miR-20a-5p, miR-20b-5p complexed with DOTAP or DOTAP alone. After 20 hr, the peritoneal cells were harvested, stained, and analyzed. The absolute number of neutrophils, monocytes, LPM and SPM, gated as in Figure 4.7. *$p < 0.05$, **$p < 0.01$. n=5 independent experiments.

4.3 Discussion

GU-rich sequences were identified in the original description of ssRNA sequences derived from HIV-1 signaling through TLR7 (11) and supported in studies testing a range of sequences (127). Other studies, resulting from the finding that influenza virus genomic RNA can signal through TLR7, revealed that poly-U, but not poly-A, -C or -G could activate TLR7 (10). Sequence specificity independent from GU-content was then found in studies of experimental small interfering RNA (siRNA) which were found to induce, rather than the expected suppression, of interferon production in dendritic cells (128). This induction was found to be TLR7-dependent. Interestingly in that study while a 19-nucleotide sequence containing the necessary 9-nucleotide motif was highly stimulatory, reducing the length to 16 or 12 nucleotides (still containing the 9 bases) greatly reduced the response.

Indeed we found that the location of the specific nucleotides was critical (e.g. the CU vs UC in miR-148a-3p vs miR-148b-3p). Furthermore, while the motif we found in miR-20a-5p (UGCUUAU) was GU-rich, the Us as well as the C were critical for TLR7 activation. In addition, we identified two short 10-nucleotide miRNA sequences, UGCUUAUAGU and GUGCUUAUAG, containing this motif, that could strongly stimulate TNF-α production. These are the shortest ssRNA sequences known to activate TLR7 described.

We speculate that within the innate immune system TLR7 can identify ssRNAs, including miRNAs, as PAMPs through prevalence analysis of their sequence motifs and flanking context. Certain motifs, such as our identified motif UGCUUAU present in miR-20a-5p, can lead to activation given that enough key flanking residues are also present. Therefore UGCUUAUAGU and GUGCUUAUAG, but not GUGCUUAUA, could activate TLR7 signaling. In addition, our study showed that within the naturally occurring miR-17-5p family (miR-20a-5p, miR-20b-5p, miR-

17-5p, miR-93-5p) as well as the site-directed substitutions based on miR-20a-5p, some nucleotide substitutions within the core UGCUUAU motif still preserved signaling (e.g. UCCUUAU in 20a-mut9 and UGCUGUU in miR-93-5p), whereas others abrogated the TLR7-dependent signaling (e.g. CGCUUAU in 20a-mut4). These findings indicate that TLR7 recognizes miRNAs with tolerance for certain amount of nucleotide substitutions within the core motif. Our results with miR-135a/b-5p and miR-148a/b-3p pairs further emphasizes the sequence dependence. Thus, TLR7 activation by miRNAs depends on both core sequence motifs and flanking context of the miRNAs, and has tolerance for nucleotide substitutions within the motif of the miRNAs.

However, the nature of how this motif and context of miRNAs leads to TLR7 signaling remains unclear. Crystal structural study of rhesus monkey TLR7 and human TLR8 indicates that both have two binding sites, a first site for small ligands and a second site for small ssRNA (32,129). The imidazoquinolines Imiquimod (TLR7 only) and Resiquimod (TLR7 and TLR8), can activate the receptors through binding the first site, whereas ssRNA stimulation requires both sites to be occupied. Interestingly for TLR7, when crystalized with guanosine and poly-U 19-mer, the structure revealed guanosine binding the first site, and shortened lengths of poly-U (predominantly 4-mers) binding the second; additional experiments revealed that ssRNA binding to the second site increasing the affinity for guanosine at the first site (32). Crystallization of TLR8 with two different ssRNAs revealed uridine at the first site and “UG” at the second site (129). Interestingly again the intact ssRNAs were not detected, and these products had to result from ssRNA breakdown, even though purified recombinant molecules were used. The same result occurred even when using phosphorothioated ssRNA. As these utilized recombinant proteins and added ligands, the actual mode of potential degradation of the ssRNA ligands and then binding to TLR7 or TLR8 have not been described. Furthermore, the structure of mouse TLR7 has not been reported.

The transcription of pro-inflammatory molecules after TLR7-dependent miR-20a-5p stimulation likely involves multiple signal transduction kinases as well as transcription factors. In our study, we found that an inhibitor of IKK-1/2 (thus preventing IκBα phosphorylation and NF-κB activation), PI3K, and MLK3 (thus inhibiting MAPKs) led to significantly attenuated transcription of cytokines expression following miRNA treatment. This is consistent with known modulators and effectors of TLR signaling.

Feng et al. reported that that TLR7 stimulating miRNAs were capable of leukocyte infiltration when injected into the peritoneal space (28). In our study, we found that miR-20a-5p induced migration of lymphocytes and monocytes when injected into the peritoneal space of mice in a TLR7-specific fashion. We also found that the effects of miR-20a-5p were significantly attenuated by the use of miR-20b-5p, which mutates the "UGCUUAU" motif to "UGCUCAU." Our in vivo data are consistent with our in vitro cytokine data and indicate that the miRNA-induced leukocyte migration is sequence and TLR7 dependent.

In addition, we identified marked different responses by the peritoneal monocyte/macrophage populations following miRNAs stimulation in vivo. Previous studies have defined two distinct subsets of peritoneal macrophages, SPMs and LPMs. Our findings demonstrated for the first time that SPMs and LPMs responded differently after miRNA stimulation in vivo. Monocytes as well as SPMs, significantly increased after peritoneal miR-20a-5p administration in TLR7-expressing mice. LPMs, in contrast, did not response to miRNA stimulation. We speculate that miRNAs stimulations can cause the influx of circulating inflammatory monocytes, which can differentiate into SPMs, into the peritoneal cavity via TLR7 activation.

In summary, we screened a wide profile of miRNAs inducing TNF-α response in multiple cell lines, and identified a large number of miRNAs capable of inducing a TLR7-dependent in-

flammatory response in microglia. We identified the unique “UGCUUAU” sequence motif of miR-20a-5p that stimulates mouse TLR7 as well as the signaling transduction kinases that promote the miRNA-stimulated transcription of cytokines. In vivo, miR-20a-5p, but not miR-20b-5p, is responsible for leukocyte migration and inflammation, depending on TLR7. Together, our data suggest that extracellular miRNAs with specific sequence motif and length are potent TLR7 activators and can trigger innate immune responses.

Chapter 5. Conclusion

5.1 Overall conclusions and future directions

The work highlighted here investigates the miRNA-induced sequence-specific activation of TLR7 both *in vitro* and *in vivo*. In the first chapter, we screened a wide profile of miRNAs for their ability to induce TNF-α and IL-6 responses in macrophages and microglial cells through TLR7. Indeed, many miRNAs can activate TLR7 *in vitro* and lead to pro-inflammatory mediator production when present in EVs. We were puzzled by the relative lack of TNF-α production following treatment with most of the let-7 family members (just low amounts with let-7a-5p, higher levels only with let-7i-5p) in our experiments, despite attempting to replication conditions (with and without DOTAP, and the use of phosphorothiolate as well as phosphodiesterase linkages. It is still not clear the reason for this difference. However, the other previously reported TLR7-activating miRNAs that were tested in detail (such as miR-21 and miR-29a), indeed activated microglia through TLR7 in our study.

In the following chapter by using closely related natural miRNA molecules, site-directed and deletion mutants, we found a short 10-nucleotide sequence capable of stimulating TLR7. This activity was dependent on a number of signaling pathways that characterize the TLR7 system. When given *in vivo*, administration of a miRNA-EV mimic could also induce leukocyte migration in a miRNA sequence-specific manner. The identification of sequence-specific miRNAs activating TLR7 raises some interesting questions. According to the current results, the GUUG motif for miR-21, GGUU for miR-29a, GUUGUGU for let-7b, UGCUUAU for miR-20a, UC for miR-148b is essential for the miRNA-TLR7 recognition (13,25-27,36). However, other questions remain. Can endosomal TLRs other than murine TLR7 be recognized by miRNAs? Can human TLR7/TLR8 also be recognized sequence-specific miRNAs? It is previously reported that TLR8

binds to uridine and the di-nucleotide UG (38). Analyses using purified TLR7 and TLR8 have revealed that their binding to G or U requires preceding interaction between TLR7/8 with oligoribonucleotides. TLR7 and TLR8 respond to GU-rich and AU-rich ORNs (127). Future studies using human TLR7 or TLR8, or investigate the possibility that other endosomal/lysosomal TLRs or pattern recognition receptors might be activated by miRNAs may be warranted. We hypothesize that sequence-specific miRNAs will bind to endosomal TLRs and lead to production of inflammatory cytokines.

TLR7 plays a vital role in the antiviral responses and the contact between a virus and uninfected TLR7-expressing cell is often sufficient to trigger the inflammatory cytokines and chemokines production without need for infection. Previous studies of the TLR7-dependent West Nile virus, Human Respiratory Syncytial virus and Ross River virus responses have shown that TLR7 represents a vital host defense mechanism against virus (130-132). The interactions of TLR7 and miRNAs studied in our project mimics the virus-TLR7 interactions and provides new insights for TLR7-associated antiviral therapies.

Our study also shows that EVs can be used as biomarkers for diagnostic purposes during the progression of neurodegenerative diseases such as PD and AD. Currently in the bench settings, there are several methods of quantification of EVs. The number of particles can be measured by Scattering/Fluorescent nanoparticle tracking analysis (optical detection of the light/fluorescent light scattered or emitted by the particle), Resistive pulse sensing (measurement of the change in conductance across a sensing pore upon passage of a particle) and Flow cytometry (optical detection of particles emitting fluorescent or scattered light). On the other hand, the mass of EVs can be achieved by BCA or Surface plasmon resonance (measurement of the change in refractive index upon absorption at a sensing interface). However, the time-

consuming isolation procedures, the need for large sample volumes or the need for bulky instrumentation still limits the implementation of EVs as clinical biomarkers of neurodegenerative diseases. Current efforts are directed at the design of integrated devices allowing for the detection and quantification of EVs in complex biofluid samples (133,134). Miniaturized microfluidic-based chips designed for both sample preparation (exosome purification (135), collection (136), lysis for RNA detection (137)) and detection (138,139) have been proposed and hold promises for the rapid detection of EVs

The miRNA sensing endosomal/lysosomal TLRs, such as TLR7, are reported to be localized in the ER and move to the endosomes or lysosomes upon stimulation. Future studies of the mechanisms controlling TLR7 localization is critical to further our understanding of miRNA-TLR7 binding and downstream signaling. Moreover, in-depth analysis of the complex processes regulating the localization and trafficking of TLR7 will provide new insights on fine-tuning immune responses in virus infection, cancer, and autoimmune diseases for the development of novel therapeutics.

It is also worth noting that the free miRNAs without DOTAP complexation did not induce TNF-α release *in vitro*, indicating miRNA mimics need to be enveloped in some type of carriers, such as DOTAP, to protect them from degradation and/or enable them to efficiently reach the endosome. Circulating miRNAs are also enveloped in some type of carriers, such as EVs. Our study provides a physiological method of delivering miRNAs for potential therapeutic purposes. TLR7 ligands are used pharmaceutically and experimentally for immune stimulation with applications to cancer, autoimmune and viral diseases, as well as vaccine efficacy (140-144). The use of small ssRNA sequences, such as the 10-mer we identified, may be useful in certain formulations and conditions.

References

1. Broz, P. and Monack, D.M. (2013) Newly described pattern recognition receptors team up against intracellular pathogens. *Nat Rev Immunol*, **13**, 551-565.
2. He, X., Jia, H., Jing, Z. and Liu, D. (2013) Recognition of pathogen-associated nucleic acids by endosomal nucleic acid-sensing toll-like receptors. *Acta Biochim Biophys Sin (Shanghai)*, **45**, 241-258.
3. Newton, K. and Dixit, V.M. (2012) Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol*, **4**.
4. Kawai, T., Adachi, O., Ogawa, T., Takeda, K. and Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*, **11**, 115-122.
5. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K. *et al.* (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science*, **301**, 640-643.
6. Hashimoto, C., Hudson, K.L. and Anderson, K.V. (1988) The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell*, **52**, 269-279.
7. Blasius, A.L. and Beutler, B. (2010) Intracellular toll-like receptors. *Immunity*, **32**, 305-315.
8. O'Neill, L.A. (2008) When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. *Immunity*, **29**, 12-20.
9. Kondo, T., Kawai, T. and Akira, S. (2012) Dissecting negative regulation of Toll-like receptor signaling. *Trends Immunol*, **33**, 449-458.
10. Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. and Reis e Sousa, C. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science*, **303**, 1529-1531.
11. Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H. and Bauer, S. (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science*, **303**, 1526-1529.
12. Yates, L.A., Norbury, C.J. and Gilbert, R.J. (2013) The long and short of microRNA. *Cell*, **153**, 516-519.
13. Fabian, M.R., Sonenberg, N. and Filipowicz, W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem*, **79**, 351-379.
14. Gibbings, D.J., Ciaudo, C., Erhardt, M. and Voinnet, O. (2009) Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol*, **11**, 1143-1149.
15. Winter, J., Jung, S., Keller, S., Gregory, R.I. and Diederichs, S. (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol*, **11**, 228-234.
16. Ksiazek-Winiarek, D.J., Kacperska, M.J. and Glabinski, A. (2013) MicroRNAs as novel regulators of neuroinflammation. *Mediators Inflamm*, **2013**, 172351.

17. Gantier, M.P. (2010) New perspectives in MicroRNA regulation of innate immunity. *J Interferon Cytokine Res*, **30**, 283-289.

18. Zhang, J., Li, S., Li, L., Li, M., Guo, C., Yao, J. and Mi, S. (2015) Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinformatics*, **13**, 17-24.

19. Sato-Kuwabara, Y., Melo, S.A., Soares, F.A. and Calin, G.A. (2015) The fusion of two worlds: non-coding RNAs and extracellular vesicles--diagnostic and therapeutic implications (Review). *Int J Oncol*, **46**, 17-27.

20. Hall, J., Prabhakar, S., Balaj, L., Lai, C.P., Cerione, R.A. and Breakefield, X.O. (2016) Delivery of Therapeutic Proteins via Extracellular Vesicles: Review and Potential Treatments for Parkinson's Disease, Glioma, and Schwannoma. *Cell Mol Neurobiol*, **36**, 417-427.

21. Lachenal, G., Pernet-Gallay, K., Chivet, M., Hemming, F.J., Belly, A., Bodon, G., Blot, B., Haase, G., Goldberg, Y. and Sadoul, R. (2011) Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Mol Cell Neurosci*, **46**, 409-418.

22. Park, S., Ahn, E.S. and Kim, Y. (2015) Neuroblastoma SH-SY5Y cell-derived exosomes stimulate dendrite-like outgrowths and modify the differentiation of A375 melanoma cells. *Cell Biol Int*, **39**, 379-387.

23. Kramer-Albers, E.M., Bretz, N., Tenzer, S., Winterstein, C., Mobius, W., Berger, H., Nave, K.A., Schild, H. and Trotter, J. (2007) Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons? *Proteomics Clin Appl*, **1**, 1446-1461.

24. Potolicchio, I., Carven, G.J., Xu, X., Stipp, C., Riese, R.J., Stern, L.J. and Santambrogio, L. (2005) Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. *J Immunol*, **175**, 2237-2243.

25. Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., Lovat, F., Fadda, P., Mao, C., Nuovo, G.J. *et al.* (2012) MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci U S A*, **109**, E2110-2116.

26. Yelamanchili, S.V., Lamberty, B.G., Rennard, D.A., Morsey, B.M., Hochfelder, C.G., Meays, B.M., Levy, E. and Fox, H.S. (2015) MiR-21 in Extracellular Vesicles Leads to Neurotoxicity via TLR7 Signaling in SIV Neurological Disease. *PLoS Pathog*, **11**, e1005032.

27. Lehmann, S.M., Kruger, C., Park, B., Derkow, K., Rosenberger, K., Baumgart, J., Trimbuch, T., Eom, G., Hinz, M., Kaul, D. *et al.* (2012) An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. *Nat Neurosci*, **15**, 827-835.

28. Feng, Y., Zou, L., Yan, D., Chen, H., Xu, G., Jian, W., Cui, P. and Chao, W. (2017) Extracellular MicroRNAs Induce Potent Innate Immune Responses via TLR7/MyD88-Dependent Mechanisms. *J Immunol*, **199**, 2106-2117.

29. Han, X., Li, X., Yue, S.C., Anandaiah, A., Hashem, F., Reinach, P.S., Koziel, H. and Tachado, S.D. (2012) Epigenetic regulation of tumor necrosis factor alpha (TNFalpha) release in human macrophages by HIV-1 single-stranded RNA (ssRNA) is dependent on TLR8 signaling. *J Biol Chem*, **287**, 13778-13786.

30. Bernard, M.A., Zhao, H., Yue, S.C., Anandaiah, A., Koziel, H. and Tachado, S.D. (2014) Novel HIV-1 miRNAs stimulate TNFalpha release in human macrophages via TLR8 signaling pathway. *PLoS One*, **9**, e106006.

31. Petes, C., Odoardi, N. and Gee, K. (2017) The Toll for Trafficking: Toll-Like Receptor 7 Delivery to the Endosome. *Front Immunol*, **8**, 1075.

32. Zhang, Z., Ohto, U., Shibata, T., Krayukhina, E., Taoka, M., Yamauchi, Y., Tanji, H., Isobe, T., Uchiyama, S., Miyake, K. *et al.* (2016) Structural Analysis Reveals that Toll-like Receptor 7 Is a Dual Receptor for Guanosine and Single-Stranded RNA. *Immunity*, **45**, 737-748.

33. Ashkar, A.A. and Rosenthal, K.L. (2002) Toll-like receptor 9, CpG DNA and innate immunity. *Curr Mol Med*, **2**, 545-556.

34. Liu, H.Y., Huang, C.M., Hung, Y.F. and Hsueh, Y.P. (2015) The microRNAs Let7c and miR21 are recognized by neuronal Toll-like receptor 7 to restrict dendritic growth of neurons. *Exp Neurol*, **269**, 202-212.

35. Salama, A., Fichou, N., Allard, M., Dubreil, L., De Beaurepaire, L., Viel, A., Jegou, D., Bosch, S. and Bach, J.M. (2014) MicroRNA-29b modulates innate and antigen-specific immune responses in mouse models of autoimmunity. *PLoS One*, **9**, e106153.

36. Fabbri, M., Paone, A., Calore, F., Galli, R. and Croce, C.M. (2013) A new role for microRNAs, as ligands of Toll-like receptors. *RNA Biol*, **10**, 169-174.

37. Iavarone, C., Ramsauer, K., Kubarenko, A.V., Debasitis, J.C., Leykin, I., Weber, A.N., Siggs, O.M., Beutler, B., Zhang, P., Otten, G. *et al.* (2011) A point mutation in the amino terminus of TLR7 abolishes signaling without affecting ligand binding. *J Immunol*, **186**, 4213-4222.

38. Miyake, K., Shibata, T., Ohto, U., Shimizu, T., Saitoh, S.I., Fukui, R. and Murakami, Y. (2018) Mechanisms controlling nucleic acid-sensing Toll-like receptors. *Int Immunol*, **30**, 43-51.

39. Sarkar, N., Panigrahi, R., Pal, A., Biswas, A., Singh, S.P., Kar, S.K., Bandopadhyay, M., Das, D., Saha, D., Kanda, T. *et al.* (2015) Expression of microRNA-155 correlates positively with the expression of Toll-like receptor 7 and modulates hepatitis B virus via C/EBP-beta in hepatocytes. *J Viral Hepat*, **22**, 817-827.

40. Ceppi, M., Pereira, P.M., Dunand-Sauthier, I., Barras, E., Reith, W., Santos, M.A. and Pierre, P. (2009) MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc Natl Acad Sci U S A*, **106**, 2735-2740.

41. Tang, B., Xiao, B., Liu, Z., Li, N., Zhu, E.D., Li, B.S., Xie, Q.H., Zhuang, Y., Zou, Q.M. and Mao, X.H. (2010) Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation. *FEBS Lett*, **584**, 1481-1486.

42. Chen, Y., Chen, J., Wang, H., Shi, J., Wu, K., Liu, S., Liu, Y. and Wu, J. (2013) HCV-induced miR-21 contributes to evasion of host immune system by targeting MyD88 and IRAK1. *PLoS Pathog*, **9**, e1003248.

43. Ahmed, F., Shiraishi, T., Vessella, R.L. and Kulkarni, P. (2013) Tumor necrosis factor receptor associated factor-4: an adapter protein overexpressed in metastatic prostate cancer is regulated by microRNA-29a. *Oncol Rep*, **30**, 2963-2968.

44. Choudhury, S.N. and Li, Y. (2012) miR-21 and let-7 in the Ras and NF-kappaB pathways. *Microrna*, **1**, 65-69.

45. Zhang, M., Liu, Q., Mi, S., Liang, X., Zhang, Z., Su, X., Liu, J., Chen, Y., Wang, M., Zhang, Y. *et al.* (2011) Both miR-17-5p and miR-20a alleviate suppressive potential of myeloid-derived suppressor cells by modulating STAT3 expression. *J Immunol*, **186**, 4716-4724.

46. Kohlhaas, S., Garden, O.A., Scudamore, C., Turner, M., Okkenhaug, K. and Vigorito, E. (2009) Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol*, **182**, 2578-2582.

47. Liu, Z., Wang, D., Hu, Y., Zhou, G., Zhu, C., Yu, Q., Chi, Y., Cao, Y., Jia, C. and Zou, Q. (2013) MicroRNA-146a negatively regulates PTGS2 expression induced by Helicobacter pylori in human gastric epithelial cells. *J Gastroenterol*, **48**, 86-92.

48. Witwer, K.W., Sisk, J.M., Gama, L. and Clements, J.E. (2010) MicroRNA regulation of IFN-beta protein expression: rapid and sensitive modulation of the innate immune response. *J Immunol*, **184**, 2369-2376.

49. Chen, X.M., Splinter, P.L., O'Hara, S.P. and LaRusso, N.F. (2007) A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. *J Biol Chem*, **282**, 28929-28938.

50. Nematian, S.E., Mamillapalli, R., Kadakia, T.S., Majidi Zolbin, M., Moustafa, S. and Taylor, H.S. (2018) Systemic Inflammation Induced by microRNAs: Endometriosis-Derived Alterations in Circulating microRNA 125b-5p and Let-7b-5p Regulate Macrophage Cytokine Production. *J Clin Endocrinol Metab*, **103**, 64-74.

51. Tili, E., Michaille, J.J., Cimino, A., Costinean, S., Dumitru, C.D., Adair, B., Fabbri, M., Alder, H., Liu, C.G., Calin, G.A. *et al.* (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol*, **179**, 5082-5089.

52. Palanisamy, V., Jakymiw, A., Van Tubergen, E.A., D'Silva, N.J. and Kirkwood, K.L. (2012) Control of cytokine mRNA expression by RNA-binding proteins and microRNAs. *J Dent Res*, **91**, 651-658.

53. Salvi, V., Gianello, V., Busatto, S., Bergese, P., Andreoli, L., D'Oro, U., Zingoni, A., Tincani, A., Sozzani, S. and Bosisio, D. (2018) Exosome-delivered microRNAs promote IFN-alpha secretion by human plasmacytoid DCs via TLR7. *JCI Insight*, **3**.

54. McCoy, C.E. (2011) The role of miRNAs in cytokine signaling. *Front Biosci (Landmark Ed)*, **16**, 2161-2171.

55. Thai, T.H., Calado, D.P., Casola, S., Ansel, K.M., Xiao, C., Xue, Y., Murphy, A., Frendewey, D., Valenzuela, D., Kutok, J.L. *et al.* (2007) Regulation of the germinal center response by microRNA-155. *Science*, **316**, 604-608.

56. Winkler, C.W., Taylor, K.G. and Peterson, K.E. (2014) Location is everything: let-7b microRNA and TLR7 signaling results in a painful TRP. *Sci Signal*, **7**, pe14.

57. Zhou, R., O'Hara, S.P. and Chen, X.M. (2011) MicroRNA regulation of innate immune responses in epithelial cells. *Cell Mol Immunol*, **8**, 371-379.

58. Wang, Z., Han, J., Cui, Y., Zhou, X. and Fan, K. (2013) miRNA-21 inhibition enhances RANTES and IP-10 release in MCF-7 via PIAS3 and STAT3 signalling and causes increased lymphocyte migration. *Biochem Biophys Res Commun*, **439**, 384-389.

59. Hong, L., Sharp, T., Khorsand, B., Fischer, C., Eliason, S., Salem, A., Akkouch, A., Brogden, K. and Amendt, B.A. (2016) MicroRNA-200c Represses IL-6, IL-8, and CCL-5 Expression and Enhances Osteogenic Differentiation. *PLoS One*, **11**, e0160915.

60. Mima, K., Nishihara, R., Yang, J., Dou, R., Masugi, Y., Shi, Y., da Silva, A., Cao, Y., Song, M., Nowak, J. *et al.* (2016) MicroRNA MIR21 (miR-21) and PTGS2 Expression in Colorectal Cancer and Patient Survival. *Clin Cancer Res*, **22**, 3841-3848.

61. Harrison, E.B., Emanuel, K., Lamberty, B.G., Morsey, B.M., Li, M., Kelso, M.L., Yelamanchili, S.V. and Fox, H.S. (2017) Induction of miR-155 after Brain Injury Promotes Type 1 Interferon and has a Neuroprotective Effect. *Front Mol Neurosci*, **10**, 228.

62. Grasso, M., Piscopo, P., Confaloni, A. and Denti, M.A. (2014) Circulating miRNAs as biomarkers for neurodegenerative disorders. *Molecules*, **19**, 6891-6910.

63. Liu, C.G., Song, J., Zhang, Y.Q. and Wang, P.C. (2014) MicroRNA-193b is a regulator of amyloid precursor protein in the blood and cerebrospinal fluid derived exosomal microRNA-193b is a biomarker of Alzheimer's disease. *Mol Med Rep*, **10**, 2395-2400.

64. Ulrich, H., do Nascimento, I.C., Bocsi, J. and Tarnok, A. (2015) Immunomodulation in stem cell differentiation into neurons and brain repair. *Stem Cell Rev*, **11**, 474-486.

65. Hernangomez, M., Carrillo-Salinas, F.J., Mecha, M., Correa, F., Mestre, L., Loria, F., Feliu, A., Docagne, F. and Guaza, C. (2014) Brain innate immunity in the regulation of neuroinflammation: therapeutic strategies by modulating CD200-CD200R interaction involve the cannabinoid system. *Curr Pharm Des*, **20**, 4707-4722.

66. Shastri, A., Bonifati, D.M. and Kishore, U. (2013) Innate immunity and neuroinflammation. *Mediators Inflamm*, **2013**, 342931.

67. Maslinska, D., Laure-Kamionowska, M. and Maslinska, S. (2012) Toll-like receptors as an innate immunity bridge to neuroinflammation in medulloblastoma. *Folia Neuropathol*, **50**, 375-381.

68. Lehnardt, S. (2010) Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. *Glia*, **58**, 253-263.

69. Griffiths, M., Neal, J.W. and Gasque, P. (2007) Innate immunity and protective neuroinflammation: new emphasis on the role of neuroimmune regulatory proteins. *Int Rev Neurobiol*, **82**, 29-55.

70. Coleman, L.G., Jr., Zou, J. and Crews, F.T. (2017) Microglial-derived miRNA let-7 and HMGB1 contribute to ethanol-induced neurotoxicity via TLR7. *J Neuroinflammation*, **14**, 22.

71. Hoss, A.G., Labadorf, A., Beach, T.G., Latourelle, J.C. and Myers, R.H. (2016) microRNA Profiles in Parkinson's Disease Prefrontal Cortex. *Front Aging Neurosci*, **8**, 36.

72. Martinez, B. and Peplow, P.V. (2017) MicroRNAs in Parkinson's disease and emerging therapeutic targets. *Neural Regen Res*, **12**, 1945-1959.

73. (2009) NIH fact sheet on frontotemporal dementia (FTD). *J Pract Nurs*, **59**, 5.

74. Hashimoto, M., Rockenstein, E., Crews, L. and Masliah, E. (2003) Role of protein aggregation in mitochondrial dysfunction and neurodegeneration in Alzheimer's and Parkinson's diseases. *Neuromolecular Med*, **4**, 21-36.

75. Priller, C., Bauer, T., Mitteregger, G., Krebs, B., Kretzschmar, H.A. and Herms, J. (2006) Synapse formation and function is modulated by the amyloid precursor protein. *J Neurosci*, **26**, 7212-7221.

76. Turner, P.R., O'Connor, K., Tate, W.P. and Abraham, W.C. (2003) Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. *Prog Neurobiol*, **70**, 1-32.

77. Hooper, N.M. (2005) Roles of proteolysis and lipid rafts in the processing of the amyloid precursor protein and prion protein. *Biochem Soc Trans*, **33**, 335-338.

78. Hernandez, F. and Avila, J. (2007) Tauopathies. *Cell Mol Life Sci*, **64**, 2219-2233.

79. Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G.M., Cooper, N.R., Eikelenboom, P., Emmerling, M., Fiebich, B.L. *et al.* (2000) Inflammation and Alzheimer's disease. *Neurobiol Aging*, **21**, 383-421.

80. Benveniste, E.N., Nguyen, V.T. and O'Keefe, G.M. (2001) Immunological aspects of microglia: relevance to Alzheimer's disease. *Neurochem Int*, **39**, 381-391.

81. Aisen, P.S. (1997) Inflammation and Alzheimer's disease: mechanisms and therapeutic strategies. *Gerontology*, **43**, 143-149.

82. Tahara, K., Kim, H.D., Jin, J.J., Maxwell, J.A., Li, L. and Fukuchi, K. (2006) Role of toll-like receptor signalling in Abeta uptake and clearance. *Brain*, **129**, 3006-3019.

83. Li, X., Melief, E., Postupna, N., Montine, K.S., Keene, C.D. and Montine, T.J. (2015) Prostaglandin E2 receptor subtype 2 regulation of scavenger receptor CD36 modulates microglial Abeta42 phagocytosis. *Am J Pathol*, **185**, 230-239.

84. Yuyama, K., Sun, H., Sakai, S., Mitsutake, S., Okada, M., Tahara, H., Furukawa, J., Fujitani, N., Shinohara, Y. and Igarashi, Y. (2014) Decreased amyloid-beta pathologies by intracerebral loading of glycosphingolipid-enriched exosomes in Alzheimer model mice. *J Biol Chem*, **289**, 24488-24498.

85. Cogswell, J.P., Ward, J., Taylor, I.A., Waters, M., Shi, Y., Cannon, B., Kelnar, K., Kemppainen, J., Brown, D., Chen, C. *et al.* (2008) Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. *J Alzheimers Dis*, **14**, 27-41.

86. Kumar, P., Dezso, Z., MacKenzie, C., Oestreicher, J., Agoulnik, S., Byrne, M., Bernier, F., Yanagimachi, M., Aoshima, K. and Oda, Y. (2013) Circulating miRNA biomarkers for Alzheimer's disease. *PLoS One*, **8**, e69807.

87. Lei, X., Lei, L., Zhang, Z., Zhang, Z. and Cheng, Y. (2015) Downregulated miR-29c correlates with increased BACE1 expression in sporadic Alzheimer's disease. *Int J Clin Exp Pathol*, **8**, 1565-1574.

88. Yang, G., Song, Y., Zhou, X., Deng, Y., Liu, T., Weng, G., Yu, D. and Pan, S. (2015) MicroRNA-29c targets beta-site amyloid precursor protein-cleaving enzyme 1 and has a neuroprotective role in vitro and in vivo. *Mol Med Rep*, **12**, 3081-3088.

89. Long, J.M. and Lahiri, D.K. (2011) MicroRNA-101 downregulates Alzheimer's amyloid-beta precursor protein levels in human cell cultures and is differentially expressed. *Biochem Biophys Res Commun*, **404**, 889-895.

90. Tili, E., Mezache, L., Michaille, J.J., Amann, V., Williams, J., Vandiver, P., Quinonez, M., Fadda, P., Mikhail, A. and Nuovo, G. (2018) microRNA 155 up regulation in the CNS is strongly correlated to Down's syndrome dementia. *Ann Diagn Pathol*, **34**, 103-109.

91. Xie, H., Zhao, Y., Zhou, Y., Liu, L., Liu, Y., Wang, D., Zhang, S. and Yang, M. (2017) MiR-9 Regulates the Expression of BACE1 in Dementia Induced by Chronic Brain Hypoperfusion in Rats. *Cell Physiol Biochem*, **42**, 1213-1226.

92. Zhang, X., Huang, X., Fang, C., Li, Q., Cui, J., Sun, J. and Li, L. (2017) miR-124 Regulates the Expression of BACE1 in the Hippocampus Under Chronic Cerebral Hypoperfusion. *Mol Neurobiol*, **54**, 2498-2506.

93. Ito, N., Sakai, A., Miyake, N., Maruyama, M., Iwasaki, H., Miyake, K., Okada, T., Sakamoto, A. and Suzuki, H. (2017) miR-15b mediates oxaliplatin-induced chronic neuropathic pain through BACE1 down-regulation. *Br J Pharmacol*, **174**, 386-395.

94. Du, X., Huo, X., Yang, Y., Hu, Z., Botchway, B.O.A., Jiang, Y. and Fang, M. (2017) miR-124 downregulates BACE 1 and alters autophagy in APP/PS1 transgenic mice. *Toxicol Lett*, **280**, 195-205.

95. Lang, A.E. and Lozano, A.M. (1998) Parkinson's disease. Second of two parts. *N Engl J Med*, **339**, 1130-1143.

96. Davie, C.A. (2008) A review of Parkinson's disease. *Br Med Bull*, **86**, 109-127.

97. Reynolds, A.D., Glanzer, J.G., Kadiu, I., Ricardo-Dukelow, M., Chaudhuri, A., Ciborowski, P., Cerny, R., Gelman, B., Thomas, M.P., Mosley, R.L. *et al.* (2008) Nitrated alpha-synuclein-activated microglial profiling for Parkinson's disease. *J Neurochem*, **104**, 1504-1525.

98. Reynolds, A.D., Kadiu, I., Garg, S.K., Glanzer, J.G., Nordgren, T., Ciborowski, P., Banerjee, R. and Gendelman, H.E. (2008) Nitrated alpha-synuclein and microglial neuroregulatory activities. *J Neuroimmune Pharmacol*, **3**, 59-74.

99. Domert, J., Sackmann, C., Severinsson, E., Agholme, L., Bergstrom, J., Ingelsson, M. and Hallbeck, M. (2016) Aggregated Alpha-Synuclein Transfer Efficiently between Cultured Human Neuron-Like Cells and Localize to Lysosomes. *PLoS One*, **11**, e0168700.

100. Hansen, C., Angot, E., Bergstrom, A.L., Steiner, J.A., Pieri, L., Paul, G., Outeiro, T.F., Melki, R., Kallunki, P., Fog, K. *et al.* (2011) alpha-Synuclein propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation in cultured human cells. *J Clin Invest*, **121**, 715-725.

101. Reyes, J.F., Rey, N.L., Bousset, L., Melki, R., Brundin, P. and Angot, E. (2014) Alpha-synuclein transfers from neurons to oligodendrocytes. *Glia*, **62**, 387-398.

102. Rostami, J., Holmqvist, S., Lindstrom, V., Sigvardson, J., Westermark, G.T., Ingelsson, M., Bergstrom, J., Roybon, L. and Erlandsson, A. (2017) Human Astrocytes Transfer Aggregated Alpha-Synuclein via Tunneling Nanotubes. *J Neurosci*, **37**, 11835-11853.

103. Emmanouilidou, E., Melachroinou, K., Roumeliotis, T., Garbis, S.D., Ntzouni, M., Margaritis, L.H., Stefanis, L. and Vekrellis, K. (2010) Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J Neurosci*, **30**, 6838-6851.

104. Alvarez-Erviti, L., Seow, Y., Schapira, A.H., Gardiner, C., Sargent, I.L., Wood, M.J. and Cooper, J.M. (2011) Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission. *Neurobiol Dis*, **42**, 360-367.

105. Maciotta, S., Meregalli, M. and Torrente, Y. (2013) The involvement of microRNAs in neurodegenerative diseases. *Front Cell Neurosci*, **7**, 265.

106. Junn, E., Lee, K.W., Jeong, B.S., Chan, T.W., Im, J.Y. and Mouradian, M.M. (2009) Repression of alpha-synuclein expression and toxicity by microRNA-7. *Proc Natl Acad Sci U S A*, **106**, 13052-13057.

107. Su, C., Yang, X. and Lou, J. (2016) Geniposide reduces alpha-synuclein by blocking microRNA-21/lysosome-associated membrane protein 2A interaction in Parkinson disease models. *Brain Res*, **1644**, 98-106.

108. Liu, Y., Liao, S., Quan, H., Lin, Y., Li, J. and Yang, Q. (2016) Involvement of microRNA-135a-5p in the Protective Effects of Hydrogen Sulfide Against Parkinson's Disease. *Cell Physiol Biochem*, **40**, 18-26.

109. Beraud, D., Twomey, M., Bloom, B., Mittereder, A., Ton, V., Neitzke, K., Chasovskikh, S., Mhyre, T.R. and Maguire-Zeiss, K.A. (2011) alpha-Synuclein Alters Toll-Like Receptor Expression. *Front Neurosci*, **5**, 80.

110. Lee, E.Y., Park, K.S., Yoon, Y.J., Lee, J., Moon, H.G., Jang, S.C., Choi, K.H., Kim, Y.K. and Gho, Y.S. (2012) Therapeutic effects of autologous tumor-derived nanovesicles on melanoma growth and metastasis. *PLoS One*, **7**, e33330.

111. Thery, C., Amigorena, S., Raposo, G. and Clayton, A. (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*, **Chapter 3**, Unit 3 22.

112. Xiang, X., Poliakov, A., Liu, C., Liu, Y., Deng, Z.B., Wang, J., Cheng, Z., Shah, S.V., Wang, G.J., Zhang, L. *et al.* (2009) Induction of myeloid-derived suppressor cells by tumor exosomes. *Int J Cancer*, **124**, 2621-2633.

113. Weischenfeldt, J. and Porse, B. (2008) Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. *CSH Protoc*, **2008**, pdb prot5080.

114. van Scheppingen, J., Iyer, A.M., Prabowo, A.S., Muhlebner, A., Anink, J.J., Scholl, T., Feucht, M., Jansen, F.E., Spliet, W.G., Krsek, P. *et al.* (2016) Expression of microRNAs miR21, miR146a, and miR155 in tuberous sclerosis complex cortical tubers and their regulation in human astrocytes and SEGA-derived cell cultures. *Glia*, **64**, 1066-1082.

115. Sun, Y., Luo, Z.M., Guo, X.M., Su, D.F. and Liu, X. (2015) An updated role of microRNA-124 in central nervous system disorders: a review. *Front Cell Neurosci*, **9**, 193.

116. Brubaker, S.W., Bonham, K.S., Zanoni, I. and Kagan, J.C. (2015) Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol*, **33**, 257-290.

117. Yelamanchili, S.V., Lamberty, B.G., Rennard, D.A., Morsey, B.M., Hochfelder, C.G., Meays, B.M., Levy, E. and Fox, H.S. (2018) Correction: MiR-21 in Extracellular Vesicles Leads to Neurotoxicity via TLR7 Signaling in SIV Neurological Disease. *PLoS Pathog*, **14**, e1007068.

118. Harrison, E.B., Hochfelder, C.G., Lamberty, B.G., Meays, B.M., Morsey, B.M., Kelso, M.L., Fox, H.S. and Yelamanchili, S.V. (2016) Traumatic brain injury increases levels of miR-21 in extracellular vesicles: implications for neuroinflammation. *FEBS Open Bio*, **6**, 835-846.

119. Liu, X.J., Zheng, X.P., Zhang, R., Guo, Y.L. and Wang, J.H. (2015) Combinatorial effects of miR-20a and miR-29b on neuronal apoptosis induced by spinal cord injury. *Int J Clin Exp Pathol*, **8**, 3811-3818.

120. Alexandrov, P.N., Dua, P., Hill, J.M., Bhattacharjee, S., Zhao, Y. and Lukiw, W.J. (2012) microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). *Int J Biochem Mol Biol*, **3**, 365-373.

121. Cheng, L., Quek, C.Y., Sun, X., Bellingham, S.A. and Hill, A.F. (2013) The detection of microRNA associated with Alzheimer's disease in biological fluids using next-generation sequencing technologies. *Front Genet*, **4**, 150.

122. Cheng, L., Doecke, J.D., Sharples, R.A., Villemagne, V.L., Fowler, C.J., Rembach, A., Martins, R.N., Rowe, C.C., Macaulay, S.L., Masters, C.L. *et al.* (2015) Prognostic serum miRNA biomarkers associated with Alzheimer's disease shows concordance with neuropsychological and neuroimaging assessment. *Mol Psychiatry*, **20**, 1188-1196.

123. Du, M., Giridhar, K.V., Tian, Y., Tschannen, M.R., Zhu, J., Huang, C.C., Kilari, D., Kohli, M. and Wang, L. (2017) Plasma exosomal miRNAs-based prognosis in metastatic kidney cancer. *Oncotarget*, **8**, 63703-63714.

124. Gui, Y., Liu, H., Zhang, L., Lv, W. and Hu, X. (2015) Altered microRNA profiles in cerebrospinal fluid exosome in Parkinson disease and Alzheimer disease. *Oncotarget*, **6**, 37043-37053.

125. Ghosn, E.E., Cassado, A.A., Govoni, G.R., Fukuhara, T., Yang, Y., Monack, D.M., Bortoluci, K.R., Almeida, S.R., Herzenberg, L.A. and Herzenberg, L.A. (2010) Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc Natl Acad Sci U S A*, **107**, 2568-2573.

126. Okabe, Y. and Medzhitov, R. (2014) Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell*, **157**, 832-844.

127. Forsbach, A., Nemorin, J.G., Montino, C., Muller, C., Samulowitz, U., Vicari, A.P., Jurk, M., Mutwiri, G.K., Krieg, A.M., Lipford, G.B. *et al.* (2008) Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. *J Immunol*, **180**, 3729-3738.

128. Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougerolles, A. *et al.* (2005) Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med*, **11**, 263-270.

129. Tanji, H., Ohto, U., Shibata, T., Taoka, M., Yamauchi, Y., Isobe, T., Miyake, K. and Shimizu, T. (2015) Toll-like receptor 8 senses degradation products of single-stranded RNA. *Nat Struct Mol Biol*, **22**, 109-115.

130. Davidson, S., Kaiko, G., Loh, Z., Lalwani, A., Zhang, V., Spann, K., Foo, S.Y., Hansbro, N., Uematsu, S., Akira, S. *et al.* (2011) Plasmacytoid dendritic cells promote host defense against acute pneumovirus infection via the TLR7-MyD88-dependent signaling pathway. *J Immunol*, **186**, 5938-5948.

131. Neighbours, L.M., Long, K., Whitmore, A.C. and Heise, M.T. (2012) Myd88-dependent toll-like receptor 7 signaling mediates protection from severe Ross River virus-induced disease in mice. *J Virol*, **86**, 10675-10685.

132. Town, T., Bai, F., Wang, T., Kaplan, A.T., Qian, F., Montgomery, R.R., Anderson, J.F., Flavell, R.A. and Fikrig, E. (2009) Toll-like receptor 7 mitigates lethal West Nile encephalitis via interleukin 23-dependent immune cell infiltration and homing. *Immunity*, **30**, 242-253.

133. Im, H., Shao, H., Weissleder, R., Castro, C.M. and Lee, H. (2015) Nano-plasmonic exosome diagnostics. *Expert Rev Mol Diagn*, **15**, 725-733.

134. Peterson, M.F., Otoc, N., Sethi, J.K., Gupta, A. and Antes, T.J. (2015) Integrated systems for exosome investigation. *Methods*, **87**, 31-45.

135. Lee, K., Shao, H., Weissleder, R. and Lee, H. (2015) Acoustic purification of extracellular microvesicles. *ACS Nano*, **9**, 2321-2327.

136. Wierz, M., Pierson, S., Gargiulo, E., Guerin, C., Moussay, E. and Paggetti, J. (2019) Purification of Leukemia-Derived Exosomes to Study Microenvironment Modulation. *Methods Mol Biol*, **1884**, 231-245.

137. Taller, D., Richards, K., Slouka, Z., Senapati, S., Hill, R., Go, D.B. and Chang, H.C. (2015) On-chip surface acoustic wave lysis and ion-exchange nanomembrane detection of exosomal RNA for pancreatic cancer study and diagnosis. *Lab Chip*, **15**, 1656-1666.

138. Rho, J., Chung, J., Im, H., Liong, M., Shao, H., Castro, C.M., Weissleder, R. and Lee, H. (2013) Magnetic nanosensor for detection and profiling of erythrocyte-derived microvesicles. *ACS Nano*, **7**, 11227-11233.

139. Shao, H., Chung, J., Balaj, L., Charest, A., Bigner, D.D., Carter, B.S., Hochberg, F.H., Breakefield, X.O., Weissleder, R. and Lee, H. (2012) Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med*, **18**, 1835-1840.

140. Ye, J., Wang, Y., Liu, X., Li, L., Opejin, A., Hsueh, E.C., Luo, H., Wang, T., Hawiger, D. and Peng, G. (2017) TLR7 Signaling Regulates Th17 Cells and Autoimmunity: Novel Potential for Autoimmune Therapy. *J Immunol*, **199**, 941-954.

141. Pero, S.C., Sun, Y.J., Shukla, G.S., Carman, C.L., Krag, C.C., Teuscher, C., Krementsov, D.N. and Krag, D.N. (2017) Vaccine draining lymph nodes are a source of antigen-specific B cells. *Vaccine*, **35**, 1259-1265.

142. Lim, S.Y., Osuna, C.E., Hraber, P.T., Hesselgesser, J., Gerold, J.M., Barnes, T.L., Sanisetty, S., Seaman, M.S., Lewis, M.G., Geleziunas, R. *et al.* (2018) TLR7 agonists induce transient

viremia and reduce the viral reservoir in SIV-infected rhesus macaques on antiretroviral therapy. *Sci Transl Med*, **10**.

143. Chi, H., Li, C., Zhao, F.S., Zhang, L., Ng, T.B., Jin, G. and Sha, O. (2017) Anti-tumor Activity of Toll-Like Receptor 7 Agonists. *Front Pharmacol*, **8**, 304.

144. Borducchi, E.N., Liu, J., Nkolola, J.P., Cadena, A.M., Yu, W.H., Fischinger, S., Broge, T., Abbink, P., Mercado, N.B., Chandrashekar, A. *et al.* (2018) Antibody and TLR7 agonist delay viral rebound in SHIV-infected monkeys. *Nature*, **563**, 360-364.