MICRORNAS AS MAJOR PLAYERS IN TLR LIGAND-INDUCED TOLERANCE, CROSS-TOLERANCE AND EXPERIMENTAL PERIODONTAL DISEASES

By

MD ABU NAHID

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To my parents who have always been supportive, encouraging, and extremely patient. To my wife who has sacrificed a lot and given me love, encourage and support. To my mentors who have given me guidance, support and wisdom.

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Md Abu Nahid

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Toll-like receptors (TLRs) are the prime sensors for microbial components leading to the production of cytokines that are controlled by various mechanisms. Monocytes pretreated with lipopolysaccharide (LPS) exhibit a state of hyporesponsiveness to homologous and heterologous TLR ligands, referred to as tolerance and cross-tolerance, which play a broader role in innate immunity. Peptidoglycan (PGN)-primed monocytes also showed similar tolerance or cross-tolerance. microRNAs (miRNAs) are small RNA molecules that negatively regulate gene expression by targeting 3'untranslated region (3' UTR) of specific mRNAs. So far, LPS- or PGN-induced tolerance mechanism has not been examined regarding miRNA expression kinetics. To investigate the significance of miRNA expression, we first examined LPS-induced miRNAs including miR-146a. LPS-induced THP-1 monocytes showed a gradual increase of miR-146a expression, negatively correlated with TNF-α and adaptor kinases. Thus, the characteristics upregulation of miR-146a toward subsequent LPS challenge in THP-1 monocytes were studied. LPS-primed THP-1 monocytes showed higher miR-146a expression, negatively correlated with TNF-α production and adaptor proteins, suggesting miR-146a plays role in tolerance. Transfection of primary miR-146a (pri-miR-146a) in THP-1 monocytes mimicked LPS priming, whereas miR-146a inhibitor largely abolished LPS-tolerance. These findings

support the critical role of miR-146a in LPS-induced tolerance. IRAK1 and TRAF6 are used by all MyD88-dependent TLR pathways and are the targets of miR-146a. Once miR-146a is upregulated, other TLR pathways using these same adaptors are also affected. Thus, we demonstrated that miR-146a was responsible for LPS-induced cross-tolerance against TLR2 ligand, TLR5 ligand, and whole bacteria. We then investigated miR-146a expression in response to periodontal pathogenic bacteria both *in vivo* and *in vitro*. We also observed a dramatic increase of miR-132 expression at an early stage of PGN treatment of THP-1 monocytes that remained at higher level over 48 hours. Upregulated miR-132 was demonstrated to be responsible for PGN-induced tolerance through affecting IRAK4, the first adaptor recruited to MyD88 in TLR signaling. Our studies suggest that miR-146a and miR-132 play important roles in innate immunity, and modulating their levels can act as a therapeutic intervention for limiting or boosting TLR pathways during over activation of innate immune system.

CHAPTER 1
INTRODUCTION

Innate Immunity and Toll-like Receptors

Innate immunity is the first line defense mechanism that recognizes, responds to, and resolves invading pathogens or their conserved molecular patterns that are common to broad pathogen classes, commonly known as pathogen-associated molecular patterns (PAMPs). For the past few decade there has been an incredible expansion in our understanding of the molecular components of innate immunity and their physiological function in host defense (1). Recognition of microorganisms is linked to a chain of events that promote inflammation, activation of innate immune responses and priming of adaptive immune responses. During microbial invasion, danger signals are effectively detected through several families of innate immune receptors. These receptors collectively survey the extracellular space, endolysosomal compartments, and cytoplasm for signs of infection or tissue damage. The specificities of these receptors are fixed in the germline and are able to recognize a diverse array of pathogens (2-4). Toll-like receptors (TLRs) represent one of the most studied pathogen-detection systems in terms of their known ligands, downstream signaling pathways, and functional relevance. Key to the central role in host defense is that the TLRs are expressed by various cells, including antigen-presenting cells. The subcellular localization of TLRs has important consequences for ligand accessibility and can affect downstream signaling events especially for the recognition of nucleic acids. As these receptors have a central role in linking pathogen recognition to the induction of innate immunity, inflammation and eventually adaptive immunity, understanding the regulation of the signaling cascade is important.

To date, thirteen different TLRs (TLR1-TLR13) have been identified in mammals that recognize microbial cell wall or pathogen-specific nucleic acids (5, 6). TLRs possess an

extracellular leucine-rich repeat domain (type 1 membrane protein) and a cytoplasmic conserved Toll/IL-1R domain. The extracellular domain recognizes a bewildering range of microbial ligands, such as bacterial and fungal cell wall components, bacterial lipoproteins and highly conserved microbial proteins (4). The molecular basis of such diverse ligand binding remains poorly understood, although the elucidation of several recent structures of ligand-receptor complexes suggest that not all TLRs use the same ligand-binding interface to recognize different ligands (7, 8). In contrast, the cytoplasmic portion responds to ligand activation by recruiting adaptor kinases to enable signal transduction, most notably through activation of nuclear-factor-kappa B (NF-κB) transcription factor to culminate in potent transcriptional responses (4, 9). Therefore, activation of NF-κB by TLRs is a critical event in the pathway to inflammation.

TLRs show specificity to an individual or a set of microbial components by forming either as homodimeric or heterodimeric structures (9). Besides stimulation of TLR4 by lipopolysaccharide (LPS, endotoxin), a wide variety of bacterial products, DNA and RNA viruses, fungi and protozoa are recognized by other TLRs. For example, heat shock protein can signal via TLR4, Gram-positive peptidoglycan (PGN) activates TLR1 and 2, TLR3 recognizes viral double-stranded RNA, TLR5 recognizes bacterial flagellin, and single-stranded RNA viruses signal via TLR7 or TLR8. Since the discovery of the various TLRs, it has become clear that they act in concert in the signaling cascade following ligand-specific stimuli. The resultant production and release of cytokines demonstrates a different spectrum for each TLR, and the regulation of these cytokines is important in innate immunity to control the inflammatory response and damage. It is now known that TLR activation induces various regulatory molecules including microRNAs (miRNAs), that may participate in various mechanisms to control excessive inflammation. It is now known that TLR activation induces various regulatory

molecules including microRNAs (miRNAs) that may control excessive inflammation by various mechanisms and have been focused here in this Chapter 1.

Endotoxin Tolerance and Cross-tolerance

LPS, glycolipid of the outer cell membrane of Gram-negative bacteria, is one of the most potent stimulators of innate immune responses. The immune system detects and responds to LPS via TLR4 and activates various transcription factors leading to strong production of proinflammatory cytokines, such as TNF-α and IL-6, primarily aimed to control growth and the dissemination of invaders and subsequently curtailing the immune response as needed (10). However, pathological dysregulation of signaling components or transcription factors is linked to an excessive inflammatory response and can cause tissue damage, autoimmune diseases, and possibly cancer (11). Thus, cytokines production needs to be tightly regulated in a balanced immune system. Cytokine overproduction is observed in septic patients and can be produced in experimental animal models with the injection of a high dose of LPS. Injection of a high dose of LPS induces pathological symptoms resembling those of the septic patient (12). Importantly, neutrophils and monocytes from septic patients are refractory to subsequent LPS exposure and no longer produce the comparable levels of inflammatory mediators (13). This mechanism, referred to as endotoxin tolerance (also called LPS hyporesponsiveness or refractoriness), prevents overstimulation from the continuous exposure to the same and related danger signals. Although endotoxin tolerance is claimed to be a specific phenomenon, *in vivo* or *in vitro* LPS-primed cells show hyporesponsiveness to heterologous zymogen, staphylococci or streptococci as well as many other non-LPS ligands. This is known as LPS-induced cross-tolerance and has also been observed in association with cells from septic patients (14). Similarly, other TLR ligands such as PGN, lipoteichoic acid, Pam_3CSK_4CysSerLys4 (Pam_3CSK_4), LPS from *Porphyromonas gingivalis*, and flagellin, plus cytokines such as TNF-α and IL-1β, have been

shown to induce homologous tolerance in monocytes/macrophages and, interestingly, can substitute for each other and sometimes mediate cross-tolerance both *in vitro* and *in vivo* (15).

Endotoxin tolerance has an early and late phase response with different characteristics following initial injection of LPS (16). The early phase is antibody independent entailing a transiently occurring refractory state. In contrast, the late tolerance appears to be mediated by anti-endotoxin antibodies directed against both surface "O" and common core antigens, which blunt the release of common core antigens (16). Thus, endotoxin is considered to provoke both innate and adaptive immunity to a certain extent, where pathogenicity mostly occurs via the lipid A component of LPS.

During endotoxin tolerance, some metabolic changes including inflammatory cytokine production are decreased during repeated LPS exposure. For example, animals injected with subtoxic LPS dose show an increased survival rate against inflammatory damage (17, 18). These studies have been conducted for decades attempting to unravel the underlying mechanisms associated with innate immune cells to identify a more effective therapeutic intervention against bacterial infection. LPS-induced tolerance and/or cross-tolerance are thus thought to have important implication in innate immunity, but how they are established is not yet completely understood. TLR2 ligand-induced tolerance has not been as extensively studied as LPS tolerance. Consequently, the mechanism of PGN-induced tolerance and cross-tolerance is less well understood. Therefore, the study of the TLRs regulation in LPS-primed or PGN-primed immune cells will help to elucidate its role against various microbial insults or whole bacteria.

-Tolerance Model and Associated Cytokine Production

In Vitro Tissue Culture Model Studies

Although studies on endotoxin tolerance have been conducted extensively in animal studies, both *in vivo* and *ex vivo*, most studies on the mechanism of innate immune cell

desensitization derive from experiments using primary cells and immortalized cell lines *in vitro* (Table 1-1). Macrophages from endotoxin-tolerant hosts have a low level of cytokine production after repeated exposure to LPS *in vitro*. Suppression of cytokine production kinetics after LPS challenge is observed for primary cells, such as human monocytes and rabbit or mouse peritoneal macrophages, as well as a variety of human and murine cell lines. The spectrum of cytokines downregulated in desensitized cells in *in vitro* tissue culture involves the same mediators shown to be suppressed *in vivo;* TNF-α levels are most reproducible in these studies, while the data for other cytokines are more inconsistent as reviewed in detail by Lehner and Hartung in 2002 (19). Depending on the experimental setting, downregulation of TNF-α has been described and is associated with the decrease, increase or unchanged status of the release of IL-1, IL-6, IL-8, IL-10, and prostaglandin E2 (PGE2) after LPS challenge(20). Most controversial data are related to the regulation of IL-1. Whereas studies with the human cell line THP-1 reveal downregulation of IL-1 both at mRNA and protein level in response to repeated LPS stimulation (21, 22), experiments conducted with human or mouse primary cells have shown no changed or even increased IL-1 production in response to a second LPS challenge (23-25).

Downregulation of TNF-α is correlated with the decreased mRNA levels, suggesting that cytokine release is controlled at the transcriptional level in cells (26, 27) and in human (28), mouse (24, 25), and rabbit primary cells (29). However, the mRNA level does not always correlate with the protein level as shown by Zuckerman et al. who observed increased mRNA levels in LPS-pretreated cells despite inhibition of TNF release (30). Another controversial issue is the expression of inducible nitric oxide (NO) synthase and NO production after secondary LPS challenge, reported be either suppressed (31, 32) or enhanced (33, 34), depending on the experimental settings. In line with these data, it has been demonstrated that depending on the

concentration of the primary LPS stimulus, either suppression or enhancement of NO production can be observed (35).

In summary, *in vitro* exposure of cells to LPS results in suppression of TNF-α release during subsequent LPS stimulation. Cells desensitized *in vitro* show many features that are similar to macrophages isolated from endotoxin-tolerant mice as well as human (19). Although, *in vitro* studies have certain limitations compared to *in vivo* system, much of our recent knowledge concerning the mechanism of monocytes/macrophage desensitization is derived from *in vitro* experiments. Thus, monocytes, macrophages or macrophage-like cell lines are considered the main cellular actors in endotoxin tolerance for *in vitro* models, consistent with the pioneering work of Beeson (36), and later demonstrated *in vivo* using macrophage-transfer experiments by Freudenberg and Galanos (37). Endotoxin tolerance also affects other myeloid cells, for example dendritic cells (DCs) and neutrophils, as well as non-immune cells, for example intestinal endothelial cells (38). As noted, not all proinflammatory cytokines have been reported to behave in a fashion similar to that of TNF-α (Table 1-1). TNF-α is stably downregulated in all tolerized models and is thus considered to be the most reliable marker of endotoxin tolerance.

In Vivo Animal Model Studies

Endotoxin tolerance can be experimentally induced in healthy humans and animals. One of the earliest experimental reports of endotoxin tolerance came from Paul Beeson in 1946 (39). In his report, repeated intravenous injection of typhoid bacterial pyrogen in rabbits caused a progressive reduction in the febrile response. Patients recovering from infections with *Salmonella typhimurium* (fever causing agent) (40) and *Plasmodium vivax* (malaria causing agent) (41) were observed to produce a reduced pyrogenic response to LPS administration compared to that of healthy individuals. As previously reviewed (42), a similar febrile response

to LPS was also reported in patients with pyelonephritis and urinary tract infections (43). Similar clinical features were also observed in an *ex vivo* study where monocytes from septic patients showed reduced levels of IL-lα, IL-lβ, TNF-α, and IL-6 production in response to LPS challenge (44). Alveolar macrophages and blood neutrophils from these septic patients were also shown to be less responsive to LPS challenge. Of note, the reduction of cytokine was more pronounced in patients infected with Gram-negative bacteria compared to those with Gram-positive bacteria (42).

To study the *in vivo* tolerance phenomenon, both animal and tissue culture models have been used to conduct experiments. A sublethal dose of LPS injection protects animals from a subsequent and otherwise lethal dose of LPS. Studies with mice have demonstrated monocytes/macrophages as the principal cells responsible for the induction of endotoxin tolerance *in vivo* (42). It has been shown that in general LPS primed animals regain responsiveness by eight days. In some experiments, rats primed with LPS survive from the lethal dose of LPS challenge due to diminished level of cytokine production. High serum levels of cytokines including TNF-α observed 90 minutes after the administration of LPS (20), are markedly decreased in tolerant animals and similar cytokine responses are observed in *in vitro* tissue culture experiments.

Beside animal studies, investigators have used human models to study endotoxin tolerance. In the human study, a ‘‘tolerance time frame’’ was described using circulating monocytes isolated from healthy individuals (45). This study showed that a short exposure to LPS in humans is sufficient to induce a refractory state to further LPS challenges (45). Other investigators have also found that circulating monocytes from healthy individuals exhibit a state of endotoxin hyporesponsiveness after five days of repetitive LPS injections at low doses (46).

Subsequently, *in vivo* endotoxin tolerance models confirm that low dose LPS pretreated mouse macrophages and human monocytes loss the ability to respond to further LPS challenge in a partial or complete manner (45, 47, 48). The key readout for endotoxin tolerance in these cells is the dramatic reduction of proinflammatory cytokines including TNF-α as summarized in Table 1-2.

Ex Vivo Model Studies

Innate immune cells are the main contributor of LPS tolerance; this understanding stems from *ex vivo* studies showing impaired cytokine production in these cells isolated from LPS-primed animals and then challenged *in vitro*. An *ex vivo* study using liver macrophage (Kupffer cells) isolated from LPS-primed rabbits were not capable to produce endogenous pyrogen (IL-1) *in vitro* (49). Peritoneal resident or thioglycolate-elicited macrophages from LPS-primed mouse and rat displayed reduced levels of TNF-α or IL-1(50) response upon LPS challenge *in vitro*. A similar impairment of IL-12 and IFN-γ production by spleen cells from LPS-primed mice was also observed (51). This *ex vivo* cytokine production by tolerized cells has been reviewed by Lehner and Hartung (19) and is summarized in Table 1-3. As discussed by Bundschuh et al.(52), the reduction of cytokine response is a common feature of various macrophage populations (bone marrow, peritoneal, alveolar, and spleen cells) isolated from endotoxin-primed mice. Beside animals, human monocyte hyporesponsiveness was also reported after LPS challenge (53, 54). *Ex vivo* studies are important for understanding the tolerization phenomenon in animals including humans.

Proposed Mechanisms of Endotoxin Tolerance

In the past few years, our understanding of the molecular mechanisms underlying hyporesponsiveness of innate immune cells after exposure to LPS has expanded considerably. A potential mechanism for tolerance to LPS is the downregulation of the cell surface receptor

molecules in the activated cells. Signaling through the TLR4 pathway is one of the predominant molecular mechanisms for the detection of Gram-negative pathogens and their cell wall components, such as LPS, by host immune cells. As reviewed recently (55), TLR4 employs signaling through two distinct adaptors pathway, MyD88 and TRIF. Most of the tolerance mechanisms have been studied in relation to MyD88 and relatively little is known about TRIF in inflammation. Thus, defects in TLR4 signaling have been observed at the level of the receptor, adaptors, signaling molecules, and transcription factors. Although Larsen and Sullivan reported that LPS pre-exposure decreased the number of LPS binding sites on monocytes (56), the expression of the LPS co-receptor CD14 was unchanged or sometimes amplified in subsequent LPS-challenge (57-59). Similar studies were reported by Labeta et al. (57) and Ziegler-Heitbrock et al. (60) on Mono-Mac-6 cells and also by McCall (57) on blood neutrophils. In their studies, CD14 was unchanged in tolerized cells. Furthermore, it was unlikely that tolerance was mediated via expression of CD14 because anti-CD14 antibody-treated THP-1 monocytes still showed LPS tolerance (61). Because LPS-mediated signaling through TLR4 also requires physical association of MD-2, its level has been examined. MD-2 levels were reported to remain either unchanged by pretreatment of C57BL/6J mouse peritoneal macrophages with LPS from *S. typhimurium* (62) or slightly increased in LPS-tolerant human monocytes(63). In contrast, Adib-Conquy and Cavaillon showed downregulated MD-2 mRNA in *E. coli* LPS-treated PBMCs (64).

Because TLR4 is the principal TLR receptor for LPS, downregulation of TLR4 cell surface expression has been examined as a possible mechanism of LPS tolerance in many studies as reviewed and summarized by Fan and Cook (65). The expression of TLR4 has been reported as either increased or decreased after LPS priming depending on cell types and experimental settings. Specific studies have also reported that induction of LPS tolerance is not only

associated with TLR4 expression (66, 67). Similarly, TLR2 receptor expression is reported to be unchanged after LPS induction (65). Thus, it appears that signaling molecules downstream of TLR4 signaling might be involved in LPS-induced tolerance. Following this induction, hyporesponsiveness in response to LPS pre-exposure has been shown to be associated with altered expression of G-protein(68), phospholipase Cγ1, and phosphatidylinositol-3 kinase (69). West et al. have reported compromised protein kinase C activation in LPS-primed cells (70), and receptor independent stimulation of the protein kinase C by phorbol myristate acetate (PMA) treatment could regain the suppression of cytokine production (19). Other researchers have described reduced signal transduction via both the mitogen-activated-protein kinase (MAPK) cascade (71, 72) and inhibitor of NF-κB kinases, causing an impaired transcription of NF-κB- and Ap-1-regulated genes (22, 71).

As another mechanism for the suppression of NF-κB-dependent gene expression in LPS tolerance, an increase in the expression of the p50 subunit of NF-κB have been observed in *S. typhimurium* LPS refractory cells (Mono-Mac-6) (59). This upregulation leads to a predominance of transactivation-inactive p50/p50 homodimers, that bind to NF-κB motifs in several promoters of proinflammatory cytokines, leading to the suppressed transcription of these genes (59, 73). This is evident by experiments showing that p50-deficient mice are resistant to LPS-tolerance (74) and many other cell-based studies support this finding (75-77). Thus, a shift in subunit composition of NF-kB favoring p50 is a proposed mechanism for endotoxin tolerance. LaRue and McCall have reported that decreased LPS-induced transcription of IL-1β in LPS-primed THP-1 cells can be regulated potentially by IκB-α (21). Suppression of IL-1 receptor-associated kinase (IRAK) activation and association with MyD88 is also observed in LPS-tolerized cells(78), supporting the idea that very early steps in TLR4-signaling upstream of NF-

κB are affected after LPS treatment. Further support for this finding is from the study showing that the induction of cross-tolerance to LPS occurred via the involvement of IL-1 receptor but not the TNF-α receptor (71). Intriguingly, the signal transductions of IL-1R, TLR4, and TLR2 employ similar signaling molecules (79, 80). Recent studies have demonstrated that pre-exposure to peptidoglycan or Pam_3CSK_4 that signals via TLR2 resulted in hyporesponsiveness to TLR4-mediated LPS signaling and vice versa (81). These findings suggest that common signaling molecules such as MyD88, IRAK, TNF receptor-activated factor 6 (TRAF6), or NF-κB-inducing kinase are suppressed in TLR ligand-primed cells. Thus, rather than diminishing TLR4 surface expression, inhibition of common signaling pathways of the IL-1R/TLR family, is mostly accountable for tolerance. This was evident by the pre-exposure of macrophages to the TLR2-dependent stimulus mycoplasmal lipopeptide MALP-2 suppressed TNF-α release without affecting TLR4 expression during LPS induction (62). Alterations in expression of the most common signaling molecules IRAK1 and TRAF6 have been reported in endotoxin tolerance due to their central role in LPS signaling downstream of TLRs (82).

Currently, four members of the IRAK family (IRAK1, IRAK2, IRAK4, and IRAKM) have been identified. All but IRAKM possess kinase activity and mediate TLR signaling. Knockdown of IRAK1 and IRAK4 has been shown to reduce cytokine response (83, 84) and thus shows their importance in TLR signaling and thereby LPS tolerance. IRAK4 knockout mice are phenotypically similar to mice lacking adaptor protein MyD88 (84). Animals with a deletion of IRAK1 are partly resistant to LPS shock (85). In contrast, IRAKM negatively regulates LPS signaling (86). Similarly, other negative regulators of signal transduction have been described previously (42, 55), and some of the important factors are shown in Table 4. Among them, only the decrease of IRAK4 has been confirmed in endotoxin tolerance of both humans and mice.

Changes in IRAK1 expression in LPS tolerance has been the focus of recent investigations (81, 82). In these studies, IRAK1 is consistently decreased after LPS treatment in various cell lines and primary cells. Li et al.(78) and Boone et al.(87) have also observed LPS tolerance in monocytes caused by impairment of IRAK1 and TRAF6 kinase activity. In another study, de Nardo et al. have shown that the knockdown of IRAK4 renders immune cells much less responsive to TLR agonists, indicating that IRAK4 is also a pivotal component for TLR signaling (83).Similarly, PGN-induced tolerance has been found to be associated with the impairment of these kinases (88) and has been discussed in Chapter 5.

Despite many studies on innate immune cell hyporesponsiveness in response to LPS or PGN pretreatment, the mechanism of suppression of cytokine production remains unclear. Because there is sound evidence for a contribution of many of the aforementioned factors, it is likely that innate immune cell hyporesponsiveness is the consequence of the coordinated action of many factors induced by the primary TLR ligand stimulus and depending on the experimental model used to examine tolerance (species, cell types, experimental settings). To extend the understanding of the mechanism, we have investigated other factors such as miRNA-mediated hyporesponsiveness as well as relative contributions of these factors (81, 82).

MicroRNAs as Novel Regulators of Gene Expression

miRNAs comprise a large family of short single-stranded approximately 21-nucleotide-long RNAs that have emerged as critical players in the life science fields. miRNAs have revolutionized our comprehension of the post-transcriptional regulation of gene expression that act via hybridization to the 3’ untranslated region (UTR) of target mRNA molecules, leading to the degradation of mRNA or repression of translation, a process also called RNA interference (RNAi). miRNAs are well conserved in eukaryotic organisms and are considered to be an important component for genetic regulation (89). miRNAs are found primarily in multicellular

organisms and also in unicellular algae including *Chlamydomonas reinhardtii* (89). About two hundred miRNAs are expressed in lower metazoans and plants, but approximately a thousand are predicted in humans. Although miRNAs are similar in structure to small interfering RNAs (siRNAs), there are distinctions between the two molecules. While miRNAs are transcribed from an endogenous gene or gene cluster and primary transcript contains a hairpin structure, siRNAs are often chemically synthesized and exogenously introduced into animal cells to observe its biological function resulting in gene knockdown. Most miRNAs bind their target mRNAs by partially complementing sites in their 3'-UTR, coding sequences (89), and even in the 5'-UTR(90). siRNAs are by design perfectly complementary to their targets, often at the coding region, leading to mRNA degradation.

An increase in the number of miRNA targets identified has led to functional studies, which demonstrate that miRNAs are involved in the regulation of almost every cellular process investigated (91). Changes in miRNA expression are critical for many biological processes including development and cell differentiation. Given the emerging roles of miRNAs in modulating immune response, it is likely that any dysregulation of miRNA expression may contribute to many human pathologies, including malignancies, chronic inflammation, and autoimmune diseases (92-94). These observations are probably not surprising as bioinformatics predictions indicate that mammalian miRNAs can regulate ~60% of all protein-coding genes(95). Deep-sequencing technologies have delivered a sharp rise in the rate of novel miRNA discovery. The current release of miRBase 17 (http://www.mirbase.org) contains 19,724 distinct mature miRNA sequences in over 153 species (96). For humans, this database lists more than 1100 predicted miRNA sequences. Although it was initially believed that miRNAs were encoded in intergenic regions, it is now known that the majority of human miRNA loci is located within

intronic regions or noncoding transcription units and is transcribed in parallel with other transcripts (97-99).

The following three approaches have been used for the identification of miRNA genes. The first approach is through forward genetics where mutations are identified that produce a certain phenotype. This approach was used to identify the first two miRNAs, lin-4 and let-7 observed in *Caenorhabditis elegans* (97, 100, 101). A second approach is using directional cloning to construct a cDNA library for endogenous small RNAs (102). However, a potential limitation of this approach is that some miRNAs expressed under specific conditions, in specific cell types, or at very low levels may be difficult to identify. The third approach is bioinformatics predictions, which is becoming increasingly more powerful and indispensible to provide a thorough catalogue of miRNA genes in sequenced genomes. Several techniques have been developed to detect miRNAs. Northern blot analysis has been widely used but may not be sensitive enough to detect miRNAs expressed at low levels. In the past few years, miRNA arrays or PCR arrays have been used for miRNA expression profiling. To validate miRNA array data, the quantitative real-time PCR assay is becoming the most commonly used method as it provides increased sensitivity and cost-effectiveness. However, to bypass the dependency of bioinformatics to predict miRNA-mRNA interactions, at least two methods, known as HITS-CLIP (103) and PAR-CLIP (104), have been developed to directly identify protein-RNA interactions via covalently crosslinking the Ago protein-miRNA-mRNA complexes. The sequences of relevant miRNA-mRNA interactions are then determined by deep sequencing.

Biogenesis and Maturation of miRNA

Functional miRNAs are processed from long endogenous primary transcripts (pri-miRNAs), which are transcribed either from independent miRNA genes or from introns of capped and polyadenlyated protein-coding RNA polymerase II transcripts. A single pri-miRNA

often contains one or more mature miRNA. Pri-miRNAs are processed by the sequential action of a pair of type III RNA endonucleases Drosha (in the nucleus) and Dicer (in the cytoplasmic compartment). Both Drosha and Dicer are bound to proteins containing double-stranded RNA-binding domains. The Drosha–DGCR8 complex processes pri-miRNAs into 60-70-nucleotide long hairpin-shaped precursor known as pre-miRNAs. In animals, pre-miRNAs are transported from the nucleus to the cytoplasm by exportin 5. Pre-miRNAs undergo a further round of processing by Dicer to yield an approximately 22-bp RNA duplex miRNA-miRNA*. However, a relatively low number of miRNAs can bypass the general miRNA processing order, and their maturation can be independent of Drosha/DGCR8, such as miR-320 or miR-484(105), or of Dicer, such as erythropoiesis-related miR-451(106, 107). Drosha/DGCR8-independent miRNAs include mirtrons and tailed mirtrons, which mature to pre-miRNA via mRNA splicing and exonuclease trimming (108, 109) as described in *C. elegans* and *D. melanogaster*.

Following processing by Dicer, the double-stranded mature miRNA is loaded onto the Argonaute family of proteins (Ago1-4) based on their intrinsic thermodynamic preference or affinity. Normally one strand is then preferentially selected to function as mature miRNA or the guiding strand, while the complementary strand (miRNA*) is released and degraded. However, both strands of the pre-miRNA hairpin can give rise to mature and functional miRNAs (89, 110). The most studied mammalian Ago is Ago2, which is also the key component of the so-called RNA-induced silencing complex (RISC). The RISC-loaded miRNA binds to its target mRNAs causing degradation or translational repression. Recent data show that a second protein GW182 is recruited to play a key role in the translational silencing and/or target mRNA degradation pathway (111, 112). Interestingly, both Ago2 (113) and GW182 (114, 115) are known targets of human autoantibodies, thus may be important in autoimmunity.

Changes in miRNA Expression byTLR Ligand Stimulation

The importance of miRNAs in regulating differentiation and function of immune cells is underlined by their unique expression. LPS stimulation can clearly modulate miRNA expression as demonstrated by microarray analysis (116). Although a subset of miRNAs has emerged, subtle differences in their expression profiles depend on the TLR agonist used, stimulation time, method of detection, and probably most importantly, the cell types examined.

The first LPS-induced miRNA profiling was performed by Taganov et al. in 2006 on THP-1 human monocytes (116). In their study, they observed the upregulation of miR-146a, miR-155, and miR-132, where miR-146a expression was validated using real-time PCR. Our laboratory confirmed the increase in miR-146a during LPS stimulation with levels increased up to 100-fold over 24 to 48 hours (81, 82, 117). An increase in miR-146a expression was also observed by other inflammatory TLR agonists as well as cytokines, including IL-1β and TNF-α. Consistently, miR-146a is also highly expressed by whole bacterial stimulation and infection (118). The induction of miR-146a expression was demonstrated to be controlled by NF-κB (116), and it is now known that expression of many other LPS-induced miRNAs is also dependent on this transcription factor. In contrast, miR-132 (and miR-212) was shown to be regulated by cyclic AMP-response-element-binding protein (CREB) as well as p300 transcriptional co-activator in Kaposi's sarcoma-associated herpesvirus (KSHV) infected endothelial cells (119). PGN-induced CREB also upregulated miR-132 (Nahid et al. submitted). Thus, like other innate immune genes, induction of miRNAs is apparently dependent on certain transcription factors and may vary greatly among cell types. Currently, a key issue in the field of miRNA research in innate immunity is the apparent variability of miRNAs induced in different cells by the same or different TLR ligands. Multiple miRNAs are induced in innate immune cells, where miR-146a has been consistently observed in many experimental settings. Table 1-5 summarizes the

miRNAs with changes in expression induced by TLR signaling in a number of independent studies (120). Note that TLR ligand-induced miRNAs are restricted not only in innate immune cells but also in other cell types. For example, miR-146a is induced in lung epithelial cells A549 in response to IL-1β (121). Induction of miR-146a expression has been reported by activating surface TLR, but not by endosomal TLR signaling (TLR3, 7 or 9). These observations indicate that miR-146a plays a role in regulating the innate immune response predominantly to bacterial pathogens. However, certain viruses, such as vesicular stomatitis virus (VSV), can also induce miR-146a (122).

In murine bone marrow-derived macrophages, miR-155 is upregulated in response to the TLR3 ligand, such as poly-inositolcytidine [poly(I:C)], and IFN-β in a MAPK JNK-dependent manner (123). Moreover, miRNAs are upregulated *in vivo* in response to bacterial components and are implicated in many inflammatory diseases. In the lung, miR-214, miR-21, miR-223, and miR-142-3p, are upregulated one to three hours after treatment with LPS (124). Interestingly, there is evidence that the expression of certain miRNAs can decrease following TLR activation indicating the balance among miRNA in physiological settings (Table 1-5). Similar to other TLR-responsive genes, miRNAs can be classified as early or late response genes; miR-146a (82) and miR-132 (Chapter 5) are highly induced 2-4 hours after stimulation, whereas other miRNAs such as miR-21 are induced later (125). Therefore, even when the same transcription factor NF-κB is activated, their processing and maturation depend on other unknown factors during TLR signaling. In addition to the induction of certain miRNAs, new mechanisms are being discovered that negatively regulate miRNA induction by TLR signaling. For example, anti-inflammatory cytokine IL-10 can inhibit the expression of miR-155 in response to LPS but has no effect on the expression of other miRNAs, such as miR-21 or miR-146a (126). However, less is known

regarding how TLR signaling can decrease miRNA expression. This function may be through transcriptional repression or post-transcriptional mechanisms that destabilize miRNA transcripts. Despite the wealth of information regarding miRNA induction, there has been a tendency in the field of miRNA biology to document their levels of change without effectively analyzing the functional consequences of these changes. The consequence of changes of a few miRNAs expression (such as miR-146a, miR-132, and miR-212) has been discussed in Chapter 2, 3, & 5 in the context of TLR-ligand induced with implications in innate immunity and specifically in controlling TLR signaling.

Molecular Interaction between miRNA and TLR Signaling Proteins

miRNAs represent a ubiquitous feature of all cells, as they are implicated in both developmental and functional studies of innate immune cells. However, it is more interesting to decipher the impact of TLR ligand associated miRNA activity on the innate immune system. It is acknowledged that innate receptors are attributed to immune cells or that particular TLRs are confined to specific cell types, making them adapt for selected functions. Recent reports have shown that TLRs themselves can be directly targeted by miRNAs. TLR4 expression is regulated by *let-7i* in cholangiocytes and contributes to epithelial immune responses against *C. parvum* infection (127). In another report, TLR2 mRNA is regulated by miR-105. The expression of miR-105 is higher in oral keratinocytes derived from patients who respond weakly to TLR2 agonist with low levels of cytokine induction, presumably owing to decreased TLR2 expression (128). This finding indicates that there might be a reciprocal relationship between TLR2 signaling and miR-105 expression. These data all point to the regulation of certain TLRs by miRNAs underscoring their importance in constitutive TLR expression; an exception to this is the recent report showing TLR4 as a direct target of LPS-induced miR-146a (129).

Rather than shutting down the TLR signaling pathway completely by abolishing receptor expression, the trend for miRNA function is to decrease TLR signaling activity by targeting the downstream signaling molecules. For example, IRAK1 and TRAF6 are two central adaptor kinases in the downstream signaling cascade and they are targeted by miR-146 (82, 116). These adaptor proteins are important components of the MyD88-dependent pathway for NF-κB activation in many cell types, including THP-1 monocytes. Taganov et al. have postulated that miR-146a can negatively regulate the MyD88-NF-κB signaling pathway after microbial infection (116), consistent with our recent reports (81, 82). IRAK2, a kinase that can compensate IRAK1 for the persistence of NF-κB activation, is also targeted by miR-146a (122), although the relevance of this observation for TLR signaling remains unknown. Thus, several studies have linked miR-146a expression to NF-κB signaling within the innate immune system.

miR-155 expression is also induced by TLR signaling and can downregulate these signaling pathways by targeting key signaling molecules. For example, inhibition of miR-155 activity in DCs resulted in an increase of components of the p38 MAPK pathway (130). The reason is that TAK1-binding protein 2 (TAB2), a signaling molecule that activates MAPK kinases downstream of TRAF6, has been confirmed as a direct target of miR-155 (130). MyD88 has also been identified as a target of miR-155 in the study of miR-155 expression induced by *Helicobacter pylori* (131). Moreover, MyD88 is targeted by miR-155 in foam cells, which induces miR-155 expression when overloaded with lipid such as oxidized low-density lipoprotein (LDL) (132).

In another study, miR-145 is known to target MAL (bridging adaptor for TLR2- and TLR4-mediated MyD88-dependent signaling) (133), although it remains to be determined whether the expression of miR-145 is also regulated during TLR2 or TLR4 signaling. However,

MAL undergoes proteasomal degradation following TLR2 and TLR4 stimulation (134). Therefore, perhaps an additional level of control for MAL expression exists through miR-145. Finally, Bruton's tyrosine kinase Btk, involved in the MyD88-dependent signaling pathways to NF-κB activation, is a target of miR-346 (135), which is highly induced by LPS stimulation of rheumatoid arthritis synovial fibroblasts. Further investigation is needed to determine whether miR-346-mediated regulation of Btk mRNA also occurs in other cells, including monocytes/macrophages.

Although many proteins are involved in TLR signaling, few are the known targets of miRNA. For example, IRAK4 is the important adaptor; however, TLR-induced miRNAs that target IRAK4 have not been identified. These adaptor proteins are common components of several TLR signaling pathways, suggesting that once a TLR is triggered (such as TLR4-LPS interaction), miRNA-mediated targeting of common signaling proteins could silence signaling through multiple TLRs, which has been discussed in Chapter 3 in the context of LPS-induced cross-tolerance. Because, most pathogens can engage many TLRs, miRNAs could help to limit robust pro-inflammatory responses by immune cells after a pathogen is encountered. If this is the case, many miRNAs work together alone or with various other mechanisms to control the expression of TLR signaling components. The combination of these mechanisms could result in timely and appropriate decrease and controlling of the pro-inflammatory response.

TLR Ligand-induced miRNA

miR-146a

The innate immune system provides an important defense against invading pathogens. miRNAs have been implicated in both the development and function of innate immune cells. Inflammatory ligand-stimulated monocytes show upregulation of many miRNAs including miR-146 (116). Two isoforms of miR-146 exist—miR-146a and miR-146b—which are encoded on

human chromosomes 5 and 10, respectively (or, in mice, chromosomes 11 and 19, respectively). The mature sequences for miR-146a and miR-146b differ by only two nucleotides, although they share the same seed sequence. Many studies have linked miR-146a expression to NF-κB signaling whereas it is less clear with miR-146b. Studies show that miR-146a is quickly induced upon activation of human monocytes (116), and that LPS-induced miR-146a targets IRAK1 and TRAF6. This finding suggests the role of miR-146a in controlling cytokine and TLR signaling through a negative feedback regulatory loop. Consistent with this finding, miR-146a contributes to the establishment of endotoxin tolerance and cross-tolerance in monocytes to regulate TNF-α production (Figure 1) as discussed in detail in Chapter 2. In human Langerhans cells (LCs), miR-146a is constitutively expressed at high levels, as compared with interstitial dendritic cells (136). In these cells, high miR-146a expression is controlled by the transcription factor PU.1 in response to TGF-β1, a key signal for epidermal LC differentiation, which does not appear to influence myelopoiesis or DC subset differentiation. Thus, constitutively high miR-146a expression may represent a novel mechanism to desensitize LCs to inappropriate TLR signaling at epithelial surfaces through decreasing NF-κB signal strength downstream of TLRs.

In contrast, upon stimulation with IL-1β, human lung alveolar epithelial cells A549 show a very rapid increase in miR-146a expression (121). Unlike THP-1 monocytes, A549 cells do not show miR-146a expression in response to LPS. Such an increase in miR-146a expression downregulates the IL-1β-induced proinflammatory chemokines IL-8 and RANTES. Surprisingly, miR-146a has a negative effect on this chemokine production only at a high level of IL-β (~10 ng/ml) treatment, indicating that this negative feedback pathway is important during severe inflammation, and it highlights how the role of miRNAs can be exquisitely cell-type-specific. A molecular cascade involving miR-146a, the miR-146a negative regulator promyelocytic

leukemia zinc finger protein PLZF, and the miR-146a target CXCR4 is also shown to be active during megakaryopoiesis (137). This regulatory pathway involves enhanced expression of PLZF, which in turn inhibits miR-146a transcription. Therefore, CXCR4 expression is increased, which is necessary for megakaryocyte differentiation and maturation.

In addition to its role in innate immunity, miR-146a also plays an important role in adaptive immunity and is involved in T-cell fate determination in mice. Lu and colleagues have reported that miR-146a is prevalently expressed in T_{Reg} cells and is critical for T_{Reg} functions (138). Indeed, deficiency of miR-146a has resulted in increased numbers but impaired function of T_{Reg} cells such as breakdown of immunological tolerance with massive lymphocyte activation and tissue infiltration in several organs (138). Recent work on miR-146a knockout mice has shown that miR-146a plays a key role as a molecular brake on inflammation, myeloid cell proliferation, and oncogenic transformation (139). Knockout of the miR-146a gene in C57BL/6 mice leads to histologically and immunophenotypically defined myeloid sarcomas and some lymphomas (140).

miR-155

A link between miR-155 and the innate immune response is highlighted from studies showing its increased expression in response to LPS and lipoprotein in monocytes or macrophages and in the splenocytes of mice injected with LPS (116, 123, 141). However, an increase in miR-155 expression has not been validated by quantitative real-time PCR in THP-1 cells after LPS stimulation (81, 82). In contrast to miR-146a, miR-155 expression is upregulated after activation of the innate response in murine macrophages by poly(I:C) (TLR-3 ligand) and CpG (TLR-9 ligand) (123). This finding suggests a role of miR-155 in the regulation of antimicrobial defense. Tili et al. (141) have shown that in mouse RAW264.7 cells, miR-155 expression can oscillate after TNF-α treatment, with an initial drop at 30 min followed by an

increase at 60 min indicating that its expression is directly or indirectly controlled by NF-κB activity (141). Thus, miR-155 can exert both positive and negative actions on the expression of NF-κB signaling proteins, IKKβ and IKK, as well as the Fas-associated death domain protein FADD and the receptor interacting serine-threonine kinase Ripk1 (141, 142). This observation is further extended by studies showing that Eμ-miR-155 transgenic mice have higher levels of TNF-α when exposed to LPS and are more susceptible to septic shock (141). Recently LPS-induced strong but transient miR-155 expression has been reported in mouse bone marrow cells, indicating the possible role of miR-155 in granulocyte/monocyte expansion (143). As reviewed by Lindsay (144), studies of the effect of long-term miR-155 overexpression suggest the involvement of miR-155 in the development of acute myeloid leukemia (AML). For example, transfected miR-155 in hematopoietic stem cells (HSCs) engraft into lethally irradiated mice have shown pathological features resembling myeloid neoplasia (143). miR-155 involvement has also been shown to be associated with B-cell malignancies (145). This finding has led investigators to speculate that upregulated miR-155 provides a possible association between the inflammatory response and cancer.

miR-155 is also known to play a role in the adaptive immune system such as development of B cells. Thai et al. (146) and Vigorito et al. (147) have described the association of miR-155 in B-cell production of isotype-switched, high-affinity IgG1 antibodies and during the development of B-cell memory. In their study, B cells lacking miR-155 failed to generate high-affinity IgG1 antibodies. miR-155 is also involved in the differentiation of T cells as demonstrated in miR-155 knockout mice, which have an impairment in Th1 and Th2 cell polarization, correlating with the predominant production of Th2 rather than Th1 cytokines (148).

miR-132 and miR-212

miR-132 has been shown to be induced by LPS (82, 116). Although miR-132 and miR-212 are produced from the same primary transcript and have the same seed sequence, miR-212 induction by LPS has not been reported. The difference in expression between miR-132 and miR-146a in response to other immune-related stimuli has been reported, suggesting different transcriptional or posttranscriptional regulation. For example, miR-132 is highly induced in response to PMA (phorbol myristate acetate), whereas no response is observed for the level of mature miR-146a. MicroRNA-132/-212 has been reported to be a CREB responsive gene; recently, miR-132 has been shown to regulate neuronal morphogenesis and the dendritic plasticity of cultured neurons by controlling the expression of the GTPase-activating protein p250GAP (149, 150). miR-132 may also be responsible for limiting inflammation in the brain as reported in mice(151). On the other hand, miR-212 is known to act as a tumor suppressor (152). Outside of the brain, miR-132 can also modulate inflammation induced by an early stage of herpesvirus infection including KSHV (119). However, no detailed expression kinetics of miR-132 or miR-212 have been described in response to innate immune ligands and no known target for TLR signaling molecules have been found to date. A detailed examination to understand the mechanistic role of those miRNAs in innate immunity has been described in Chapter 5.

miR-let-7i, miR-let-7e, and miR-125b

Expression of TLR4 in epithelial cells is finely regulated and alterations of TLR4 expression have been reported in intestinal and airway epithelial cells following microbial infection (127). As a possible mechanism, Chen et al. have shown that in human biliary epithelial cells (cholangiocytes), miRNA let-7i (one of the isoform of miR-let-7) is downregulated in response to *C. parvum* or LPS whereas TLR4 is upregulated (127). Their observation suggests

that let-7i regulates TLR4 expression *in vitro*. These data further suggest that miRNA-mediated post-transcriptional regulation is critical for innate immune cell response to microbial infection.

Interestingly, another let-7 isoform let-7e is upregulated in response to LPS(153). LPS-stimulated macrophages produce miRNAs that can control the expression of signaling molecules involved in TLR pathways (153). In this case, LPS signals activate Akt1, and Androulidaki et al. (153) have shown that let-7e and miR-181c are upregulated whereas miR-155 and miR-125b is downregulated in an Akt1-dependent manner. Their transfection studies have revealed that let-7e represses TLR4 and that miR-155 represses SOCS1, two proteins critical for LPS-driven TLR signaling, which are thought to regulate endotoxin sensitivity and tolerance. Thus, $Akt1^{-/-}$ macrophages have exhibited increased responsiveness to LPS in culture and consistently $Akt1^{-/-}$ mice do not develop endotoxin tolerance *in vivo*. Overexpression of let-7e and suppression of miR-155 in $Akt1^{-/-}$ macrophages can restore tolerance to LPS in culture and in animals, indicating that Akt1 regulates the response of macrophages to LPS by controlling miRNA expression (153). miR-125b has been shown to directly regulate TNF-α 3'-UTR. Thus, the oscillatory behavior of these miRNAs may play an important role in the regulation of TNF-α expression during LPS stimulation to maintain homeostasis.

Immune Cell Development Involves miR-150, miR-181a, and miR-223

Certain miRNAs are implicated in lymphocyte development and are expressed in a stage-specific fashion affecting key transcription factors (92). For example, miR-150 is highly expressed in resting mature lymphocytes (B cells and T cells), but not in their progenitors, and its expression declines in subsequent differentiation into the effector Th1 and Th2 subsets (154, 155). *In vivo* studies using a combination of loss- and gain-of-function gene targeting approaches for miR-150 have identified its physiological function in hematopoietic development (155). The consequence of miR-150 overexpression in mouse HSCs leads to a selective defect in B-cell

development at the pro- to pre-B transition. This observation is supported by using transgenic mice with moderate ectopic but ubiquitous expression of miR-150 which behaves comparable to normal mice, but B-cell development is severely impaired while change in T-cell development is less pronounced (156). In this study, there is an increased cell death of the *in vitro*-cultured pro-B cells due to ectopic expression of miR-150. Conversely, miR-150 knockout mice are morphologically normal and fertile but have expanded number of peritoneal B-1 cells, accompanied by fewer conventional B-2 cells (155).

The functional significance of miRNA during hematopoiesis has been observed by specific disruption of the key components, such as Ago2 and Dicer, which are involved in miRNA biogenesis. In one study, a conditional deletion of Dicer in HSCs renders these cells unable to reconstitute the hematopoietic system, while knockout of Ago2 results in impaired B-cell and erythroid differentiation that leads to the expansion of immature erythroblasts (157). Furthermore, T-cell-specific deletions of Dicer results in fewer T cells in the thymus and periphery (92, 158, 159). Dicer deficiency in B lymphocytes has also been shown to diminish B-cell survival and the antibody repertoire (160). miRNAs are thus thought to play a critical role in the biology of various immune cells. Recently, several experimental studies have reported the association of miRNA in the development of immune cells. Early studies by Chen et al. have demonstrated that miR-181a is selectively expressed in thymus-derived B cells and expressed at a lower level in the heart, lymph nodes, and bone marrow (161). In bone marrow-derived B cells, miR-181a expression is decreased during B-cell development from the pro-B to pre-B cell stage (161). In addition, miR-181a may have a regulatory role in lymphocyte development due to the fact that expression of miR-181a in HSCs and progenitor cells lead to an increase in $CD19^+$ B cells and a decrease in $CD8^+$ T cells to peptide antigens (162). miR-181a also known to influence

T-cell development and function (92), supported by the expression of miR-181a which augments T-cell receptor (TCR) signaling strength (162). Overexpression of miR-181 correlates with higher T cell sensitivity in immature T cells, indicating that the positive role of miR-18a in intrinsic antigen sensitivity "rheostat" during T cell development (162). In contrast, miR-181a knockdown results in lower TCR signal strength and in the inhibition of positive and negative selection in an *in vitro* fetal thymic organ culture model. miR-181a causes repression of several phosphatases, including SHP-2, PTPN22, DUSP5 or DUSP6 (162). Due to multitarget regulatory affect, miR-181a seems to be crucial for TCR signal strength and T cell sensitivity to antagonists and finally influence B-cell lineage selection as well as T-cell development and activation (92).

Granulopoiesis is regulated in part by miR-223 (163). miR-223 knockout mice somewhat unexpectedly have a two-fold increase in granulocytes, but they are hypersensitive to activating stimuli and display increased fungicidal activity (164). The miR-223 knockout mice also suppress activation of neutrophils and hence miR-223 is important in linking differentiation with function in the granulocytic lineage during homeostatic granulopoiesis. Expression of miR-223 is regulated by a circuit consisting of two transcription factors C/EBP, which activates, and NFI-A, which represses transcription (165). These transcription factors are reported to control the expression of miR-223 during granulocytic differentiation, which in turn controls the development of granulocytes (163).

Viruses and miRNAs

miRNAs are important tools for viruses to modulate gene expression. To date, more than 200 miRNAs have been identified in virus, predominantly in herpesviruses, but additionally in polyomaviruses, ascoviruses, and adenoviruses; this has been extensively reviewed recently by Skalsky and Cullen (166) and Plaisance-Bonstaff and Renne (167). DNA viruses can encode single (e.g. simian virus 40 and adenovirus) or several miRNAs (e.g. herpesvirus). In contrast,

the RNA viruses, such as yellow fever virus, human immunodeficiency virus and hepatitis C virus (HCV) do not seem to encode miRNAs. There is no known viral proteins found in miRNA processing and thus viral miRNA biogenesis appears to be dependent solely on cellular factors (166). Recently, it has become clear that some host miRNAs protect against viral infection, while some viruses have been shown to produce miRNAs of their own that regulate both viral and host genes (167). As the functional significance of viral miRNAs is beginning to emerge, it is clear that viral miRNAs can target both its own and cellular transcripts and thus viruses can utilize miRNA to evade host immune responses. Viral miRNAs, like other viral factors, are involved in cellular reprogramming to regulate the latent-lytic switch, support viral replication by promoting cell survival, proliferation, and/or differentiation and modulate immune responses (166, 167). To date, the most fully characterized cellular targets of viral miRNAs are those of the KSHV miRNAs. KSHV is known to encode 12 miRNA genes (168, 169). It has also been shown that one of these KSHV-miRNA, miR-K12-11, has 100% seed sequence identity with human miR-155, thus likely cross-regulates the same endogenous targets as miR-155 (169). BACH1, a transcriptional repressor involved in regulating oxidative stress has been identified as a common cellular target of these miRNAs (168, 169). *In vivo* study with the dysregulation of miR-155 expression has been shown to be linked to hematopoietic malignancies as well as alterations in lymphocyte development and innate and adaptive immune responses (146, 148). Given the role of miR-155 in many malignancies (170), the exploitation of existing miR-155-regulated pathways by viruses may contribute to viral oncogenesis.

Cellular miRNA expression is intensely influenced by viral infection, which can be attributed to both host antiviral defenses and viral factors altering the cellular environment. One such example is EBV inducing miR-146a expression in B cells (171). The EBV latent membrane

protein LMP1 also induces miR-29b, which results in miR-29b mediated downregulation of the T-cell leukemia gene TCL1, a protein with roles in cell survival and proliferation (172). EBV produces miRNAs including miR-BART2, which targets the EBV-DNA polymerase BALF5 during infection and contributes to viral maintenance and latency (173, 174). HCMV-encoded miR-UL112 represses the expression of MICB (MHC-class-I-polypeptide-related sequence B), which is required for natural killer cell-mediated killing of virus-infected cells (175). Sometimes host miRNA can also be inhibited by certain virus. For example, miR-17-5p and miR-20a, are suppressed by HIV-1 infection. miR-17-5p and miR-20a target PCAF (p300/CBP-associated factor), a cellular histone acetylase and proposed cofactor of the HIV-1 Tat transactivator (176). The consequence of cellular mRNAs downregulation seems to create a host environment supporting for the viral life cycle. Human miR-32 has been shown to downregulate the replication-essential viral proteins encoded by open reading frame 2 and later produce a negative effect on the replication of retrovirus PFV-1 (primate foamy virus type 1) (177). The downregulation of these viral genes results in slower PFV1 replication.

Another example illustrating the interesting and complex relationship of virus and miRNA includes HCV. Pedersen et al. have reported that IFN-β stimulation of hepatic cells results in the production of at least eight miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, and miR-448) that have perfect seed sequence complementarity to HCV mRNAs (178). Additionally, miR-122, a miRNA that is required for HCV replication, is significantly reduced in response to IFN-β treatment (178). Thus, the host response to HCV appears to utilize miRNAs to suppress viral mRNAs as well as downregulating miR-122 to inhibit viral replication. VSV is another interesting virus that its infection in mouse macrophages induces overexpression of miR-146a in a TLR-MyD88-independent but a RIG-I-NF-κB-dependent

manner (122). The VSV-induced miR-146a downregulates VSV-triggered type I IFN production and, thus promoting VSV replication in macrophages (122). Similarly, miR-132 is upregulated by KSHV infection, which limits the production of IFN-β and assists in viral gene replication (119). The resulting effect helps the virus to survive, thus allowing infection of the host to continue.

miRNA and Disease

miRNAs and Cancer

miRNAs confer a layer of post-transcriptional regulation via fine-tuning of gene expression in developmental processes, cell proliferation, metabolism, cell differentiation, and morphogenesis. miRNAs are also known to promote or suppress malignant processes in a similar manner to classical oncogenes and tumor suppressors as reviewed in recent articles (179, 180).

Tumor suppressor miRNAs

Tumor suppressor miRNAs are miRNAs, which target mRNAs encoding for proteins that promote tumor initiation and progression. Thus, the loss of function of a tumor suppressor miRNA by genomic deletion, mutation, epigenetic silencing, and/or miRNA processing ultimately leads to an inappropriate increase in the levels of the respective mRNA target, which in turns initiates or contributes to the malignant transformation. For example, the miR-15a/16-1 cluster downregulates the antiapoptotic gene BCL2 and deletion or downmodulation of these miRNAs results in increased cell survival promoting leukemogenesis and lymphomagenesis in hematopoietic cells (181). Let-7 family members are downregulated in several human tumors including lung and breast cancer (182). Let-7 family members act as tumor suppressors by targeting various well-characterized oncogenes, such as the Ras family, HMGA2, and c-MYC, and other key components of cell cycle and cell proliferation (183, 184). A growing body of evidence suggests that restoration of let-7 expression may be a useful therapeutic option in

human cancer (183). Downregulation of miR-29 members has been reported in various human cancers including aggressive chronic lymphocytic leukemia, lung cancer, prostate cancer, rhabdomyosarcoma, and invasive breast cancer (179). Their tumor suppressor activity is assumed to act through targeting the T-cell leukemia/lymphoma 1, the BCL2 family member MCL1, the cyclin-dependent kinase CDK6, and the transcriptional repressor YY1.

Oncogenic miRNA

miRNAs are classified as oncogenes when their target mRNAs code for tumor suppressor proteins. Overexpression or amplification of these miRNAs is followed by downmodulation of the target tumor suppressor protein, leading ultimately to initiate malignant transformation. In normal cells, miR-155 is highly expressed both in activated B and T cells and in monocytes/macrophages, playing a critical role in hematopoiesis and normal immune functions. The oncogenic ability of miR-155 is associated with an upregulation of c-MYC by an unknown mechanism. Overexpression of miR-155 has been reported in Burkitt lymphoma, Hodgkin disease, non-Hodgkin lymphoma, CLL, AML, lung cancer, and breast cancer (179). Overexpression of miR-155 in vivo induces granulocyte/monocyte expansion with features of myeloproliferative disorders. Indeed, miR-155 is overexpressed in the bone marrow of patients with certain subtypes of AML (143). The miR-17-92, a polycistronic cluster containing six tandem precursors (miR-17, miR-18a, miR-19a, miR-20a, miR19b-1, and miR-92), is one of the best-characterized oncogenic miRNAs. Multiple reports have shown that overexpression of miR-17-92 promotes cell proliferation, inhibits differentiation, increases angiogenesis, and sustains cell survival favoring malignant transformation (185). c-MYC and E2F1/3 transcription factors are known to directly activate miR-17-92 transcription (186). Interestingly, VEGF is able to induce high levels of miR-17/18/20 components in the angiogenic process, and it has been demonstrated that the miR-17-92 is a novel target for p53-mediated gene repression (187).

Overexpression of miR-21 in glioblastoma cells inhibits apoptosis, whereas its silencing by antisense oligonucleotides inhibits cell growth, triggers activation of caspases, and increases apoptotic cell death by targeting tumor suppressor genes such as PTEN, PDCD4 (programmed cell death 4), and TPM1 (tropomyosin 1) (188).

miRNA and Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease of synovial tissues, often with irreversible joint destruction. In RA synovial tissues, infiltrated immune cells (macrophages and lymphocytes) play a substantial role in RA pathogenesis including proliferation of the lining cells, and production of inflammatory cytokines. The pathogenesis of RA has not been fully elucidated, and early diagnosis is important to avoid permanent joint damages. Recent studies have supported an important role for miRNAs in RA. Pauley et al. reported the increased expression of some miRNAs (miR-155, miR-146a, miR-132, and miR-16) in PBMCs from RA patients (189). Surprisingly, elevated miR-146a in RA does not negatively correlate with TNF-α production and with levels of its targets IRAK1 and TRAF6 in contrast to observation in *in vitro* system. The reason of this is still unclear. miR-146-expressing cells in synovial tissues were found to be predominantly CD68+ macrophages as well as CD3+ T cells and CD79+ B cells (190). It will be interesting to determine whether immune cells with high miR-146a content from RA patients will show any *ex vivo* tolerance to microbial ligand. In another report miR-155 is intensely expressed in the RA synovial fibroblasts (191). Over-expression of this miRNA can down-regulate the expression of MMP3 (matrix metalloproteinases), thus leading to modulation of joint inflammation. Nakamachi et al. observed a significantly greater decrease in the miR-124a expression level of RA synovial fibroblasts regulates the proliferation of RA synovial fibroblasts by suppression of the production of the CDK-2 (cyclin-dependent kinase 2) and MCP-1 (monocyte chemotactic protein-1) proteins (192). miR-346 is up-regulated in LPS-

activated RA synovial fibroblasts, which indirectly regulates the IL-18 releasing mechanism through the inhibition of Bruton's tyrosine kinase (Btk) in response to LPS (135). In RA synovial tissue, miR-146, miR-155, and miR-346 are up-regulated and miR-124a is down-regulated by inflammatory cytokines, and these altered expressions of miRNAs contribute to the proliferation of RA synovial fibroblasts, inflammatory response, and production of MMPs, and subsequently to joint destruction. miR-223 is also expressed in T-lymphocytes from RA patients, especially naïve CD4+ lymphocytes (193). Synovial tissue in RA patients is infiltrated with macrophages and naïve CD4+ lymphocytes, therefore, miR-223 might also participate in RA pathogenesis. It has been reported that miR-223 is also the critical regulator of osteoclastogenesis, suggesting that miR-223 might play a role in bone destruction in RA (191). Recently, the importance of IL-17 in RA pathogenesis has been well discussed. Niimoto et al. (194) demonstrated that six miRNAs, let-7a, miR-26, miR-146a/b, miR-150, and miR-155, are significantly up-regulated in the IL-17 producing T cells. In the past several years, while the expression analyses of miRNA in RA have increased, reproducible functional analysis is largely missing with the exception that miR-146a has been consistently reported as upregulated in different cell types. The functional role of miRNA in arthritis should be clarified to explore potential development of a novel treatment.

miRNA and Bacteria-Associated Disease

The expression of miRNA has been discussed in response to microbial ligand. However, the link between the miRNA expression and disease progression is just beginning to be explored. Evidence showed that miR-155 and BIC, the gene that encodes miR-155, were induced in primary human monocytes at four hours post-infection with *Francisella novicida* in a TLR2/MyD88 dependent manner. miR-155 positively regulated pro-inflammatory cytokine release by affecting its target SHIP in human monocytes infected with *F. novicida* (195). Surprisingly, the infectious *F. novicida* (highly virulent type A SCHU S4 strain) caused lower

miR-155 expression, indicating the role of miR-155 in this disease. Another study showed that miR-155 expressions regulated by *Helicobacter pylori* (known to cause peptic ulcer and cancer) both *in vitro* and *in vivo* (196). RNAi-mediated knockdown (KD) of the Foxp3 in T cells abolished miR-155 expression, indicating a direct link between Foxp3 and miR-155 in human T cells. miRNA is also thought to play role in inflammatory disease. One such predominant proinflammatory diseases is periodontal diseases. Periodontal diseases is an aggressive immune and inflammatory diseases affects the connective tissue surrounding the teeth, leading to tooth loss, and is a globally distributed chronic disease in humans. An experimental periodontal disease can be induced by a complex of bacteria *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola.* Recently, many studies demonstrated the presence of those bacterial genomic DNAs in atherosclerotic lesions (197-199), indicating that these bacteria may cause systemic infection. Despite several reports of the role of miRNAs in the regulation of innate and adaptive immune responses, miRNA-mediated regulation of oral infection with periodontal pathogens during experimental periodontal disease has not been documented. It has been also shown that tooth development is tightly controlled by discrete sets of miRNAs where they regulate epithelial stem cell differentiation (200). Accordingly, it will be interesting to determine the link between periodontal infection and miR-146a expression and this will be discussed in Chapter 4.

Summary

miRNAs play important roles in the control of gene expression involved in many cellular activities and have a critical role in the regulation of innate immune system. An example of miR-146a targeting of key signaling proteins in the MyD88-dependent signaling pathway highlights the importance of miRNA in innate immunity (Figure 2), which is also discussed in Chapter 3. Although many studies have shown the induction of miRNAs by TLR ligands, functional data showing the exact effects of miRNAs on TLR responses are still required. Thus, it will be

interesting to study the functional consequence of miRNA expression both *in vivo* and *in vitro* during bacterial infection and the mechanism through which they affect innate immunity. It remains to be determined whether dysregulation of miRNAs is causal to the development and progression of inflammatory diseases. Finally, revealing the modest regulation of TLR signaling by miRNAs will provide promising drug discovery targets against various inflammatory diseases.

Part of this Chapter has been accepted for publication as a review article entitled "MicroRNA in TLR signaling and endotoxin tolerance" by Nahid MA, Satoh M, Chan EKL in Cellular and Molecular Immunology. We apologize to the many authors whose work could not be cited directly because of space limitations.

Table1-1. In vitro cytokine dysregulation in endotoxin-tolerized cells from different cell and tissue types. Adopted and modified from reviews (19, 20, 55)

Cytokine	Changes in expression	Cells/Tissues using in *in vitro* studies
TNF-α	↓	human PBMCs(201, 202), human monocytes(55, 201), human DC(203), human peritoneal PMΦ(28), rabbit PMΦ(29), mouse PMΦ(20, 33, 55), human THP-1 cells(21, 61, 204), mouse RAW264.7 cells(205, 206)
IL-1β	↓	human THP-1 monocytes (21, 61, 204)
	↑	human PBMCs (207), human PMϕ (28), mouse PMϕ (208)
IL-6	↓	human PBMCs (209), human monocytes (210), human PMϕ (28), mouse PMϕ (72, 76)
IL-10	↓	human PBMCs(55, 209)
	↑	human monocytes(55, 210), human MonoMac6 cell line(26)
IL-12	↓	human monocytes (203), human DC (203)
IL-1RA	↑	human THP-1 cells (202)
G-CSF	↓	human PBMCs (209), mouse PMϕ (63)
	↑	human PMϕ (28)
TGFβ	↑	human PMϕ (202, 211)

PBMCs, peripheral blood mononuclear cells; PMϕ, peritoneal macrophages; G-CSF, granulocyte-colony stimulating factor; ↑, increase expression; ↓, decrease expression

Table 1-2. Changes in cytokine expression reported in in vivo endotoxin tolerance conditions. Adopted and modified from reviews (19, 212)

Cytokine	Changes in expression	*In vivo* studies of endotoxin tolerance
TNF-α	↓	human (213-215), pig (216), rabbit (217), guinea pig (218), rat (219), mouse(212, 220)
IL-1β	↓	human (214), rabbit (217), mouse (220)
	↑	mouse (30)
IL-6	↓	human (213, 214), guinea pig (218), mouse (220, 221)
	↑	human (215)
IL-8	↓	human (213-215)
IL-10	↓	rat (222), mouse (223)
IL-12	↓	mouse (51)
G-CSF	↓	human (214, 215), mouse (220, 224)
IFNγ	↓	mouse (51, 225)
TGFβ	↑	mouse (46)

Table 1-3. Cytokine dysregulation in endotoxin-tolerized cells reported in *ex vivo* studies. Adopted and modified from reviews (19, 55).

Cytokine	Changes in expression	*Ex vivo* studies
TNF-α	↓	human blood(42, 226), human PBMCs(53, 55), rabbit PBMCs(217), mouse PMΦ(52), rat PMΦ(50), rat Kupffer cells(227)
IL-1β	↓	human blood(226), human PBMCs(53, 55), rabbit PBMCs(217), mouse PMΦ(228)
	↑	human PBMCs (215)
IL-6	↓	human blood(226), human PBMCs(53, 55), rat PMΦ(229), rat Kupffer cells(230)
	↑	human PBMCs (215)
IL-12, IFNγ	↓	mouse spleen cells (51)

Table 1-4. Upregulation of negative regulators of TLR signaling pathways during LPS priming. Adopted and modified from reviews (20, 65)

Regulatory proteins	Function	LPS primed condition	Refs
IRAKM	Prevents dissociation of IRAK1 and IRAK4	increased	(86)
A20	Prevents ubiquitinylation of TRAF6	increased	(87)
SOCS1	Inhibit JAK-STAT signaling cascade	increased	(231)
MyD88s	Unable to induce IRAK phosphorylation	increased	(232)
sTREM-1	Anti-inflammatory role in mice	increased	(233)
TRAF4	Interacts and counteracts TRAF6 and TRIF	increased	(234)
Tripartite-motif protein,TRIM30α	Prevents downstream activation of NF-κB and cytokine induction through degrading TAK1	increased	(235)

MyD88s, a splice variant of MyD88; SOCS1, suppressor of cytokine signaling 1; sTREM, soluble triggering receptor expressed on myeloid cells;

Table 1-5. TLR ligand-induced miRNAs. Adopted and modified from a review by O'Neill et al.(120).

miRNA	TLRs	Cell type	Other miRNA inducers
Upregulated			
miR-146a	TLR2,3,4 & 5	THP-1 cells (116), Mϕ (139), BMDMs (139), T cells (236)	EBV (237), VSV (122), RIG-I (122), TNF-α (116), IL-1β (121)
miR-132	TLR4 & 9	THP-1 cells (116), human monocytes and Mϕ (119), BMDMs and splenocytes (151)	KSHV (119)
miR-212	unknown	unknown	KSHV (119), EtOH (238)
miR-155	TLR2,3,4 & 9	BMDMs (126), THP-1 cells (116), monocytes(195), Mϕ (239), DCs (240), B cells (241), Treg cells (242)	Helicobacter pylori (243), KSHV (168), EBV (244), TNF-α and IFN-γ (245)
miR-21	TLR4	Inflamed lung tissue (246), RAW264.7 cells and BMDMs (125), B cells (247), H69 cholangiocytes (248)	Cryptosporidium parvum (248), EBV LMP1 (249)
miR-223	TLR4	Inflamed lung tissue (124), DCs (130)	ND
miR-147	TLR2,3 & 4	BMDMs, RAW264.7 cells, THP-1 cells, and alveolar Mϕ (250)	ND
miR-9	TLR2,4,7 &8	Human monocytes and granulocytes (251)	IL-1β (252)
miR-125b	TLR4	H69 cholangiocytes (248), RA synovial fibroblasts (135), LPS-tolerized THP-1 cells (253)	C. parvum (248)
let-7e	TLR4	Peritoneal Mϕ (153)	ND
miR-27b	TLR4	Human Mϕ (254)	ND
Downregulated			
miR-125b	TLR4	Splenocytes (254), BMDMs (153), DCs (130)	ND
let-7i	TLR4	H69 cholangiocytes (127)	C. parvum (127)
miR-98	TLR4	H69 cholangiocytes (255)	C. parvum (255)

BMDM, bone marrow derived macrophages; EBV, Epstein-bar virus; EtOH, ethyl alcohol; KSHV, Kaposi's sarcoma herpesvirus; LDL, low density lipoprotein; LMP1,latent membrane protein 1; ND, not determined; RA, rheumatoid arthritis; RIG-I, retinoic acid inducible gene I; VSV, vesicular stomatitis virus

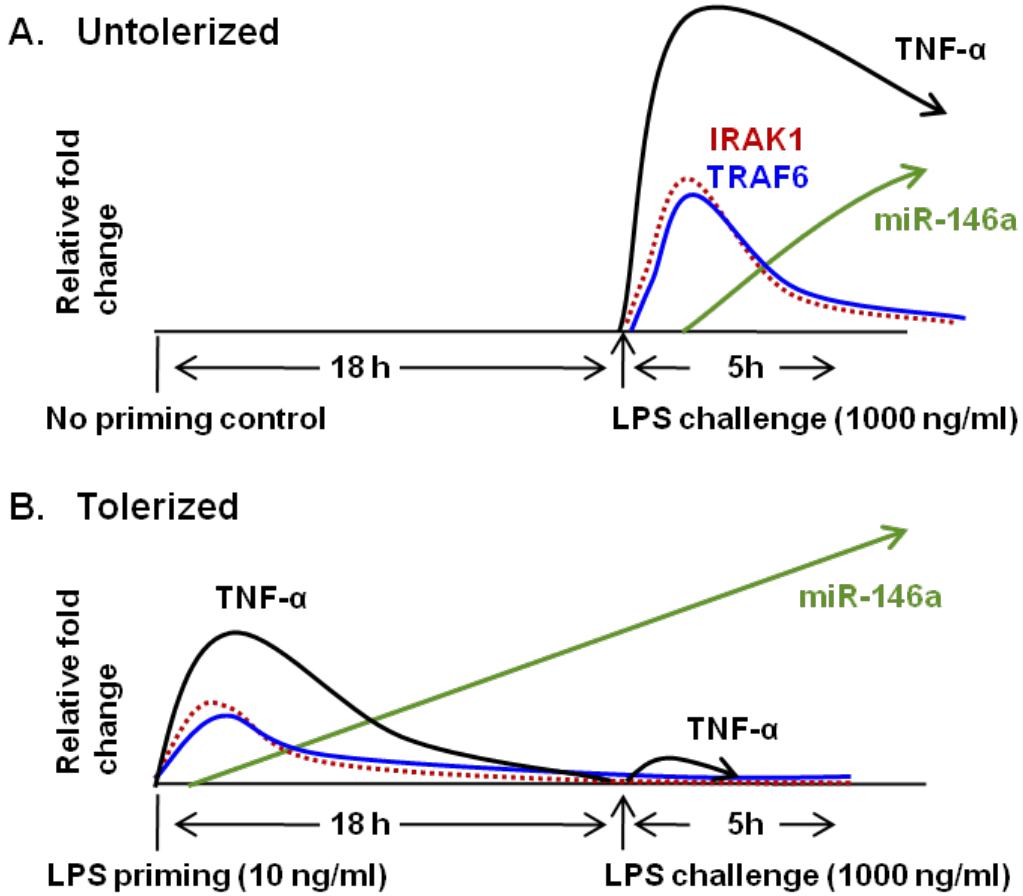

Figure 1-1. A schematic summary of the response to LPS stimulation in THP-1 cells. (A) Unprimed cells challenged with high dose LPS at 1000ng/ml produces a prominent TNF-α response. There are respective increases in both IRAK1 and TRAF6 which peaked at 2-4 h. The increase in miR-146a expression starts at 2 h and continue to increase in the presence of LPS. (B) Tolerized cells are generated by priming with low dose LPS at 10ng/ml leading to rapid and transient TNF-α, IRAK1, and TRAF6 response. TNF-α production decreases as soon as miR-146a starts to increase. Tolerized cells do not respond to high dose LPS challenge unlike the untolerized control, which is responsive to LPS at this stage. The sustained level of miR-146a at 18 h apparently blocks the otherwise robust TNF-α response(82).

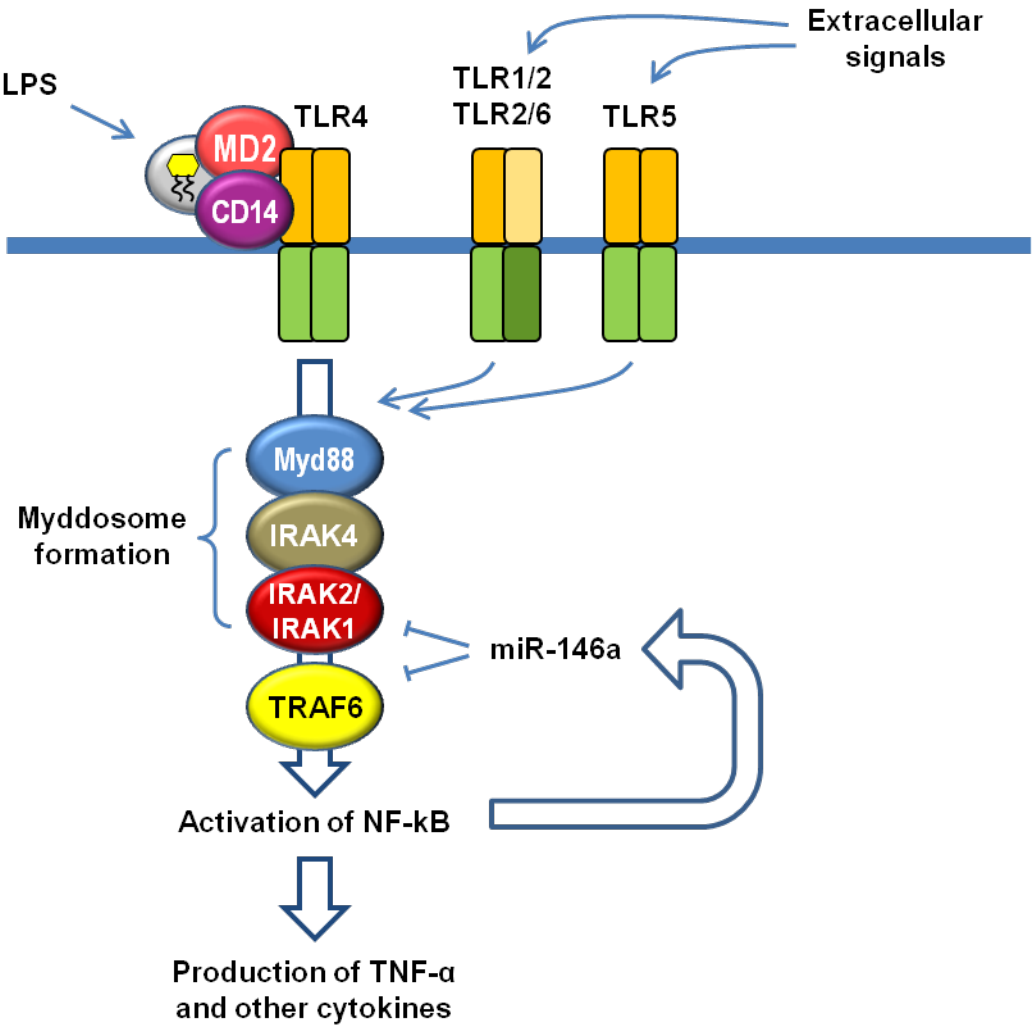

Figure 1-2. A model of the role of miR-146a in LPS-TLR4-mediated signal transduction contributing to endotoxin-tolerance and cross-tolerance. LPS binds to the LPS-binding protein, which in turn is coupled to CD14 on the cell surface of monocytes. Subsequently, LPS-CD14 interacts with TLR4 and forms a complex with another accessory protein MD-2. The TLR4 signaling cascade is initiated after binding with the adaptor protein MyD88. The activation leads to the helical assembly of the so-called myddosome complex(256), involving 6 MyD88, 4 IRAK4, and 4 IRAK2/1 molecules, which in turn recruits TRAF6. This chain of events triggers activation and translocation of NF-κB and results in the transcription of cytokines such as TNF-α and miR-146a. As shown, miR-146a downregulates expression of IRAK1/2 and TRAF6 (82, 116, 122) and the high level of expressed miR-146a blocks subsequent LPS and other TLR ligand challenges. See text for other miRNAs are also induced by LPS; MD2, myeloid differentiation protein-2.

CHAPTER 2
MIR-146A IS CRITICAL FOR ENDOTOXIN-INDUCED TOLERANCE. IMPLICATION IN INNATE IMMUNITY

Background

Innate immunity plays an important role in providing the primary defense against invading pathogenic microorganisms by identifying their conserved components known as pathogen-associated molecular patterns. During infection, pathogen-associated molecular patterns are recognized by the host through several conserved pattern recognition receptors presented on innate immune cells such as monocytes/macrophages and dendritic cells. Toll-like receptors (TLRs) are the best characterized and evolutionary conserved pattern recognition receptors, and they play a central role in the initiation of innate immune response by binding to their respective ligands. TLRs have conserved Toll and IL-1 receptor domain in the cytosolic region, which activates common signaling pathways, most notably through activation of NF-κB transcription factor.

Lipopolysaccharide (LPS or endotoxin) is the principal component of the outer membrane of Gram-negative bacteria. LPS-induced TLR4 signal transduction activates NF-κB, leading to the production of pro-inflammatory cytokines such as IL-1β and TNF-α (10). Pathological dysregulation of NF-κB is linked to inflammatory diseases such as sepsis, autoimmune diseases, and possibly cancer (11). Neutrophils and monocytes from sepsis patients are refractory to subsequent LPS challenge and no longer produce these cytokines (13). This phenomenon, referred to as endotoxin tolerance, is also a mechanism to prevent overstimulation from the continuous exposure to same danger signals in the environment. Endotoxin tolerance has been established for decades *in vivo* (39) and has also been extensively investigated in vitro using primary monocytes/macrophage cells and cell lines (30, 60, 200, 257). To understand the endotoxin tolerance mechanism, changes of cell surface molecules, signaling proteins, pro-

inflammatory and anti-inflammatory cytokines, and other mediators have been studied. Despite intense investigations for decades into the hyporesponsiveness associated with innate immune cells in response to LPS priming, there is no consensus yet on the primary mechanism responsible for its development (10).

microRNA (miRNA) is a new class of regulators of gene expression that acts at the post transcriptional level via an RNA interference mechanism (102). In mammals, miRNA biogenesis involves the initial transcription by RNA polymerase II of primary miRNAs, which are sequentially cut by two RNase III enzymes, Drosha and Dicer, and create ~23-nucleotide double-stranded RNA duplexes (258). Eventually, the mature miRNA guide strand is loaded into the miRNA-induced silencing complex, where it guides the recognition and translational repression or degradation of target mRNAs. miRNAs have emerged to play important roles in many biological processes ranging from cellular development and differentiation to tumors . Recently, miRNAs have been shown to be involved in innate immunity. During the activation of an innate immune response, a rapid increase in the expression of selected miRNAs, namely miR-146a, (102) miR-132, and miR-155 (116), miR-125a (141), and miR-9 (251) have been observed in monocytic cell lines or mouse macrophages in response to LPS, but their biological activities are still obscure, and studies are needed on their kinetics and subsequent putative role in innate immunity. Initial studies on miR-146a expression in response to microbial components and cytokines, including IL-1β indicate that it is involved in innate immunity against bacterial pathogens and is also implicated in inflammatory diseases. Interestingly, this response does not seem to be restricted to inflammatory cells, because miR-146a expression has been observed in lung epithelial cell (144). Further analysis to determine the biological role of miRNA-146a reveals that its expression is NF-κB-dependent and regulates production of cytokines such as IL-

1β and TNF-α in innate immunity (116). IL-1 receptor-associated kinase (IRAK1) and TNF receptor-associated factor 6 (TRAF6), which are important in TLR4 and pro-inflammatory cytokine (IL-1) signaling, have been established as molecular targets for miR-146a (116). More importantly, IRAK1 and TRAF6 are known to be part of the common signaling pathway derived from TLR-2, -4, and -5 and the IL-1β receptor, leading to speculation that increased miR-146a expression might act in a negative feedback pathway. Previously, Li et al. (78) and Boone et al. (87) observed LPS tolerance in monocytes caused by impairment of IRAK1 and TRAF6 kinase activity, respectively. Considering its ability to regulate TRAF6 and IRAK1, we hypothesize that miR-146a is involved in endotoxin tolerance. However, association between miR-146a overexpression and endotoxin tolerance has not been examined.

The aim of this study was to investigate the unique expression pattern of miR-146a compared with other LPS-responsive miRNAs and to observe its role in tolerance in innate immune cells to subsequent LPS exposure. Our findings suggest that miR-146a contributes to controlling TNF-α production and provides innate immunity to evade recurrent bacterial infection through establishing endotoxin tolerance.

Experimental Procedure

Cell Culture and LPS Stimulation

Human THP-1 cells, an undifferentiated promonocytic cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained by twice weekly passage in RPMI 1640 medium containing 25 mm HEPES and l-glutamine (BioWhittaker, Lonza, Walkersville, MD), 10% (v/v) fetal bovine serum (Mediatech Inc., Manassas, VA), and 100 units/ml penicillin-streptomycin (Mediatech) at 37 °C with 5% CO_2. Log phase cells were used in all experiments and cultured at a density of 10^6 cells/ml.

To determine the kinetics of LPS-induced cytokine production *in vitro,* fresh THP-1 cells were suspended in complete RPMI 1640 culture medium and seeded at 10^6 cells/ml in a 24-well plate. Cells were stimulated with 0, 10, 100, and 1000 ng/ml highly purified *Salmonella enterica* serotype Minnesota LPS (Sigma) prepared in the same culture medium by serial dilution of a 500 μg/ml stock solution in tissue culture grade phosphate-buffered saline (PBS). Cells were harvested, and supernatants were collected at 2, 4, 8, 12, or 24 h after incubation and stored at -80 °C until assayed for cytokine levels. Cell pellets were washed in PBS and stored in RNAlater at 4 °C for total RNA isolation in subsequent analysis.

***In Vitro* Induction of Endotoxin Tolerance**

An LPS tolerance cell model using the monocytic cell line THP-1 was adapted from methods described previously with some minor modifications (21, 61, 204). Briefly, before starting the tolerance assay, THP-1 cells were cultured for ~4 days until cells were in log phase and a concentration at 10^6 cells/ml, and viability was checked by trypan blue staining to be >99%. THP-1 cells ($5x10^5$ cells/ml) were transferred in fresh complete medium in new 5- or 25-ml flasks. The cells were incubated with low dose of LPS (10 ng/ml) for 18 h. In some studies, cells were primed with 10, 100, or 1000 ng/ml LPS. After two washes with tissue culture grade PBS, cells were resuspended in complete culture medium alone or with LPS (0, 10, 100, or 1000 ng/ml, final concentration) and distributed in 24-wells plate and returned to the CO_2 incubator for an additional 5 h at 37 °C. Supernatants were harvested and stored at -80 °C until assayed for TNF-α.

Quantification of miRNA and mRNA Expression Level by qRT-PCR

Total RNA of THP-1 cells was isolated using the *mir*Vana isolation kit (Ambion, Austin, TX) following the manufacturer's protocol. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology Inc., Wilmington, DE), and

equal amounts of each RNA (6.7 ng for miRNA and 33 ng for mRNA) were used for real-time RT-PCR (qRT-PCR) analysis. miRNA analysis was performed using the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix, and TaqMan microRNA Assay primers for human miRNAs (Applied Biosystems, Foster City, CA). For mRNA analysis, a High Capacity cDNA RT Kit (Applied Biosystems) and TaqMan mRNA assay primers for IRAK1 and TRAF6 were used. The cycle threshold (*Ct*) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined, and miRNA expression values were calculated using RNU44 as endogenous control (Applied Biosystems) following the $2^{-\Delta\Delta Ct}$ method (259). mRNA expression values were quantified in the same way after normalization to 18S RNA.

THP-1 Cell Transfection

Pre-miR miRNA precursor molecules (miR-146a mimic) and anti-miR miRNA inhibitors (miR-146a inhibitor) were purchased from Ambion and dissolved in nuclease-free water, and the resulting 20 μm stock was stored in aliquots at -80 °C prior to use. One day before the transfection, cells were transferred in fresh culture medium at a concentration of $5x10^5$ cells/ml. The following day, THP-1 cells ($5x10^5$ cells/well) were transfected with 40 nm of precursor or inhibitor using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. miR-146a mimic-transfected THP-1 cells were incubated for 24 h followed by washing two times with complete growth medium. The washed cells were treated with 1000 ng/ml LPS for 3 h. For miR-146a inhibitor experiment, transfected THP-1 cells were incubated for 24 h followed by washing with complete growth medium and addition of 10 ng/ml LPS for an additional 16 h. Cells were then washed with complete growth medium and challenged with 1000 ng/ml LPS for 3 h. Supernatants from cell cultures were collected and assayed for TNF-α secretion, and cell pellets were used for RNA isolation and real-time PCR analysis.

ELISA for TNF-α

Supernatants were collected from cell cultures at different time points after stimulation with various concentrations of LPS. Secreted TNF-α protein in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA) using an Opt EIA TNF-α kit as recommended by the manufacturer (BD Biosciences). Absorbance was measured at 405 nm using a microplate reader (Model 680, Bio-Rad). A_{405} was converted into concentrations using standard curves of recombinant human TNF-α.

IL-1β and IL-6 Assays

Cultured supernatants from LPS treated THP-1 monocytes, were analyzed using the human cytokine/chemokine Milliplex kit (Millipore) according to the manufacturer's protocol to quantitatively detect IL-1β and IL-6. Samples were analyzed on a 200 system (Luminex, Austin, TX).

Western Blot Analysis

LPS-tolerized and untolerized THP-1 cells (5 $x10^6$/condition) were collected 2 h after LPS challenge, pelleted at 1000 x g for 10 min and lysed on ice for 10 min in 1 ml of lysis buffer (50 mm HEPES, pH 7.6, 150 mm NaCl, 1 mm EDTA, 1% Nonidet P-40 (v/v), 20 mm β-glycerophosphate, 1 mm sodium orthovanadate, 1 mm sodium fluoride, 1 mm benzamidine, 5 mm para-nitrophenyl phosphate, 1 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture from Roche Diagnostic, Indianapolis, IN). Soluble lysates were quantitated for protein concentration using a Bio-Rad protein assay kit, separated by SDS-PAGE (10% acrylamide, w/v) along with a broad range molecular weight marker (Bio-Rad), and electroblotted to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked overnight at 4 °C with 5% (w/v) nonfat milk in 1xPBS/0.05% (v/v) Tween 20 (PBS-T) and were probed with primary rabbit anti-IRAK1 or anti-TRAF6 at 1:200 (Santa Cruz Biotechnology,

Santa Cruz, CA). The membranes were washed three times with PBS-T and incubated for 1 h with goat anti-rabbit IgG-horseradish peroxidase at 1:5000 (Southern Biotechnology, Birmingham, AL). After washing in PBS-T, reactive bands were visualized by Super Signal chemiluminescent reagent (Pierce).

Statistical Analysis

Data are presented in the figure as mean ± S.D. For multiple group comparisons, one-way analysis of variance ($p < 0.05$) was performed, followed by the two-sided, unpaired Student's *t* test as described by Shaffer (260). Unpaired two-tailed Student's *t* test was used to compare two independent groups. For all statistical analysis, Prism for Windows, version 5.0 (GraphPad Software, San Diego, CA) was used, and $p < 0.05$ was considered statistically significant.

Results

Kinetics of LPS-induced Expression of TNF-α, miRNAs, and Adaptor Kinases

LPS is one of the potent stimulators of monocytes and macrophages in innate immunity, and it induces the production of a diverse array of inflammatory mediators, including TNF-α both *in vitro* and *in vivo* (261). To observe TNF-α production in vitro, the most commonly employed monocytic cell line, THP-1 (262), was used in the present study. After LPS stimulation of THP-1 cells, kinetics of TNF-α production were examined. As shown in Fig. 2-1A, log phase THP-1 cells were treated with LPS at concentrations of 0, 10, 100, or 1000 ng/ml and harvested at different time points from 2 to 24 h to assess TNF-α production as measured by ELISA described under "Experimental Procedures." In supernatants from cultured LPS-stimulated monocytes, TNF-α started to appear within 2 h and reached a maximal level at 4 h of stimulation followed by gradual decrease starting at 8 h. The highest dose of LPS (1000 ng/ml) generated the highest level of TNF-α up to 3000 pg/ml at 4 h, consistent with levels reported previously (61). The progressive change in TNF-α level was validated at the mRNA level by

qRT-PCR analysis performed on RNA samples collected from the same cells shown in Fig. 2-1B. The kinetics of TNF-α mRNA expression showed similar progression compared with secreted TNF-α protein except the peak level of TNF-α mRNA was 2 h (versus 4 h peak of protein).

LPS causes up-regulation of miR-146a in THP-1 cells and monocytes as demonstrated by Taganov et al. (116) and Bazzoni et al. (251) independently, through microarray analysis. Following their observations and considering the subsequent experimental purposes in this study, kinetics of miR-146a, miR-155, and miR-132 expression were determined by qRT-PCR analysis on the same RNA samples (Fig. 2-1, C–F). The fold changes in miRNA expression were calculated by comparing the value of LPS-treated cells to that of untreated samples cultured in parallel. miR-146a showed an average increase of 7-fold after 8 h, and interestingly a gradual increase was observed up to 35-fold over 24 h (Fig. 2-1C). In contrast, miR-155 and miR-132 expression showed increases of 4- and 10-fold, respectively, after 12 h of LPS treatment, and no further increase was observed in 24 h (Fig. 2-1, D and E). No significant change in the expression of miR-16 was observed (Fig. 2-1F), and this could be considered as an additional LPS-independent control together with the internal control RNU44. Most strikingly, in response to LPS, miR-146a was the only LPS-induced miRNA that showed a gradual increase significantly over 24 h (Fig. 2-1C). In one set of experiments, miR-146a continued to elevate up to 45-fold at 48 h after LPS treatment (data not shown).

Target prediction algorithms have shown that two important adaptor molecules, IRAK1 and TRAF6, are two of the top targets for miR-146a. This was experimentally validated by Taganov et al. (116) by demonstrating miR-146a-induced reduction of luciferase expression in luciferase reporters containing 3′-untranslated repeat of these adapter molecules. Fig. 2-1, G and

H, show the respective increases in IRAK1 and TRAF6 at 2 and 4 h and decreases at 8 h and subsequent time points in line with the elevated expression of miR-146a starting at 8 h. These data are consistent with IRAK1 and TRAF6 being regulated by miR-146a in NF-κB-driven inflammatory response to LPS.

Pretreatment of THP-1 Cells with LPS Results in Reduced TNF-α Production Linked to miR-146a Overexpression

TNF-α is considered as the most commonly used marker of endotoxin tolerance due to its dramatic reduction following subsequent LPS challenge compared with primary treatment of LPS (29). Fig. 2-2 shows the ability of LPS to induce tolerance based on TNF-α production using the THP-1 cell model. THP-1 cells were primed with 10 ng/ml LPS for 18 h followed by washing with PBS and challenged with various doses of LPS ranging from 0 to 1000 ng/ml. After 5 h of incubation with challenged LPS, TNF-α protein level was analyzed by ELISA (Fig. 2-2A). TNF-α production decreased ~4-fold compared with the untolerized control after challenged with 1000 ng/ml LPS, demonstrating significant tolerance induction as expected from previous studies (61). Analysis of mRNA level of TNF-α by qRT-PCR (Fig. 2-2B) showed levels consistent with the ELISA data, and reduction of TNF-α mRNA was significant when challenged with either 100 or 1000 ng/ml LPS. Treatment with 10 ng/ml (or 1 ng/ml, data not shown) of LPS in this instance did not show discernable difference in TNF-α protein or mRNA compared with control 5 h after LPS challenge. In one experiment, THP-1 cells were primed with 10 ng/ml TNF-α for 18 h and then challenged with 1000 ng/ml LPS to determine if TNF-α could substitute LPS priming to induce LPS tolerance. The results showed that TNF-α did not induce tolerance, and it was noted that the level of miR-146a was not significantly above untreated control 18 h after TNF-α treatment (data not shown).

To understand the role of miRNA in LPS tolerance, miRNA analyses in the tolerized THP-1 cells were performed in relation to the untolerized control shown in Fig. 2-2 (C–F). As expected, miR-146a in the tolerized sample showed significantly higher expression over 18 h of initial incubation plus 5 h of challenge, whereas little or no changes in expression of miR-155, miR-132, and miR-16 were observed compared with untolerized controls. Fig. 2-2 (G and H) shows the expression of known miR-146a targets IRAK1 and TRAF6 in both tolerized and untolerized cells. The levels of IRAK1 and TRAF6 were not affected significantly up to 100 ng/ml of secondary LPS challenge compared with the untolerized controls. At 1000 ng/ml LPS challenge, there was significant reduction in levels of both IRAK1 and TRAF6. These data suggest that these adaptor molecules might be affected at the post-transcriptional level. Confirmation of the moderate reduction of IRAK1 and TRAF6 proteins was obtained in the Western blot analysis comparing LPS tolerized to control untolerized cells (Fig. 2-2I). Consequently, miR-146a may play an important role in LPS tolerance based on the inverse correlation with TNF-α production and levels of IRAK1 and TRAF6 in tolerized THP-1 cells.

Higher Doses of LPS Priming Render Efficient Tolerance and Higher miR-146a Expression

Endotoxin tolerance is usually studied at a low dose (10 ng/ml) of LPS priming. However, in nature, animals are always exposed to various doses of LPS and are refractory to LPS challenge. To obtain better understanding about the impact of the priming concentration in relation to miR-146a expression on endotoxin tolerance in vitro, a range of priming concentrations from 0, 10, 100, and 1000 ng/ml was employed followed by 1000 ng/ml LPS challenge. In one of the earlier experiments, priming with 1 ng/ml LPS did not show significant difference from control and was not included in subsequent experiments (data not shown). Priming with 10 ng/ml LPS resulted in >90% decrease ($p < 0.01$) of TNF-α production (Fig. 2-3A). Complete unresponsiveness was detected with 100 and 1000 ng/ml LPS priming. This

result is consistent with the previous study by both LaRue et al. (21) and Jacinto et al. (204) where THP-1 cells primed with higher dose of LPS were more refractory to subsequent LPS challenge. Fig. 2-3B shows gradual augmentation of miR-146a correlated with tolerance but similar changes in other miRNAs such as miR-155 and miR-132 were not observed (data not shown). These data are consistent with Fig. 1C, which showed miR-146a expression to be LPS dose-dependent. Western blot analysis confirmed the moderate, gradual reduction in IRAK1 and TRAF6 levels with the increase of miR-146a expression proportional to LPS priming dosage (Fig. 2-3C). Thus, these data showed the positive correlation of LPS priming dose with up-regulation of miR-146a and LPS tolerance.

The Elevated Expression of miR-146a Depends on Continued Exposure to LPS and is Correlated with the Requirement for LPS Tolerance

In the first LPS tolerance experiment (Fig. 2-2), the results suggested that, among the known LPS-induced miRNA analyzed, the elevated miR-146a expression levels were most correlated with LPS tolerance. When THP-1 cells were continuously exposed to LPS, the level of miR-146a continued to rise at 24 h (Fig. 2-1C) and 48 h (data not shown). To determine if the continuing increase in miR-146a level requires continued exposure to LPS, THP-1 cells were primed with 10 ng/ml LPS continuously for 18 h, then washed twice with PBS and cultured in complete growth medium for an additional 0, 12, or 22 h (LPS withdrawal). At each time point, 8×10^5 cells were challenged with 1000 ng/ml LPS for 5 h prior to analysis for TNF-α protein production in culture supernatant by ELISA (Fig. 2-4A) and qRT-PCR analysis of miR-146a expression (Fig. 2-4B). With 18 h of continuous LPS priming and 5 h of LPS challenge, the same condition used in Fig. 2A, significant reduction in TNF-α secretion and highly elevated miR-146a level were observed again as expected. Interestingly, after 12 h of LPS withdrawal, cells started to regain LPS responsiveness to TNF-α production and almost completely recovered from

tolerance after 22 h of LPS withdrawal (Fig. 2-4A). Fig. 2-4B shows that the expression of miR-146a decreased 12 and 22 h after LPS withdrawal. At 22 h after LPS withdrawal, the levels of TNF-α and miR-146a were no longer significantly different from the unprimed controls. Thus, the presence of up-regulated miR-146a due to LPS priming is important for maintaining tolerance, because it is diminished gradually with the disappearance of miR-146a in washed THP-1 cells.

Up-regulation of miR-146a Alone Can Mimic LPS Priming to Induce LPS Tolerance

LPS has been shown to induce the expression of a few regulatory miRNAs (116, 251). Similar up-regulation of miR-146a was observed in LPS-primed and LPS-tolerized THP-1 cells in this study. To determine the direct role of miR-146a in endotoxin tolerance, THP-1 cells were transfected with 40 nm miR-146a mimic followed by challenge with 1000 ng/ml LPS after 24 h. miR-146a was expressed 1,850-fold higher in miR-146a mimic transfected cells than the mock transfected cells confirming the successful transfection (Fig. 2-5A). In Fig. 2-5B, cells transfected with miR-146a mimic produced about 4 times less TNF-α in comparison to mock transfected cells 3 h after the LPS challenge. The ability of transfected miR-146a to cause tolerization was similar to the tolerance positive control (4- versus 6-fold). Besides TNF-α, other pro-inflammatory cytokines are produced through NF-κB activation and transcriptional regulation. As expected, Fig. 2-5 (C and D) shows similar changes for two other NF-κB-regulated cytokines IL-1β and IL-6. To determine the effect of overexpression of miR-146a on adaptor molecules, RNA samples from transfected cells were analyzed for the mRNA levels of TRAF6 and IRAK1, both targets of miR-146a. THP-1 cells transfected with miR-146a mimic showed a 62% reduction of TRAF6 and a 56% reduction of IRAK1 compared with mock transfected cell (Fig. 2-5E). An unrelated human gene lamin A/C was also included as an additional control showing no significant changes in expression level between miR-146a mimic

transfected and mock control. These data demonstrate the ability of miR-146a alone to induce LPS tolerance via specific negative regulation of TRAF6 and IRAK1.

Knockdown of miR-146a in THP-1 Cells Diminishes LPS Tolerance

As shown in Fig. 2-5, transfected miR-146a induced LPS hyporesponsiveness in THP-1 cells. To corroborate the role of miR-146a on LPS tolerance, the effect of miR-146a knockdown on LPS tolerance was determined by suppressing its activity with miR-146a inhibitor. THP-1 cells were transfected with miR-146a inhibitor at a concentration of 40 nm, and supernatant as well as total RNA samples were collected as described under "Experimental Procedures." Because the level of miR-146a is very low in unstimulated THP-1 cells, the knockdown effect in these cells transfected with the miR-146a inhibitor might not be easily appreciated. Thus to confirm the functional activity of the miR-146a inhibitor, the knockdown of miR-146a was monitored in miR-146a inhibitor-transfected cells to determine whether it would efficiently block miR-146a production upon LPS stimulation. As shown in Fig. 2-6A, miR-146a expression in transfected cells was down-regulated by 9-fold compared with mock transfected cells primed with LPS. Cells transfected with miR-146a inhibitor showed efficient response to LPS and produced significantly higher ($p < 0.01$) TNF-α protein compared with mock and mR-146a inhibitor alone (Fig. 2-6B). THP-1 cells transfected with miR-146a inhibitor showed a 52% increase of TRAF6 and an 80% increase in the level of IRAK1 compared with mock transfected cells (Fig. 2-6C). In contrast, the expression of an unrelated gene lamin A/C was not affected in miR-146 inhibitor-transfected cells. Thus, LPS tolerance was disrupted due to knockdown of miR-146a, which helped cells to regain LPS responsiveness. Taken together, these data demonstrate that LPS-induced miR-146a upregulation is critical to endotoxin tolerance.

Discussion

Previously, changes in miRNA expression profiles in THP-1 cells were reported primarily using only a single LPS dose at fixed time, but in this report, kinetics of the LPS-induced miRNA were analyzed using different LPS doses for up to 24 h. A distinct expression pattern was observed for each LPS-induced miRNA, but only miR-146a persisted for an extended period and at very high levels. A similar expression pattern is likely to occur in the host depending on the danger signal. Li et al. (78) and Jacinto et al. (204) reported that the IRAK1 protein level dramatically decreased within 90 min after LPS priming of monocytes and remained at a low level following 6 h of LPS challenge. Consistent with this finding, a negative correlation between the expression of miR-146a and adaptor kinases IRAK1 and TRAF6 as well as TNF-α (protein and mRNA) was demonstrated in this study. It is acknowledged that the regulation of IRAK1 and TRAF6 by miR-146a has been characterized (116) and confirmed in some subsequent studies (121, 189, 263). The new data in this report are focused on defining the role of miR-146a in endotoxin tolerance.

Endotoxin tolerance has long been recognized as an interesting mechanism that controls the intensity and duration of innate immune cell activation, but a key regulator for tolerance has not been identified (10). Nomura et al. (76) described stimulation of monocytes with LPS leading to the down-regulation of TLR4-MD2 (myeloid differentiation protein-2) from the cell surface, essentially causing them to be unresponsive during a second LPS challenge. However, in a 2002 review article, Dobrovolskaia and Vogel (47) concluded that much higher doses of LPS were required for the TLR4 receptor down-regulation than for tolerance induction, and sustained overexpression of TLR4 did not prevent tolerance induction (66). In another study, LPS tolerance has been shown to be independent of LPS co-receptor CD14, because THP-1 cells showed tolerance in the presence or absence of anti-CD14 monoclonal antibody (61). Randow et

al. (202) reported that anti-inflammatory cytokines such as IL-10 and transforming growth factor-β could lead to deactivation of macrophages in response to LPS. On the other hand, Berg et al. (264) showed that IL-10 and transforming growth factor-β knock-out mice were still capable of developing LPS tolerance. Similarly, LPS tolerance was shown to be independent of other cytokines such as interferon-γ because LPS tolerance could be induced in interferon-γ knock-out mice (265). In short, the key regulator for endotoxin tolerance has not been identified, and clearly our finding to report the critical role of miR-146a in this mechanism is novel.

miR-146a Plays a Critical Role in LPS Tolerance

Fig. 2-7 summarizes our proposed model for the critical role of miR-146a in LPS tolerance in THP-1 cells. LPS binds to the LPS-binding protein, which in turn is coupled to CD14 on the cell surface of monocytes. Subsequently, LPS-CD14 interacts with TLR4 and forms a complex with another accessory protein MD-2. The TLR4 signaling cascade is initiated after binding with adaptor protein MyD88 that acts as a bridge between TLR4 and incoming IRAK1 adaptor kinase, which further recruits TRAF6. This chain of events triggers activation and translocation of NF-κB and results in the transcription of immune-responsive genes and cytokines such as TNF-α and mRNA regulators such as miR-146a. Consequently, low dose LPS-primed THP-1 cells produce TNF-α rapidly and continue to do so for 4–6 h, and then TNF-α production decreases as soon as regulatory miR-146a starts to increase. Then, 18 h post-priming, a profound difference between miR-146a expression and TNF-α secretion is established, and because the up-regulated miR-146a acts negatively on IRAK1 and TRAF6 mRNA the cells become tolerized (Fig. 2-7A). Tolerized cells do not respond to high dose LPS challenge due to the high level of miR-146a unlike the untolerized control, which is responsive to LPS at this stage (Fig. 2-7B).

Priming time is important to render monocytic cells to be tolerant. LaReu et al. (21) have observed moderate tolerance in THP-1 cell as early as 6 h and profound tolerance after 18 h of

LPS priming. Consistent with this time frame, even THP-1 cells stimulated with low dose LPS (10 ng/ml) showed high level of miR-146a expression. Furthermore, Jacainto et al.(204), and this report showed that LPS tolerance correlated with the increase in LPS-priming dose and complete tolerance (zero TNF-α production) was observed when 100 ng/ml or higher concentration of LPS was used; these high concentrations of LPS were linked with the high up-regulation of miR-146a confirming its substantial role in LPS tolerance.

The biological significance of other miRNAs induced by LPS such as miR-132 and miR-155 on LPS tolerance is not clear. They might have direct or indirect roles on LPS tolerance together with miR-146a. Thus, transfection studies were performed to examine the effects of overexpression of miR-146a alone on LPS tolerance. Transfection of miR-146a-mimic into THP-1 cells resulted in dramatic reduction of TNF-α indicating its negative regulatory effect on TNF-α production and subsequent LPS challenge. In the transfected cells, miR-146a level was pronounced, although TRAF6 and IRAK1 mRNA were not completely abolished. Thus, miR-146a down-regulated IRAK1 and TRAF6 mRNA at the post-transcriptional level completely consistent with a previous report (116). Krutzfeldt et al. (266, 267) introduced the usage of chemically modified miRNA inhibitor known as antagomirs to define the biological functions of miRNAs. In this report miR-146a knockdown in THP-1 cells resulted in recovery from LPS tolerance and restored TNF-α secretion in response to LPS challenge (Fig. 6B). Thus, the findings fully support the dominant role of miR-146a in LPS tolerance compared with other negative regulators previously suggested as discussed above.

miR-146a Expression in LPS Stimulated Cells Contributes to Cell Survival

LPS is likely the most potent natural danger known to evoke the immune system with a systemic production of pro-inflammatory cytokines and chemokines, which recruit and activate immune cells leading to subsequent elimination of the putative infectious agent (268). More

importantly, the rapid induction of cytokines such as TNF-α, IL-1, and IL-6 is indispensable for mounting the innate response and subsequent robust adaptive immune response to defend against the first invading pathogens. Although cytokine production is important for the efficient control of growth and dissemination of invading pathogens, overproduction of these cytokines is harmful for the host, because it can be detrimental to the host physiological functions and may lead to multiple organ failure and death, a condition known as the septic shock syndrome (269). Thus, cells of the innate immune system must employ constantly a multilayered control mechanism to maintain innate immunity functional and inflammation in check. For example, in the in vivo model of LPS tolerance, rats primed with a low dose of LPS (2 μg/100 g of body weight) survived a subsequent high dose of LPS challenge (1000 μg/100 g of body weight), which is known to be 100% lethal to naive rats (20). In the present study, THP-1 showed a coordinated control mechanism in the early intense production of TNF-α at the initial stage of the LPS response, and then the level of TNF-α was gradually decreased; the magnitude and timing of this response is consistent with a published study on human mononuclear cells (270). These new data demonstrating the role of miR-146a in controlling LPS tolerance confirm it as a key factor that keeps the innate immune system in balance toward cell survival.

Implication of Endotoxin Tolerance in Innate Immunity

Up-regulated miR-146a in TLR4-mediated endotoxin tolerance reported in this study is likely to affect other pattern recognition receptor activity in innate immunity. Taganov et al. (116) observed elevated miR-146a expression in response to TLR-2, -4, or -5 stimulation by bacterial and fungal components or following exposure to TNF-α or IL-1β. On the other hand miR-146a expression was not increased through activation of TLR-3, -7, or -9 in responses to viral or bacterial nucleic acids (116). Recently, Hou et al. (122) observed overexpression of miR-146a in response to vesicular stomatitis virus infection in a mouse infection model. In their

study, miR-146a was shown to regulate type 1 interferon production by inhibiting RIG-I signaling molecules IRAK1, IRAK2, and TRAF6 (122). Interestingly, another report by Tang et al. (263) showed the down-regulation of miR-146a in a subset of human lupus patients with elevated expression of type 1 interferon. In any case, TNF-α can be produced through activation of all TLRs, except TLR3, and all these pathways involve the miR-146a target molecules TRAF6 and IRAK1 adaptor kinases (271). Hedl et al. (272) showed chronic stimulation of Nod2 caused down-regulation of IRAK1 activation and rendered intestinal macrophages tolerant to muramyl dipeptide. Muramyl dipeptide activates the NF-κB pathway via the same intermediates IRAK1 and TRAF6, and thus LPS induction of miR-146a is likely to induce cross-tolerance to Nod2 pathway. Therefore, endotoxin tolerance associated with up-regulated miR-146a (NF-κB driven) may have a broader role involved in regulating TNF-α, a key player of the cytokine network, induced by a number of pattern recognition receptors indicated in innate immunity.

Innate immune response to the invading microorganism is influenced by miR-146a. In A549 epithelial cells, Perry et al. (121) reported the IL-1β-induced release of IL-8 and RANTES (regulated on activation normal T cell expressed and secreted) that were negatively regulated by the up-regulation of miR-146a. Similarly, in LPS-treated THP-1 cells, miR-146a shows unique up-regulation that has also been observed to be responsible to cause LPS tolerance to a wide range of LPS, including *S. enterica* in this study. Interestingly, Cavaillon et al. (201) found that LPS-tolerant mice were significantly resistant to *S. enterica* or *Cryptococcus neoformans* infection for >2 months. Thus, overexpression of miR-146a associated with endotoxin tolerance may have important consequences in host innate immunity responding to a wide range of bacterial infection. More extensive studies are needed to fully explore the complete role of this miRNA especially in terms of its half-life in LPS-tolerant mice. It is known that, in response to

LPS challenge, endotoxin-tolerant monocytic cells show reduced production of IL-1α, IL-1β, and IL-6, along with TNF-α (21). Up-regulation of miR-146a in certain inflammatory conditions such as rheumatoid arthritis and sometimes in cancer cells has been observed, although the function of miR-146a is still unclear in those conditions (189, 191, 273).

In summary, multiple lines of evidence reported here provide the categorical role of miR-146a in endotoxin tolerance. This miRNA is highly up-regulated in tolerized cells and acts as a tuning mechanism to prevent an overstimulated inflammatory state. It is interesting to speculate that modulating the level of miR-146a can be used in therapeutic intervention for inflammation and protecting against sepsis.

This work was published in 2009 in *Journal of Biological Chemistry:* Nahid MA, Pauley KM, Satoh M, Chan EKL. miR-146a is critical for endotoxin-induced tolerance: IMPLICATION IN INNATE IMMUNITY. J. Biol. Chem. 2009 Dec 11;284(50):34590-9.

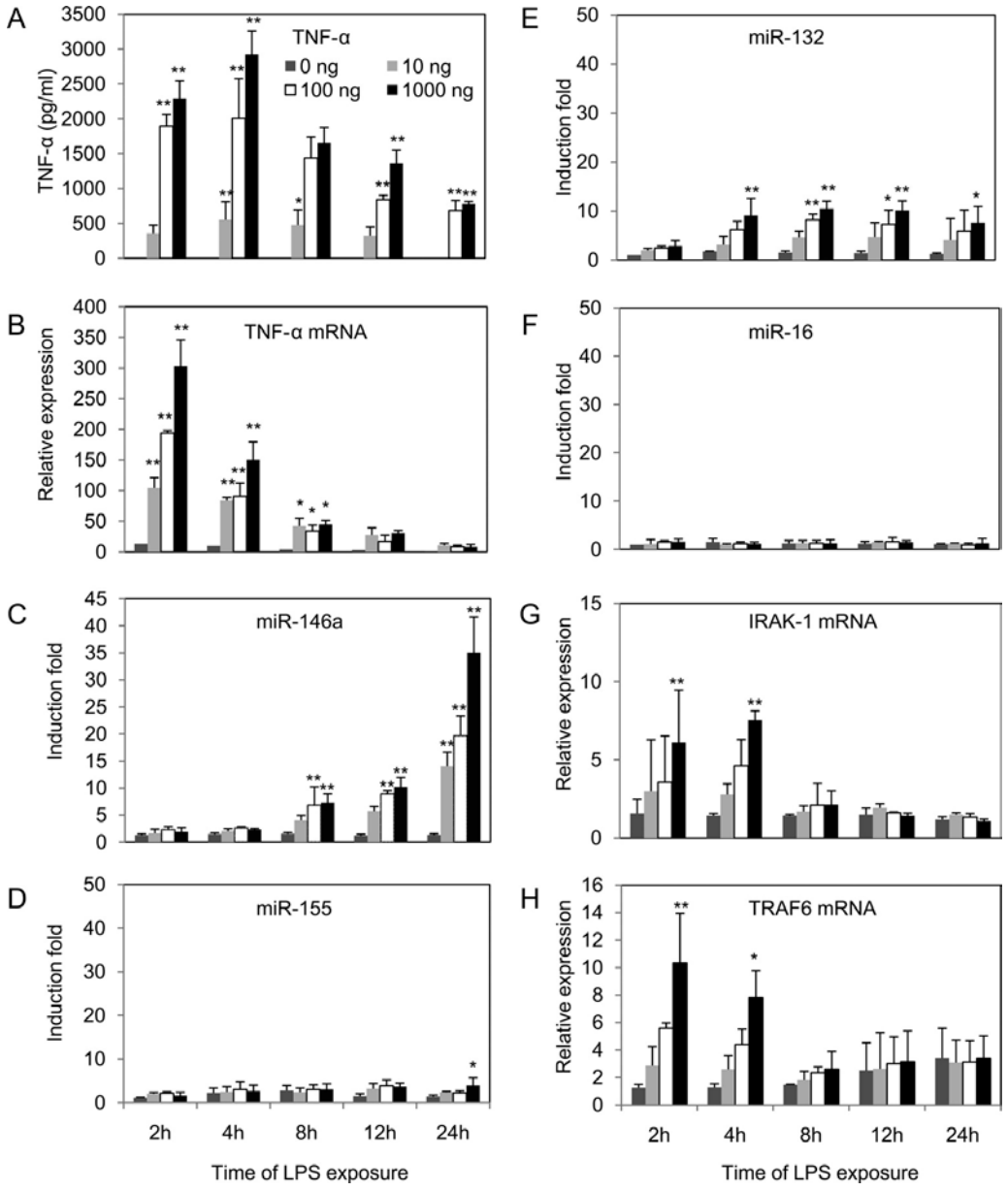

Figure 2-1. Dose- and time-dependent expression of TNF-α and adaptors TRAF6 and IRAK1 mRNA preceded that of miR-146a in LPS-stimulated monocytic THP-1 cells. THP-1 cells were treated with 0, 10, 100, or 1000 ng/ml LPS and incubated for 2, 4, 8, 12, or 24 h. Culture supernatants were collected at the indicated time points from 2 to 24 h for TNF-α protein analysis using ELISA (A). Total RNA were purified from the respective cell pellets and analyzed by qRT-PCR for the expression of TNF-α mRNA (B), miR-146a (C), miR-155 (D), miR-132 (E), miR-16 (F), IRAK-1 mRNA (G), and TRAF6 mRNA (H). mRNA and miRNA expression were normalized with control 18 S RNA and RNU44, respectively. All results are expressed as mean ± S.D. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with untreated cells.

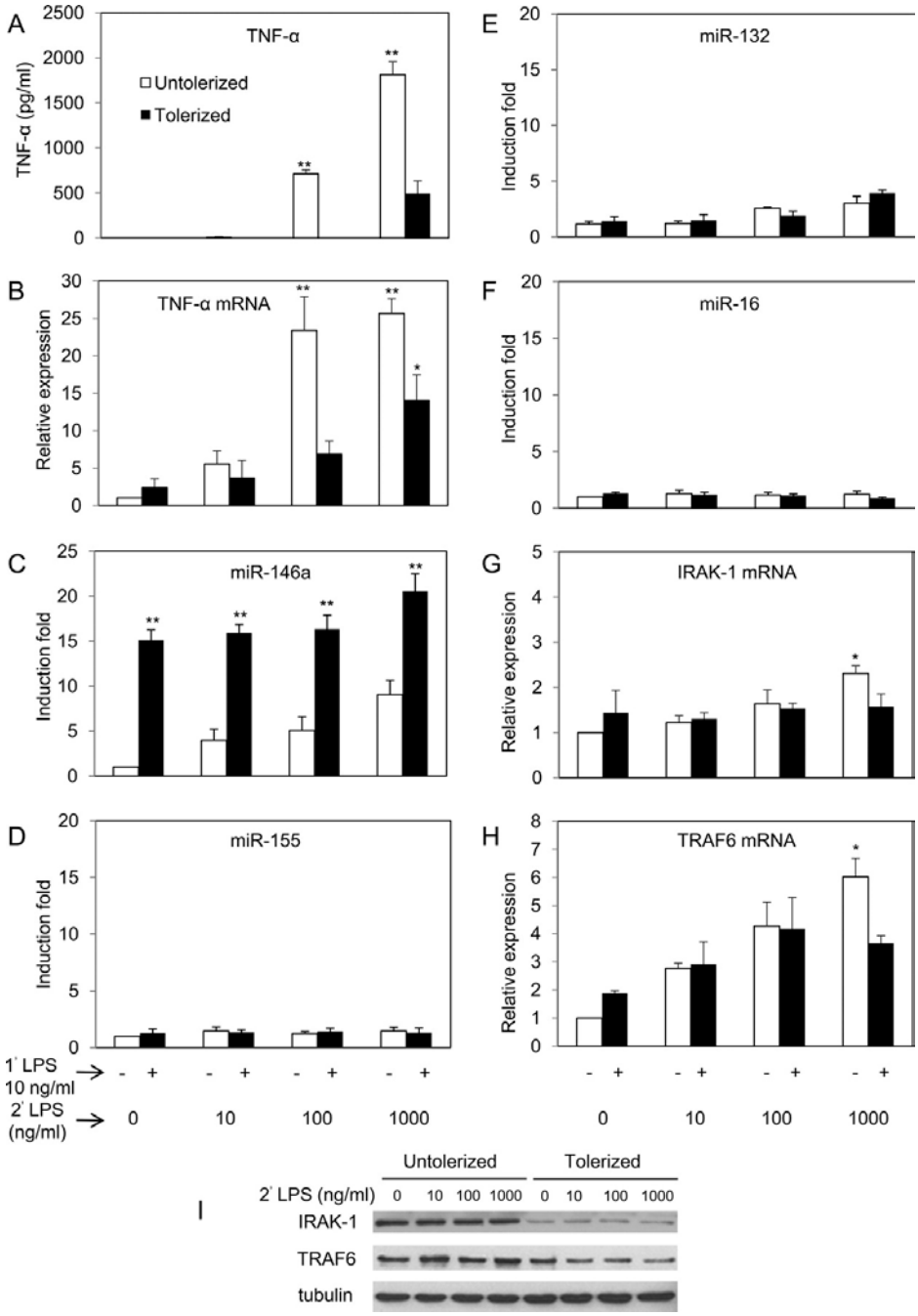

Figure 2-2. High level of miR-146a may account for LPS tolerance in the THP-1 cell model. THP-1 cells primed with 10 ng/ml LPS continuously for 18 h (tolerized, ■) and untreated controls incubated for the same time period (untolerized, □) were washed twice with PBS, and challenged with 0, 10, 100, or 1000 ng/ml LPS for 5 h. Culture supernatants and total RNA were analyzed as described in Methods (*A-H*). All results are expressed as mean ± S.D. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with untolerized THP-1 cells. Lysates of the tolerized and untolerized cells 2 h after LPS challenge were analyzed for IRAK-1, TRAF6, and tubulin expression by Western blot (*I*).

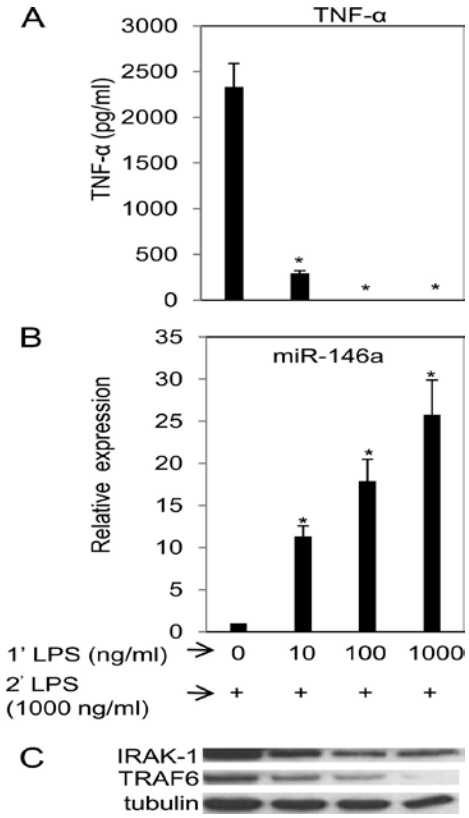

Figure 2-3. Higher priming dose of LPS induced higher miR-146a levels and more efficient suppression of TNF-α production in subsequent LPS challenge. THP-1 cells were primed with 0, 10, 100, or 1000 ng/ml LPS continuously for 18 h, washed twice with PBS, and challenged with 1000 ng/ml LPS. Supernatants and cell pellets were collected 5 h later as described under "Experimental Procedures" for TNF-α protein determined by ELISA (A), miR-146a expression analysis in total RNA (B). Data points and error bars represent mean ± S.D. of three independent experiments. *, $p < 0.01$ compared with untreated cells. Western blot analysis for IRAK1, TRAF6, and tubulin in the cell lysates collected 2 h after LPS challenge.

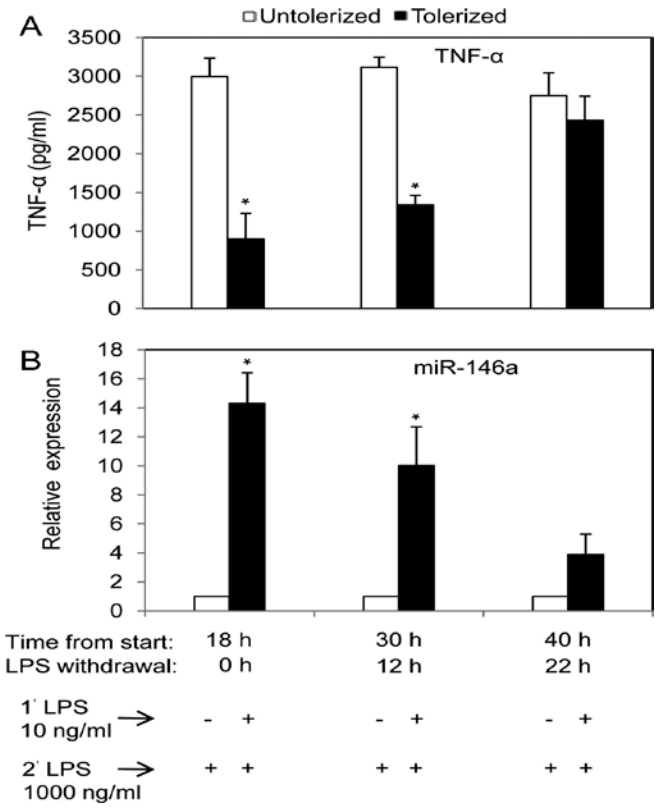

Figure 2-4. Reduction in miR-146a expression in LPS tolerized THP-1 cells inversely correlated with TNF-α production. THP-1 cells were cultured with (tolerized) or without (untolerized) 10 ng/ml LPS continuously for 18 h. Cells were then washed twice with PBS and cultured in complete growth medium for an additional 0, 12, or 22 h (LPS withdrawal). At each time point, 8×10^5 cells were challenged with 1000 ng/ml LPS for 5 h prior to analysis of TNF-α protein production in culture supernatant by ELISA (A) and qRT-PCR analysis of total RNA for miR-146a expression (B). Values are expressed as mean ± S.D. from three independent experiments. *, $p < 0.01$ compared with LPS untreated cells.

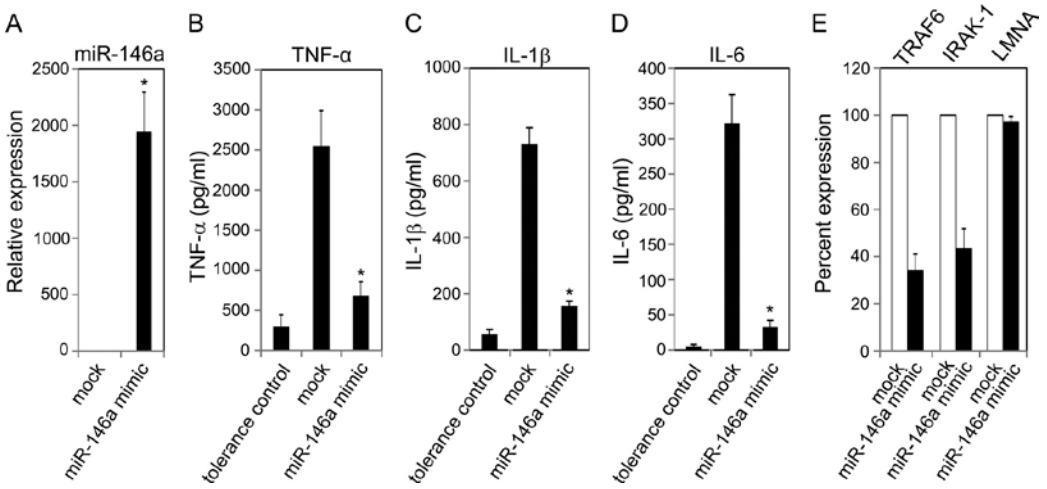

Figure 2-5. Transfected miR-146a alone could mimic LPS priming in LPS tolerance assay. THP-1 cells transfected with 40 nM miR-146a mimic followed by incubation for 24 h, mock transfected cells, and 10 ng/ml LPS primed cells (tolerance positive control) were washed with complete growth medium and challenged with 1000 ng/ml LPS for 3 h. A, RNA isolates were prepared from cell pellets of mock and transfected cells followed by qRT-PCR for miR-146a expression normalized with RNU44. B, ELISA analysis of TNF-α protein in supernatants tolerance-positive control, mock transfected, and miR-146a mimic transfected cells. C and D, multiplex analysis of IL-1β and IL-6 proteins in supernatants of tolerance-positive control, mock transfected, and miR-146a mimic-transfected cells. E, the same RNA samples were also analyzed by qRT-PCR for mRNA expression of miR-146a targets TRAF6, IRAK-1, and an unrelated gene lamin A/C (LMNA, negative control). Data are representative of three independent experiments and expressed as mean ± S.D. *, $p < 0.01$ compared with mock transfected cells.

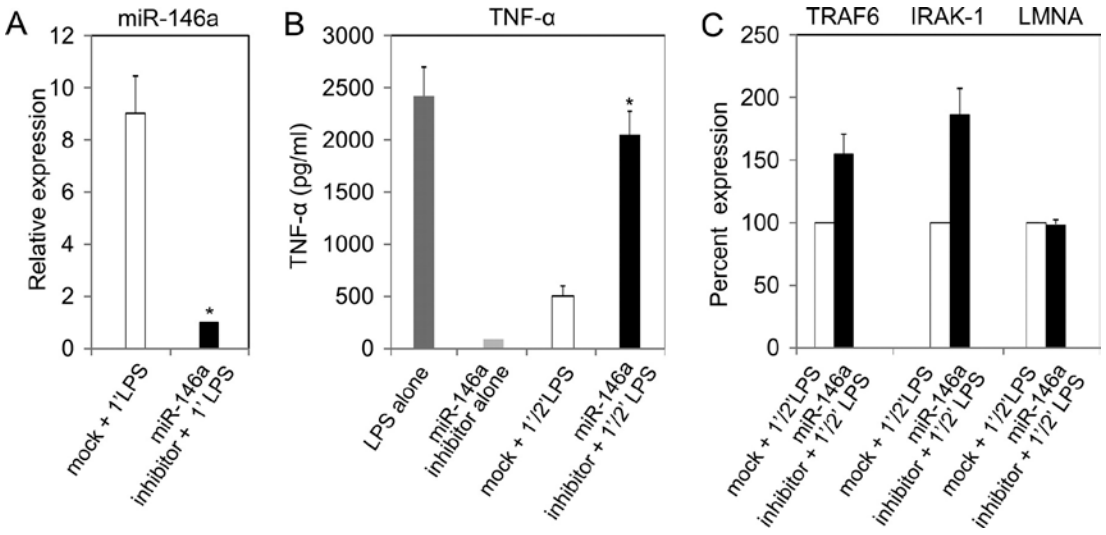

Figure 2-6. Blockade of miRNA-146a expression abrogated LPS-induced tolerance in THP-1 cells. THP-1 cells transfected with 40 nm miR-146a inhibitor for 24 h, along with mock transfected, were washed with complete growth medium and primed with 10 ng/ml LPS for 16 h. Cells were then washed with complete growth medium and challenged with 1000 ng/ml LPS for 3 h. Additional controls included were cells transfected with miR-146a inhibitor alone and cells treated with 1000 ng/ml LPS alone for 3 h (LPS alone, TNF-α-positive control). A, total RNA from cell pellets of mock and miR-146a inhibitor-transfected cells, primed with 10 ng/ml LPS for 16 h, were analyzed by qRT-PCR for miR-146a expression. B, TNF-α protein in supernatants of TNF-α (+) control, miR-146a inhibitor alone (without LPS), mock transfected, and miR-146a inhibitor-transfected, were measured by ELISA. C, total RNA were analyzed for TRAF6, IRAK-1, and an unrelated gene lamin A/C (LMNA) expression in both mock and miR-146a inhibitor-transfected cells by qRT-PCR. Data are expressed as mean ± S.D. of three independent experiments. *, $p < 0.01$ compared with mock transfected cells.

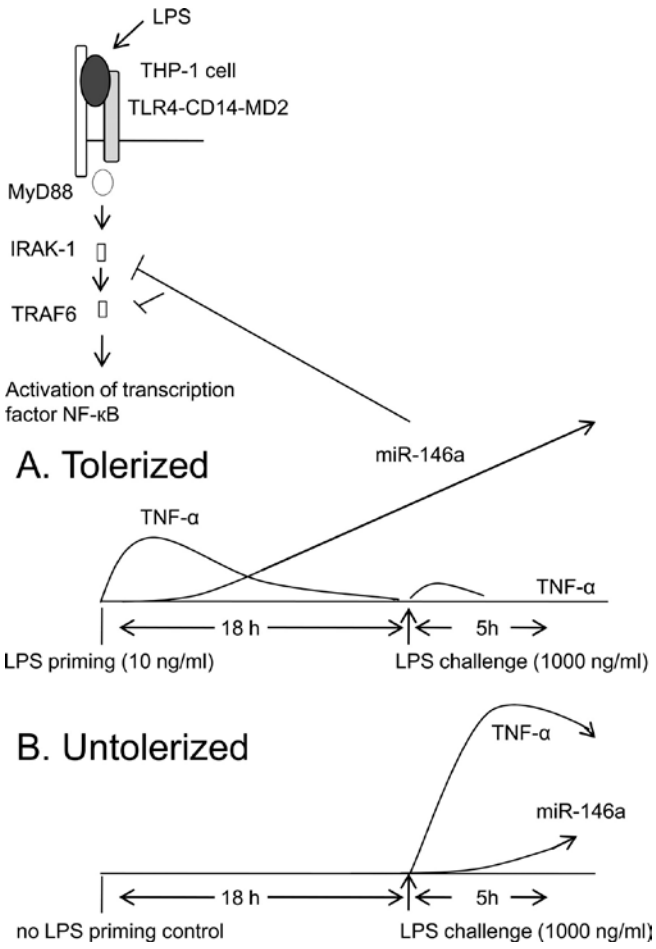

Figure 2-7. A model of the role of miR-146a in LPS-TLR4-mediated signal transduction in tolerized (A) versus untolerized (B) cells. See text under "Discussion." MD2, myeloid differentiation protein-2.

CHAPTER 3
MECHANISTIC ROLE OF MICRORNA-146A IN ENDOTOXIN-INDUCED DIFFERENTIAL CROSS-REGULATION OF TLR SIGNALING

Background

Innate immunity is the primary defense mechanism that recognizes, responds to, and resolves invading infectious microbes or their conserved components, known as pathogen-associated molecular patterns (PAMPs). During microbial invasion, danger signals are effectively recognized by the host's innate immune system through several conserved pattern-recognition receptors. TLRs represent one of the best characterized pathogen-detection systems. Once they are activated by PAMPs, they signal to transcription factor NF-κB to produce proinflammatory cytokines. TLRs possess an extracellular leucine-rich repeat domain (type 1 membrane protein) and cytoplasmic conserved Toll/IL-1R domain. The extracellular domain recognizes microbial ligands (274), whereas the cytoplasmic portion responds by recruiting adaptor kinases to enable signal transduction, most notably through activation of NF-κB transcription factor (9). Therefore, activation of NF-κB by TLRs is a critical event on the road to inflammation, as in LPS-induced systemic inflammation mediated by TLR4.

To date, many TLRs (TLR1–TLR13) have been identified in mammals (5, 6) based on their microbial cell wall specificity or pathogen-specific nucleic acids. TLRs recognize the ligands as either homodimeric or heterodimeric (9). For example, TLR4 binds to LPS, TLR1/2 or TLR2/6 to lipopeptides, TLR5 to flagellin (subunits of bacterial flagella), and TLR3, TLR7, or TLR9 to nucleic acid. Several PAMPs, including LPS, can stimulate TLR4. However, LPS is the fundamental pathogenic cell wall component of Gram-negative bacteria and is considered one of the most potent immunostimulatory components. The immune system responds to LPS with a systemic production of pro- and anti-inflammatory cytokines (via activating transcription factor NF-κB), such as TNF-α, IL-6, and IL-10, primarily aimed to control growth and dissemination of

invaders and subsequently curtail the immune response as needed (10). On the contrary, pathological dysregulation of NF-κB is linked to substantial systemic inflammatory damage that gives rise to sepsis, multiorgan failure, autoimmune diseases, and possibly cancer (11, 262, 275). Interestingly, during bacteremia, neutrophils and monocytes from septic patients assume a refractory state to subsequent LPS challenges and no longer produce inflammatory mediators (13, 42). This phenomenon, referred to as endotoxin tolerance (also called LPS hyporesponsiveness or refractoriness), is a host mechanism aimed at limiting inflammatory damage caused by overactivation of the immune system to continuous exposure to Gram-negative bacteria or their products. LPS refractoriness to subsequent LPS challenges (homologous tolerance) has been investigated extensively *in vitro* using monocyte/macrophage primary cells and cell lines and *in vivo* using animal models as well as being observed in humans (42, 45, 47, 48, 71, 226, 276). LPS priming of the immune cells also results in diminished cytokine response after subsequent stimulation with non-LPS heterologous TLR ligands (226, 276-278). This is known as LPS-induced cross-tolerance and has also been observed in association with cells from septic patients (14). Similarly, such other TLR ligands as peptidoglycan (PGN), lipoteichoic acid (LTA), $Pam_3CSK_4CysSerLys_4$ (Pam_3CSK_4), and flagellin, plus such cytokines as TNF-α or IL-1β, have been shown to induce homologous tolerance in monocytes/macrophages and, interestingly, they can substitute for each other and sometimes mediate cross-tolerance both *in vitro* and *in vivo* (15). LPS-induced tolerance and/or cross-tolerance are thought to play a broader role in host innate immunity, but how it is established is still not completely understood. Therefore, the study of the TLRs regulation in LPS-primed immune cells will help to explicate its role against various microbial insults or whole bacteria, although no detailed or consensus mechanism has been identified to demonstrate

how LPS-primed immune cells become hyporesponsive to homologous and/or heterologous ligands, a phenomenon also known as differential cross-regulation (10).

microRNAs (miRNAs) have emerged as a new layer of gene expression regulators that act at the posttranscriptional level via the RNA interference mechanism (102). In mammals, the progression of miRNA biogenesis involves the initial transcription of genomic DNA by RNA polymerase II into primary miRNAs, which are sequentially processed by two RNase III enzymes, Drosha (in the nucleus) and Dicer (in the cytoplasm), to become ~23-nucleotide noncoding dsRNA duplexes (258). Eventually, the duplex is loaded into the RNA-induced silencing complex with a guide strand to target mRNAs primarily at their 3′-untranslated regions. This consequence leads to inhibition of translation and/or a decrease in mRNA stability as a result of accelerated decapping and deadenylation (89, 279). miRNAs have been revealed to play important roles in many biological systems, ranging from the development and differentiation of cells to tumors (102), including those in the mammalian immune system (92, 93). During an inflammatory response to microbial insults, many of the miRNAs, including miRNA (miR)-146a induced by TLRs, can negatively regulate the activation of inflammatory pathways in myeloid cells (116, 122, 125, 239, 250), although during the course of their expression kinetics, their biological activities in innate immunity are largely unknown. In initial studies on miR-146a expression kinetics observed in response to cognate ligands for TLRs, the cytoplasmic sensor retinoic acid-inducible gene I (also known as DDX58) and proinflammatory cytokines, including IL-1β. Thus, miR-146a is thought to play a role in innate immunity against pathogenic insults as well as inflammatory diseases (116, 122). Further analysis to determine the biological significance of miR-146a reveals that its expression is NF-κB–dependent, consistent with the presence of an NF-κB binding site in the promoter region, and regulates production of such

cytokines as IL-1β and TNF-α in innate immunity by affecting signaling molecules (116). IL-1R–associated kinase-1 (IRAK1) and TNFR-associated factor 6 (TRAF6) are important signaling adaptor kinases in the downstream of TLR4 signal transduction, and they promote inflammation sustained by proinflammatory cytokines, including TNF-α. Interestingly, these two adaptor kinases are the direct molecular targets for miR-146a, as shown by Taganov et al. (116). Subsequently, our recent *in vitro* study has demonstrated the mechanistic role of miR-146a in endotoxin tolerance. During this study of LPS tolerance, miR-146a was shown to increase continuously and remain at a high level, exerting negative effects on IRAK1 and TRAF6 mRNA at the posttranscriptional level (82). Previously Li et al. (78) and Boone et al. (87), respectively, observed LPS tolerance in monocytes due to impairment of IRAK1 and TRAF6 kinase activity in TLR4 signaling. Interestingly, IRAK1 and TRAF6 are not only used by TLR4 for signaling, but also by other TLRs, such as TLR2, TLR5, TLR7, TLR8, and TLR9, as well as the IL-1β receptor (271). Therefore, they are considered the common and central adaptor kinases, and should their activity be diminished, cellular refractoriness may happen by other TLRs (except TLR3) signaling. This leads to the speculation that increased miR-146a expression during an LPS-primed state might play a part in a negative feedback pathway for other ligand–TLR interactions. Considering the ability of miR-146a to regulate TRAF6 and IRAK1, shared by all TLRs (except TLR3), we hypothesize that it is involved in endotoxin-induced cross-tolerance against various microbial component sensed by all other TLRs. Thus, the aim of this study was to investigate the effect of the unique expression pattern of miR-146a in LPS-primed monocytic cells on differential TLR cross-regulation. Our findings suggest that overexpression of miR-146a contributes to controlling proinflammatory cytokine production and confers cross-tolerance to

innate immune cells and thus modulates our innate immunity to evade recurrent similar or different bacterial infections or both.

Materials and Methods

Cell Culture and Innate Immune Ligand Stimulation

Human THP-1 cells, an undifferentiated promonocytic cell line, were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained by twice weekly passage in RPMI 1640 medium containing 25 mM HEPES and l-glutamine (BioWhittaker, Walkersville, MD), 10% FBS (Mediatech, Manassas, VA), and 100 U/ml penicillin-streptomycin (Mediatech) at 37°C with 5% CO_2. Log phase cells were used in all experiments and cultured at the density of 10^6 cells/ml. To determine the kinetics of ligand-induced cytokine production in vitro, fresh THP-1 monocytes were suspended in complete RPMI 1640 culture medium and seeded at 10^6 cells/ml in a 24-well plate. Cells were stimulated with the following innate immune ligands: 100 ng/ml LPS (TLR4 ligand) from *Salmonella enterica* serotype Minnesota Re595 (LPS Se; Sigma-Aldrich, St. Louis, MO), 100 ng/ml Pam_3CSK_4 (a synthetic bacterial lipoprotein and proinflammatory ligand for TLR2/TLR1), 500 ng/ml PGN (*Escherichia coli* 0111:B4, TLR2 ligand), 1 μg/ml LPS from *Porphyromonas gingivalis* (LPS Pg, TLR2 ligand), 1 μg/ml LTA (TLR2 ligand) from *Staphylococcus aureus*, 100 ng/ml recombinant flagellin (TLR5 ligand) from *S. typhimurium*, 10 μg/ml muramyl dipeptide (MDP), 10 ng/ml TNF-α, 100 ng/ml IL-1β, and 10 ng/ml IFN-γ (BD Biosciences, Franklin Lakes, NJ). Additionally, to observe the similar TLR ligand stimulatory effect in primary cells, mouse peritoneal-derived macrophages were obtained from 3-mo-old female C57BL/6 mice, which were injected with 0.5 ml 4% (w/v) sodium thioglycollate, 3 d prior to sacrifice. Primary cells were seeded at $x10^6$/ml in complete DMEM containing 10% FBS in 24-well plates followed by washing after 5 h, using complete growth DMEM to remove nonadherent cells. Adherent macrophages were stimulated with LPS

and PGN (0-10 μg/ml) for 6, 12, and 24 h. All of the TLR1, TLR2, and TLR5 ligands were obtained from InvivoGen (San Diego, CA). Ligands were used at concentrations previously reported to induce mediators. Stocks were prepared in tissue culture-grade PBS and preserved at -20°C until needed. Cells were harvested and culture supernatants were collected at various time points over 24 h and stored at -80°C until assayed for cytokines levels. Cell pellets were washed in PBS and stored in RNAlater (Ambion, Austin, TX) at 4°C or frozen at -80°C for total RNA isolation in subsequent analysis.

***In Vitro* Induction of Homologous Tolerance and Cross-tolerance**

An LPS-induced tolerance and cross-tolerance cell model using monocytic cell line THP-1 was adapted from methods described previously (82, 204, 280), with some minor modifications. Briefly, before starting tolerance and/or cross-tolerance assays, THP-1 cells were cultured for 4 d until cells were in log phase and concentration at 10^6 cells/ml and viability was checked to be >99% by trypan blue staining. THP-1 cells were transferred to fresh complete medium in new 5- or 25-ml flasks at $5x10^5$ cells/ml. Cells were incubated with a low dose of LPS (10 ng/ml) for 18 h. In some cross-tolerance experiments, cells were primed for 18 h with PGN, Pam_3CSK_4, or flagellin. After two washes with tissue culture-grade PBS, cells in complete culture medium alone (untolerized negative control) or with the same or a different ligand were cultured in a 24-well plate. For observing LPS-induced cross-tolerance against bacteria as a secondary challenge, *E. coli* DH10B and *P. gingivalis* strain 33277 were grown until mid-log phase, then harvested and placed in sterile saline, followed by killing at 65°C for 30 min as described previously (281). Heat-killed bacteria were then washed three times with sterile saline and used at a final concentration equivalent to 1 x 10^6 CFU/ml. Similarly, to observe the TLR ligand tolerance efficiency in primary cells, mouse peritoneal macrophages were primed with LPS or PGN (100 ng/ml) in 24-well plates followed by washing and challenged with PGN, Pam_3CSK_4, or LPS.

After incubation for 5 h at 37°C in 5% CO_2, supernatants were harvested by centrifugation (1500x g at 4°C, 5 min) and immediately stored at -80°C until assayed for inflammatory mediators TNF-α, IL-1β, or IL-6.

Quantification of miRNA and Mrna Expression Level by Quantitative Real-time PCR

Total RNA of TLR ligands in treated and untreated THP-1 cells was prepared using the *mir*Vana miRNA isolation kit (Ambion) following the manufacturer's protocol. RNA yield and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Wilmington, DE), and equal amounts of each RNA ($A_{260}/A_{280} \approx 2.0$, 6.7 ng for miRNA) were used for quantitative stem-loop reverse transcription and real-time PCR (qRT-PCR) analysis. Quantification of mature miRNAs expression was performed using the TaqMan microRNA reverse transcription kit, TaqMan Universal PCR Master mix, and TaqMan microRNA assay primers of interest for human miRNAs (Applied Biosystems, Foster City, CA). For mRNA analysis, a High Capacity cDNA RT kit (Applied Biosystems) and TaqMan mRNA assay primers for IRAK1, TRAF6, and TLR4 were used with 33 ng total RNA per reaction. The cycle threshold (*Ct*) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined, and miRNA expression values were calculated using the abundant and virtually pure RNU44 as an endogenous control (Applied Biosystems) following the $2^{-\Delta\Delta Ct}$ method (259). mRNA for gene expression values were quantified in the same way after normalization to mammalian 18S rRNA.

THP-1 Cell Transfection

miR-146a functional analyses were performed using synthetic miR-146a mimic and miR-146a inhibitor obtained from Ambion and reconstituted in nuclease-free water at a concentration of 20 μM. Stocks were stored in aliquots at -80°C prior to use. One day before the transfection, cells were transferred to fresh culture medium at a concentration of 5×10^5 cells/ml. The

following day, THP-1 cells adjusted to 5 x 10^5cells/well were transfected with miR-146a mimic (20 nM) or inhibitor (40 nM) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. miR-146a mimic-transfected THP-1 cells were incubated for 24 h, followed by washing twice with complete growth medium. The washed cells were treated with different ligands at the above-mentioned concentration for 5 h. For miR-146a inhibitor experiments, transfected THP-1 cells were incubated for 24 h, followed by washing with complete growth medium. Cells were then challenged with various ligands for 5 h. Supernatants from cell cultures were collected and assayed for cytokines secretion, and cell pellets were used for RNA isolation and qRT-PCR analysis.

ELISA for Cytokine Assay

Supernatants were collected from cell cultures at different time points after being induced with various stimuli. Secreted cytokines TNF-α, IL-1β, and IL-6 in the supernatants were measured by ELISA using OptEIA cytokine kits as recommended by the manufacturer (BD Biosciences). Absorbance was measured at 405 nm using a microplate reader (model 680; Bio-Rad, Hercules, CA). A_{405} was converted to protein concentrations (pg/ml) using standard curves of recombinant human or mouse cytokines.

Western Blot Analysis

LPS-primed and unprimed THP-1 cells (5 x 10^6/condition) were collected 2 h after LPS Se, LTA, Pam_3CSK_4, PGN, LPS Pg, and flagellin challenge, pelleted at 1000 xg for 10 min, and lysed on ice for 10 min in 1 ml lysis buffer (50 mM HEPES [pH 7.6], 150 mM NaCl, 1 mM EDTA, 1% (v/w) Nonidet P-40, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 5 mM para-nitrophenyl phosphate, 1 mM DTT, 1 mM PMSF, and complete protease inhibitor mixture from Roche Diagnostic, Indianapolis, IN). Supernatants were collected after centrifugation at 13,000 rpm for 20 min at 4°C. Similarly, PGN- and Pam_3CSK_4-

primed THP-1 cell lysates were prepared 2 h after homologous or LPS challenge. Soluble lysates were quantitated for protein concentration using a Bio-Rad protein assay kit, separated by SDS-PAGE (10% acrylamide; Bio-Rad, w/v) along with Precision Plus Protein standards (Bio-Rad), and electrotransfered to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked for 1 h at room temperature or overnight at 4°C with 5% (w/v) nonfat milk in PBS/0.05% Tween 20 (PBS-T, v/v) and were probed with primary rabbit anti-IRAK1 or anti-TRAF6 Antibody at a concentration of 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed three times with PBS-T and incubated for 1 h with goat anti-rabbit IgG-HRP at a concentration of 1:5000 (SouthernBiotech, Birmingham, AL). After washing in PBS-T, reactive protein bands were visualized by SuperSignal Pico chemiluminescent reagent (Pierce).

Statistical Analysis

Data are presented in figures as mean ± SD. For multiple group comparisons, one-way ANOVA ($p < 0.05$) was performed, followed by the two-sided, unpaired Student *t* test as described by Shaffer (260). An unpaired, two-tailed Student *t* test was used to compare two independent groups. For all statistical analysis, Prism for Windows, version 5.0 (GraphPad Software, San Diego, CA) was used, and $p < 0.05$ was considered statistically significant.

Results

Analysis of Innate Immune Ligand-induced TNF-α Secretion and miRNA Expression Kinetics in Monocytic THP-1 Cells

Microbial ligands (or PAMPs) are recognized by innate immune receptors, such as lipopeptides and lipoproteins by TLR2 (282, 283), double-stranded viral RNA by TLR3 (284), LPS by TLR4 (282), and bacterial flagellin from both Gram-positive and Gram-negative bacteria by TLR5 (285). These ligands have been shown to produce a diverse array of inflammatory

mediators, including TNF-α *in vitro*, as well as *in vivo* or *ex vivo* conditions (204, 226, 261, 286, 287). To observe TNF-α production *in vitro*, the most commonly employed acute monocytic leukemia cell line THP-1 (262) was used in the current study. After TLR ligand stimulation of THP-1 cells, kinetics of TNF-α production were examined. As shown in Fig. 3-1A, log phase THP-1 cells were treated with agonists for TLR2 (or TLR1), TLR4, TLR5, and NOD2, or cytokines IL-1β and IFN-γ, and TNF-α production in supernatants at 8 or 24 h was assessed by ELISA. In supernatants from ligand-stimulated THP-1 cell culture, TNF-α was detected at a significant level in response to all ligands except MDP, IL-1β, and IFN-γ (Fig. 3-1A). TNF-α protein levels at 8 and 24 h were also correlated with mRNA levels (data not shown).

LPS induces up-regulation of miR-146a in THP-1 monocytes, as described by Taganov et al. (116) and Bazzoni et al. (251), through microarray analysis and independently confirmed by our laboratory (82). miR-146a is also induced in response to various other components from both Gram-positive and Gram-negative bacteria, as well as proinflammatory cytokines (116). Following these observations and considering the subsequent experimental purposes of this study, expression of miR-146a, miR-155, miR-132, and miR-16 were determined by qRT-PCR analysis on the same RNA samples (Fig. 3-1B–E). The fold changes in miRNA expression were calculated by comparing the value of TLR ligand-treated cells to that of untreated samples cultured in parallel. miR-146a showed significant expression after 8 h exposure of THP-1 cells to LPS Se, PGN, Pam_3CSK_4 (TLR2-TLR1 ligand), and to LPS Pg, flagellin (a major component of the bacterial flagellar filament and agonist of the TLR5 receptor), and IL-1β. In contrast, stimulation of LTA, NOD2 ligand MDP, or IFN-γ induced little or no miR-146a expression. Interestingly, there was a substantial increase at 24 h that reached up to 26-fold for LPS Se, 15- to 17-fold for Pam_3CSK_4, PGN, and LPS Pg, 10-fold for flagellin, and 7-fold for IL-1β (Fig. 3-

1B). In contrast, after 8 h, miR-155 showed an increase of 3-fold for LPS Se, 4- to 5-fold for Pam_3CSK_4, PGN, and LPS Pg, and 3-fold for IL-1β (Fig. 3-1C). Similarly, after 8 h, miR-132 expression showed increases of 10-fold for LPS Se, 15-fold for Pam_3CSK_4 and PGN, 6-fold for LPS Pg, and 3-fold for flagellin (Fig. 3-1D). No significant change in the expression of miR-16 was observed (data not shown). This last result could serve as a control miRNA that is regulated independently from TLR ligand stimulation together with the internal control RNU44. Notably, in response to the TLR ligands and IL-1β, among those examined, only miR-146a showed an increase between 8 and 24 h (Fig. 3-1B) and this was negatively correlated to TNF-α release.

Primary mouse peritoneal macrophages were also used to observe the relationship between TNF-α and miR-146a expression stimulated by different doses of LPS or PGN for 6, 12, and 24 h. LPS- or PGN-treated primary cells showed dramatic TNF-α production at 6 h, both peaking at 6 or 12 h, and reduced by 24 h (Fig. 3-1E and G). In contrast, miR-146a showed gradual increase for all the time points showed (Fig. 3-1F and H). Note that at the highest concentration of 10 μg/ml LPS or PGN, the levels of miR-146a were actually lower than when 1 μg/ml LPS or PGN was used, indicating that an upper limit could be reached with these primary cells.

Inhibition of miR-146a Increases TNF-α Production in TLR Ligand-stimulated THP-1 Cells

As shown in Fig. 3-1A versus Fig. 3-1B, there is a general inverse correlation between miR-146a expression and TNF-α production. To examine if miR-146a has any direct effect on TNF-α production, THP-1 cells were transfected with miR-146a inhibitor and then 24 h later stimulated with various TLR ligands. Because the level of miR-146a is very low in unstimulated THP-1 cells, the knockdown effect in the cells transfected with the miR-146a inhibitor might not be easily appreciated (82). Results shown in Fig. 3-2A confirm that transfection of miR-146a inhibitor prior to LPS stimulation could efficiently block and/or downregulate miR-146a

expression by >90% compared with mock-transfected cells primed with LPS. Fig. 3-2B illustrates how THP-1 cells transfected with miR-146a inhibitor showed a 52 and 60% increase in the level of TRAF6 and IRAK1 mRNA, respectively, whereas an unrelated gene, lamin A/C, was unaffected. Increases of IRAK1 and TRAF6 protein levels were confirmed by Western blot analysis (Fig. 3-2C), consistent with the changes in mRNA levels and previous reports (82, 122). Cells transfected with miR-146a inhibitor showed increased TNF-α production in response to LPS Se (62%), Pam_3CSK_4 (53%) PGN (52%), LPS Pg (42%), and flagellin (40%) (Fig. 3-2D). These data show the level to which miR-146a and adaptor kinases TRAF6 and IRAK1 affected TNF-α production in response to LPS, Pam_3CSK_4, PGN, and flagellin, but not LTA and other ligands known to not affect miR-146a production.

LPS-induced Mir-146a May Account for Cross-tolerance to a Panel of Innate Immune Ligands in THP-1 Cells

Involvement of LPS in homologous tolerance (82) as well as cross-tolerance to other TLR stimuli (62, 226, 288, 289) has been shown *in vitro* and/or *in vivo* or *ex vivo*. Fig. 3-3 shows the extent of LPS-induced cross-tolerance to a panel of innate immune ligands associated with miRNA expression in terms of proinflammatory cytokines using the THP-1 cell model. THP-1 monocytes were primed with 10 ng/ml LPS for 18 h, followed by washing with PBS and challenging with various agonists, as described in Materials and Methods. After 5 h incubation with challenged ligands, TNF-α protein level was analyzed by ELISA (Fig. 3-3A). TNF-α levels decreased significantly ($p < 0.01$) after secondary challenges with Pam_3CSK_4, PGN, LPS Pg, and flagellin in comparison with the same stimulation of naive THP-1 cells. MDP (NOD2 ligand), IL-1β, and IFN-γ did not induce TNF-α (Fig. 3-1A) regardless of the LPS priming. Analysis of TNF-α mRNA levels by qRT-PCR (Fig. 3-3B) showed levels consistent with the changes in protein data of cross-tolerized THP-1 cells, and reduction of TNF-α mRNA was

significant compared with the untolerized control. Previously, LaRue and McCall (21) reported that neutrophils from septic patients showed less LPS-induced IL-1β mRNA and protein synthesis, which was supported by their *in vitro* studies using THP-1 monocytes. Production of other proinflammatory cytokines, such as IL-6, is also known to be reduced in LPS-primed cells (20). Consistent with these reports, LPS-primed cells showed reduced IL-6 and IL-1β production in response to the innate immune stimuli, as shown in Fig. 3-3C and 3-3D. The reduced production of IL-6 and IL-1β was similar to the pattern observed for TNF-α production.

Because miR-146a was shown to be responsible for LPS tolerance (82), its levels in the cross-tolerized conditions were confirmed as it was postulated to have a role in LPS-induced cross-tolerance (Fig. 3-3E). As expected, miR-146a in the cross-tolerized sample showed higher expression during 18 h initial LPS-priming incubation plus 5 h ligand challenge, but little or no changes in the expression of miR-16 (Fig. 3-3F) were observed compared with unprimed controls. For Fig. 3-3E, the open bars (untolerized) depict miR-146a levels challenged by different ligands for a total of only 5 h, as there was no priming at 0-18 h. These results are consistent with data already presented in Fig. 3-1B for exposure of 8 and 24 h. The filled bars (tolerized) represent miR-146a levels after 18 h LPS priming and 5 h challenge with different ligands.

Figs. 3-3G and 3-3H show the mRNA expression of known miR-146a targets IRAK1 and TRAF6 in both cross-tolerized and untolerized cells, respectively. The mRNA levels of IRAK1 and TRAF6 were not affected significantly between cross-tolerized and untolerized cells; this is not unexpected, as miR-146a regulates these adaptor molecules via primarily translational repression (82, 116). Fig. 3-3I shows Western blot analysis of IRAK1 and TRAF6 protein levels comparing LPS cross-tolerized cells to control untolerized cells demonstrating moderate

reductions of ~50-75% in all cases, as expected. This level of reduction in IRAK1 and TRAF6 was consistent with “medium alone” control (untolerized cells versus cross-tolerized cells) when cells were tolerized by LPS priming but not exposed to secondary challenge. Consequently, miR-146a may play an important role in LPS-induced cross-tolerance based on the inverse correlation with proinflammatory cytokine production and the repressed levels of IRAK1 and TRAF6.

To support that the THP-1 cell-based model of endotoxin cross-tolerance can be observed in primary cells, mouse primary macrophages were also tolerized with LPS or PGN. Fig. 3-3J shows decrease in TNF-α production by 50% in LPS-LPS homologous tolerance (priming challenge) and by 75% in PGN-PGN homologous tolerance. Heterologous tolerance varies between 40% reduction in TNF-α for PGN-LPS to 52% TNF-α reduction for LPS-PGN. Fig. 3-3K shows a 5- to 10-fold increase in expression of miR-146a in LPS-tolerized cells, consistent with its potential role in endotoxin tolerance in primary cells as well.

Previously, highly elevated miR-146a expression was shown to be dependent on the continuous presence of LPS in the medium as demonstrated by its decreasing levels after 22 h LPS withdrawal (82). Accordingly, THP-1 monocytes were primed with PGN continuously for 18 h, then washed twice with PBS, and cultured in complete growth medium for an additional 0, 12, or 22 h (PGN withdrawal). With 18 h continuous PGN priming and 5 h PGN challenge, the same condition used in Fig. 3-3A, negative correlation between TNF-α secretion and miR-146a expression, was observed as expected (Fig. 3-3L-M). After 12 h PGN withdrawal, cells started to regain PGN responsiveness and almost completely recovered from tolerance after 22 h PGN withdrawal (Fig. 3-3L). Thus, upregulated miR-146a from PGN priming, similar to LPS priming (82), is important for maintaining tolerance and cross-tolerance. The half life (t1/2) of miR-146a estimated from this experiment was ~9–10 h.

LPS- or PGN-primed THP-1 monocytes show effective homologous tolerance with higher priming dose correlating with higher miR-146a levels.

The effectiveness of endotoxin tolerance in THP-1 cells correlated with an increase in priming dose up to 1000 ng/ml LPS was reported in our earlier study with secondary challenge LPS doses of up to 1000 ng/ml (82). Thus, to further advance our understanding of whether homologous tolerance can be achieved with even higher doses of LPS, a range of challenge concentrations of LPS up to 10,000 ng/ml (or PGN up to 2,000 ng/ml) was employed to primed THP-1 cells. Priming with 10 ng/ml LPS or higher concentrations resulted in an 80-90% decrease ($p < 0.01$) in TNF-α production, even at the highest dose of LPS challenge (10,000 ng/ml, Fig. 3-4A). Fig. 4B shows the results of gradual augmentation of miR-146a correlated with degree of tolerance observed in Fig. 3-4A. These data are uniformly consistent with our previous report, which found that the increase in miR-146a expression and reduction in its target IRAK1 and TRAF6 protein levels were LPS dose-dependent (82), with the exception that the current experiment further reinforces the previous conclusion that LPS tolerance was maintained even when up to a 10 times higher secondary challenge dose of LPS was employed. A similar homologous tolerance phenomenon was observed for the TLR2 ligand PGN. Fig. 3-4C illustrates how THP-1 cells primed with 10 ng/ml or a higher concentration of PGN were significantly resistant to secondary PGN challenges of up to 2000 ng/ml, and this homologous tolerance was correlated with respective priming doses and upregulation of miR-146a (Fig. 3-4D).

Cross-regulation between TLR2, TLR4, and TLR5 Ligands in THP-1 Cells

Fig. 3-5A shows that priming with 100 ng/ml PGN (TLR2 ligand) resulted in significant hyporesponsiveness to PGN (500 ng/ml), as well as to heterologous ligand LPS (100 ng/ml) where TNF-α protein production dropped by >90% compared with unprimed controls observed in ELISA. In Fig. 3-5F, qRT-PCR analysis showed about 12-fold increase of miR-146a

expression in the PGN-primed cells (similar to Fig. 3-1B), indicating again a negative correlation with TNF-α production, as shown in Fig. 3-5A. Similar cross-tolerance and correlation of TNF-α and miR-146a expression was observed for another TLR2 ligand, Pam_3CSK_4 (Fig. 3-5B, 3-5G). Flagellin is a potent simulator of innate immunity and is recognized by TLR5, which triggers defense responses both at epithelial surfaces and systemically. Fig. 3-5C showed that priming with 100 ng/ml flagellin diminished TNF-α production by 95% in a flagellin secondary challenge, as well as in challenges with heterologous ligands Pam_3CSK_4 (50%) and PGN (70%). In contrast to these results, there was no significant change of TNF-α production between flagellin-primed and control unprimed THP-1 cells against LPS (Fig. 3-5C), and this observation is consistent with data reported by Mizel and Snipes (287). Note that miR-146a expression was induced in flagellin-primed cells (Fig. 3-5D). To obtain further understanding of why flagellin-primed THP-1 cells did not show hyporesponsiveness to LPS, it was postulated that an increased expression of TLR4 in stimulated cells, as reported by Siedlar et al. (290), might override miR-146a activity. Fig. 3-5E shows that TLR4 mRNA expression measured by qRT-PCR was higher in flagellin-primed cells than in unprimed controls. A moderate reduction of IRAK1 and even less for TRAF6 was observed by Western blot analysis in PGN- and Pam3CSK4-primed THP-1 monocytes (Fig. 3-5H, 3-5I). Consequently, these *in vitro* data suggest cross-talking among TLRs, and may be attributed in part to miR-146a overexpression affecting TNF-α production in primed cells against subsequent stimulation by homologous and heterologous ligands. The fact that flagellin-primed THP-1 cells did not affect TNF-α production in the LPS challenges served as an interesting control for observed specificity; the mechanism underlying it remains unclear even with the observed increase level of TLR4 and will be a subject of future studies.

IRAK1 and TRAF6 Knockdown Reduces Inflammatory Response to TLR Ligands

IRAK1 and TRAF6, the known molecular targets for miR-146a, are important adaptor kinases that help to amplify the initial signal transduction response by all TLRs except TLR3. The absence and/or inhibition of these kinases cause reduced inflammatory response by TLR ligands both in vivo and in vitro, indicating that these molecules mediate major mechanism of endotoxin tolerance (71, 78, 82, 204, 206, 290). To directly examine the importance of IRAK1 and TRAF6 for other TLR signal transduction in THP-1 monocyte activation, IRAK1 and TRAF6 mRNA expression was targeted by specific siRNA transfection (Fig. 3-6). Twenty-four hours after transfection of THP-1 cells with 40 nM small interfering (si)RNA targeting IRAK1 (siIRAK1) resulted in a 60% decrease in IRAK1 mRNA level but no affect on the mRNA of TRAF6 or lamin A/C (Fig. 3-6A). Similarly, the TRAF6 mRNA level was reduced by 80% following transfection with siRNA targeting TRAF6 (siTRAF6, Fig. 3-6B) confirming successful transfection and knockdown. Transfected cells were treated with various ligands, and culture supernatants were collected after 24 h incubation for cytokine production analysis. THP-1 cells deficient in IRAK1 showed a reduction in TNF-α production in response to LPS Se (80%), Pam_3CSK_4 (50%), PGN (57%), LPS Pg (78%), and flagellin (60%) compared with mock-transfected cells, with LTA serving as a negative control (Fig. 3-6C). Similarly, Fig. 3-6C also shows that TRAF6 knockdown diminished TNF-α production by THP-1 cells in response to LPS Se (70%), Pam_3CSK_4 (52%), PGN (33%), LPS Pg (53%), and flagellin (35%). Similar effects were observed for IL-1β (Fig. 3-6D) and IL-6 production (Fig. 3-6E). These data are consistent with the report by Baltimore et al. (92) that IRAK1 and TRAF6 are the central adaptor kinases, the absence of which greatly affects the ability to transmit subsequent TLRs signal transduction.

Upregulation of Mir-146a Alone Can Mimic LPS Priming to Induce Cross-tolerance

LPS has been shown to induce the expression of a few regulatory miRNAs, including miR-146a (116, 206, 251), which is critical for endotoxin-induced tolerance (82). LPS-pretreated THP-1 cells showed cross-tolerance to a panel of innate immune ligands linked to miR-146a expression (Fig. 3-3A). Following this, to determine the direct functional role of miR-146a in endotoxin-induced cross-tolerance, THP-1 cells were transfected with 20 nM miR-146a mimic followed by challenges with various TLR ligands. After 24 h, miR-146a showed significantly higher expression ($p < 0.01$) in miR-146a mimic-transfected cells compared with mock-transfected cells, confirming the successful transfection (Fig. 3-7A). In Fig. 3-7B, TNF-α production by transfected THP-1 cells 5 h after challenges by LPS Se (76%), Pam_3CSK_4 (54%), PGN (30%), LPS Pg (64%), and flagellin (43%) was significantly reduced compared with the mock-transfected cells. The effect of overexpression of miR-146a on TRAF6 and IRAK1 was validated by monitoring their mRNA levels (Fig. 3-7C), which were reduced by 60 and 55%, respectively, compared with mock-transfected cells. An unrelated human gene, lamin A/C, showed no significant changes in expression level between miR-146a mimic-transfected and mock-transfected controls. Reduction of TRAF6 and IRAK1 protein levels was documented by Western blot analysis (Fig. 3-7D), consistent with previous reports (82, 122). Taken together, these data demonstrate that LPS-induced miR-146a upregulation plays a pivotal role in providing cross-tolerance against a range of innate immune ligands.

miR-146a Expression Links to Cross-tolerance Against Heat-killed Whole Bacteria

In the preceding experiments, LPS-induced cross-tolerance was observed in TLR1, TLR2, and TLR5 ligands where miR-146a played a dominant role. However, in nature, individuals are exposed to whole bacteria rather than a single microbial ligand. Fig. 3-8 shows LPS-induced cross-tolerance demonstrated using heat-killed whole bacteria. Priming THP-1 cells with low-

dose LPS (10 ng/ml) resulted in significant ($p < 0.01$) reduction of TNF-α production following the secondary challenge with heat-killed *E. coli* (75%) or *P. gingivalis* (57%) at 1 x 10^6 CFU/ml (Fig. 3-8A). Similar hyporesponsiveness by human leukocytes has been observed by de Vos et al. (226). To demonstrate the role of miR-146a alone in this cross-tolerance, TNF-α production by miR-146a mimic-transfected THP-1 cells that were subsequently challenged with heat-killed *E. coli* at 1 x 10^6 cells/ml for 5 h was examined. In Fig. 3-8B, miR-146a mimic-transfected THP-1 cells showed hyporesponsiveness to heat-killed *E. coli*, and this sensitivity is miR-146a mimic dose-dependent. Thus, miR-146a plays an important role in providing cross-tolerance in the in vitro THP-1 cell model against various TLRs ligands, as well as whole heat-killed bacteria.

Discussion

While involvement of miR-146a in innate immunity has previously been reported in response to various microbial components and cytokines, much is still not known about its biological significance. In this study, the kinetics of TLR ligand-induced miR-146a expression were analyzed to elucidate its broader role in innate immunity. Consistent with previous findings, exposure of THP-1 monocytes to various bacterial inflammatory insults, such as LPS, PGN, and flagellin, resulted in rapid and continuous expression of mature miR-146a. Similar kinetics were not observed, however, for other LPS-induced miRNAs, such as miR-155 and miR-132 (Fig. 3-1). Owing to low basal levels of miR-146a in THP-1 monocytes, production of proinflammatory cytokines such as TNF-α takes place continuously once LPS stimulation occurs. As miR-146a expression starts to rise, the expression of IRAK1 and TRAF6 is putatively inhibited via translational repression and mRNA degradation and thus removes the amplification effectors in TNF-α production (82). A similar negative correlation in TLR2, TLR4, and TLR5 microbial ligands-induced cytokine release and miR-146a overexpression was also observed in this study. TNF-α production was increased in response to the same ligands after blocking miR-

146a activity, indicating its direct or indirect effect on TLR signaling. The new data in this article are thus focused on defining the role of miR-146a in endotoxin-induced cross-tolerance to those ligands, many of which, except LTA, MDP, and IFN-γ, also induce miR-146a upregulation.

Endotoxin tolerance is known to prevent inflammatory response to homologous or heterologous ligand challenge. Upon pre-exposure to other TLR ligands from Gram-positive or Gram-negative bacteria, monocytes acquire similar hyporesponsiveness to unrelated ligands. In the case of LPS-induced tolerance, a number of mechanisms have been proposed, including downregulation of TLR4 expression (282), degradation of such adaptor proteins as IRAK1 or TRAF6 (78, 82, 204) decreasing the association of TLR4 and MyD88 (63), suppression by IRAKM and TRIM30α (86, 235), downregulation of NF-κB activity, disruption of chromatin remodeling, expression of anti-inflammatory cytokines, and, more recently, by the post-transcriptional silencing of adaptor kinase mRNA through the action of miR-146a (82). In most cases, LPS-induced cross-tolerance is thought to occur by way of a similar mechanism involved in homologous tolerance. The key regulator of cross-tolerance is poorly understood, unfortunately, and thus our study of the mechanistic role of miR-146a in LPS-induced cross-tolerance is novel.

Our proposed model for the mechanistic role of miR-146a in LPS-induced cross-tolerance in THP-1 monocytes is as follows. In this THP-1 cell model, LPS/TLR4 signaling is facilitated by LPS-binding protein and CD14 and is mediated by a TLR4/MD-2 receptor complex. The downstream of the TLR4 signaling cascade is initiated after the binding of the Toll/IL-1R domain with adaptor protein MyD88. This binding acts as a bridge between TLR4 and incoming IRAK1 adaptor kinase, which further recruits TRAF6. This series of interactions triggers overall signal amplification, activation, and translocation of the transcription factor NF-κB, which

quickly initiates the expression of immune-responsive genes and such proinflammatory cytokines as TNF-α at mRNA and protein levels peaking at 2 and 4 h, respectively (82). Although miR-146a transcription is also known to be regulated by NF-κB (116), there appears to be a delay of between 2 and 4 h in its expression, with the level clearly elevated at 8 h (82), and it remains at a high level (from 30- to 100-fold) for up to 72 h (data not shown). The increase in the miR-146a level has negative effects on mRNA expression of IRAK1 and TRAF6, which are shared in the TLR4, TLR1/6, TLR2, and TLR5 pathway. Thus, the progression of these signal transductions is controlled or inhibited by miR-146a upregulation in LPS-primed THP-1 cells. In nature, individuals are exposed to various doses of different TLR ligands at the primary or secondary level. Notably, LPS-tolerized cells can resist 100-fold higher doses of homologous secondary challenges as measured by the reduction in TNF-α production (82). Similarly, TLR2 ligands such as PGN showed hyporesponsiveness to higher doses of homologous secondary challenges where miR-146a was upregulated while adaptor kinases were downregulated. Thus, once miR-146a upregulation takes place, THP-1 monocytes then become hyporesponsive to higher doses of secondary challenges of many known TLR ligands.

Biological Significance of LPS-induced Cross-tolerance Contributed by miR-146a

In this article, the ability of LPS-primed THP-1 monocytes to induce cross-tolerance to other TLR agonists is congruent with previous *in vitro* and *in vivo* findings (226, 289). Monocytes or macrophages pretreated with LPS showed hyporesponsiveness to TLR2 ligands (LTA, PGN, Pam_3CSK_4, LPS from *P. gingivalis*) (204, 291, 292) or flagellin, as reported in this study and by others (287). Similarly, in this study, Pam_3CSK_4, PGN, and flagellin demonstrated both self-tolerance and cross-tolerance to LPS, which is consistent with previous findings (15, 204, 293). Jacinto et al. (204) observed that LPS priming caused disruption of IRAK kinases and development of cross-tolerance to LTA. However, LTA did not show cross-tolerance to LPS at a

significant level; the present studies suggest this is true in part because LTA does not induce high levels of miR-146a. Consistent with these in vitro findings, Lehner et al. (293) and Dalpke et al. (15) observed that LPS-primed mice were hyporesponsive to LTA and CpG DNA. Although LPS cross-tolerized to flagellin (Fig. 3-3E), flagellin did not prevent a strong response to LPS (Fig. 3-5C). This finding raises the possibility that flagellin and LPS tolerance may be mediated by different, distinct mechanisms. Regarding this idea, Mizel and Snipes (47) observed that flagellin tolerance was not due to decreased surface expression of TLR5 or IRAK1 degradation. Interestingly, flagellin-primed cells showed an increase of TLR4 expression (Fig. 3-5E), a finding corroborated by van Aubel et al. (294). Although it is not still clear why flagellin does not induce cross-tolerance against LPS, the latter finding provides additional evidence that there is a difference between flagellin and LPS tolerance.

Note that cross-tolerization also occurs between LPS and cytokines such as TNF-α and IL-1. Repeated injections or infusions of TNF-α in rats were shown to protect against an LD50 injection of LPS (295), suppressing LPS-induced fever (296) and cytokine production by bone marrow-derived adherent cells (297). TNF-α and LPS do not induce reciprocal tolerance in murine macrophages (71). However, THP-1 cells pretreated with TNF-α showed a partial repression of LPS-induced cell signaling, as reported by Ferlito et al. (288). This may be linked to the miR-146a expression observed in this study. The difference between murine macrophages and human cells, including THP-1 monocytes, to various ligands has been shown by Bowie et al. (235). Following this observation, Bowie concluded that TLR expression patterns are not entirely equivalent in humans and mice. Previously, however, De Nardo et al. (83) observed that LPS-primed primary mouse macrophages showed tolerance due to IRAK1 protein degradation. Accordingly, in this study, LPS- or PGN-primed mouse peritoneal macrophages showed

homologous or heterologous tolerance where miR-146a showed elevated expression in LPS-tolerized cells that can regulate the adaptor kinases. IL-1β was shown to be involved in the induction of homologous tolerance and in limiting the response of THP-1 cells to LPS, as shown by others both *in vivo* and *in vitro* (298). This new finding regarding cross-tolerance (except flagellin to LPS) is linked to miR-146a overexpression. As expected, IRAK1 or TRAF6 adaptor kinase expression was inhibited by PGN, and Pam_3CSK_4-tolerized cells provide additional support for miR-146a involvement in cross-tolerance. Interestingly, our study (data not shown) and Perry et al. (121) both found continuous miR-146a expression in IL-1β treated A549 cells, which showed negative effect on IRAK1 and TRAF6 at the posttranscriptional level. In their study, miR-146a alone has been shown to negatively regulate the release of proinflammatory chemokines, including IL-8 and RANTES in IL-1β-treated A549 cells.

IRAK1 and TRAF6, known as the proximal protein kinases in TLR signaling pathways (299), are the molecular targets for miR-146a. TLR signaling is impaired due to the degradation or translation inhibition of these adaptor kinases during innate immune activation, giving rise to endotoxin tolerance (71, 82, 204, 290, 300). Similarly, silencing of adaptor kinases rendered THP-1 monocytes hyporesponsive to a set of innate immune ligands and resulted in lower cytokine production, namely TNF-α, IL-1β, or IL-6. However, silencing of either IRAK1 or TRAF6 did not cause complete suppression of cytokines in response to the TLR ligands used in this study (Fig. 3-6). A similar pattern of LPS response was observed after knockdown either of the adaptor kinases in monocytes or macrophages, as reported by others (83, 117). These findings imply that IRAK1 and TRAF6 translational inhibition by miR-146a in LPS-primed cells contributes to homologous and heterologous tolerance at a significant level, but not in a complete manner. Such a conclusion is consistent with the failure of complete resistance to LPS-induced

death in IRAK-deficient mice (83). Additionally, a deficiency of IRAK1 impairs, but does not completely eliminate, IL-1 actions as reported by Thomas et al. (301). This suggests that other members of the IRAK family may compensate in part for the functions of IRAK1.

Notably, during LPS stimulation of THP-1 cells, a few miRNAs, including miR-146a, are upregulated. The biological significance of such miRNAs as miR-132 (116), miR-9 (251), and miR-155 (116) to LPS-induced cross-tolerance is not clear. They may participate directly or indirectly to LPS-induced cross-tolerance. The activation of NF-κB by TLRs is critical to inflammation. Among the LPS-induced miRNAs, only miR-146a has been shown to be NF-κB-dependent, and inhibition of NF-κB by PDTC, an NF-κB inhibitor, decreased miR-146a expression (116, 122). Consequently, in this report, miR-146a overexpression by miR-146a mimic transfection caused THP-1 monocytes to be less responsive to various ligands, including LPS. Krützfeldt et al. (266, 267) introduced the usage of chemically modified miRNA inhibitors, known as antagomirs, to define the biological functions of miRNAs. In this study, miR-146a knockdown in THP-1 cells showed more inflammatory response to non-LPS ligands. Interestingly, PGN-primed THP-1 monocytes recovered responsiveness correlating with the reduced levels of miR-146a in the PGN withdrawal experiment (Fig. 3-3 L-M) demonstrated its role to maintain immune homeostasis.

In addition to purified ligands, LPS-induced cross-tolerance against whole bacteria was also shown *in vitro* (Fig. 3-8). Accordingly, Cavaillon et al. (201) first reported that LPS-tolerized human monocytes were hyporeactive in TNF-α production, to heat-killed *Streptococcus pyogenes*, *S. aureus*, and zymosan (a TLR2 ligand). Reduced *ex vivo* responsiveness to whole heat-killed Gram-positive bacteria, such as *S. pyogenes* and *S. aureus* (201), has been also reported in severe sepsis. Similarly, in this report, LPS-primed monocytes showed significant

reduction of TNF-α after challenging with *E. coli* and *P. gingivalis*. Interestingly, miR-146a expression showed a negative correlation with TNF-α production, indicating its role in hyporesponsiveness to whole bacteria. This finding has also been supported by transfection of miR-146a mimic alone. Thus, this finding fully supports the dominant role of miR-146a in LPS-induced cross-tolerance compared with other negative regulators previously suggested.

Implication of Differential Cross-regulation in Innate Immunity

Various PAMPs from both Gram-positive and Gram-negative bacteria have been shown to interact with particular TLRs. Thus, while sepsis is more commonly caused by Gram-positive bacteria, it can also be caused by Gram-negative bacteria. Moreover, LPS from Gram-negatives is one of the best studied immunostimulatory components of bacteria and can induce systemic inflammation and sepsis if excessive signals occur without proper regulation. In our previous report, miR-146a was upregulated in LPS-stimulated THP-1 cells, showing it to be a regulator of proinflammatory cytokine production by establishing tolerance against LPS (82). Interestingly, both we and Taganov et al. (116) observed elevated miR-146a expression in response to TLR2 or to TLR5 stimulation by bacterial and fungal components or following exposure to TNF-α or IL-1β. Following this, the ligands that showed miR-146a expression were primarily considered in our LPS-induced cross-tolerance study. This upregulated miR-146a caused by LPS provided cross-tolerance in THP-1 cells. Analogous to LPS, both TLR2 and TLR5 ligands showed cross-tolerance to unrelated ligands provided by miR-146a expression. miR-146a induction, however, was not observed in response to a nucleic acid analog, such as polyinosinic-polycytidylic acid (116), although in a recent study Hou et al. (122) observed overexpression of miR-146a in response to vesicular stomatitis virus (VSV) infection in a mouse infection model. In that study, miR-146a was shown to regulate type 1 IFN by inhibiting retinoic acid-inducible gene I signaling molecules IRAK1, IRAK2, and TRAF6 (122). Interestingly, a report by Tang et al.

(263) showed the downregulation of miR-146a in a subset of human lupus patients with elevated expression of type 1 IFN. In THP-1 monocytes, NOD2 is highly expressed and is a receptor for PGN or its breakdown product MDP. In our study, MDP alone was unable to stimulate proinflammatory cytokine production in THP-1 cells, but, in combination with LPS, produced a synergistic effect, as shown by others (302). After 18 h MDP treatment, THP-1 cells did not show cross-tolerance to TLR2 or TLR4 ligands (data not shown), although after prolonged treatment of primary monocyte-derived macrophages with MDP, Hedl et al. (272) observed cross-tolerance against Pam_3CSK_4 and lipid A (TLR4 ligand). This discrepancy might be due to cell type differences or treatment period or both. In another study, Gutierrez et al. (303) observed that NOD2-induced NF-κB activation involving IRAK1 is likely to activate cytokine production. Thus, miR-146a is likely to downregulate NOD2 activity by affecting IRAK1 and contributes to maintaining a state of hyporesponsiveness toward commensal microflora. In any case, TNF-α can be produced through activation of all TLRs, except TLR3, and all of these pathways involve miR-146a target molecules TRAF6 and IRAK1 adaptor kinases (271). Recently, de Vos et al. (226) observed LPS-induced cross-tolerance against TLR ligands, including TLR3 and TLR7, in human volunteers. Similarly, LPS-induced cross-tolerance was observed in THP-1 cells against TLR3 ligands, such as polyinosinic-polycytidylic acid, in terms of TNF-α production (data not shown). Whether innate immunity involving miRNAs, including miR-146a, directly or indirectly regulates TLR3 and TLR7 signaling remains to be determined. Therefore, endotoxin-induced cross-tolerance associated with upregulated miR-146a may have a broader role in regulating TNF-α, a key player in the cytokine network, induced by a number of pattern recognition receptors in innate immunity. However, *in vivo* investigations, such as the phenotypic analysis of mice with targeted deletion of miR-146a, will be necessary to fully explore the role of this

miRNA in innate immunity. It is interesting to speculate whether all of these TLRs involving TRAF6 and IRAK1 were regulated by miR-146a in a comparable manner. During LPS induction, some of the miRNAs have been reported to be downregulated, including let-7i, which is thought to target TLR4 itself (127), and miR-125b, which is known to target TNF-α (141). Sometimes miRNAs are dysregulated in cancer (304) or aberrantly expressed in such inflammatory diseases as rheumatoid arthritis (273). Thus, miRNAs may form a key link between inflammation and cancer; however, the induction of specific miRNAs by TLRs, including miR-146a, as a key step in tumor progression is still unclear.

In summary, multiple lines of evidence reported in this study substantiate the indispensable role of miR-146a in endotoxin-induced cross-tolerance. miR-146a expression involved in LPS-induced cross-tolerance operates as a negative regulatory feedback (or tuning) mechanism to prevent the destructive consequences of uncontrolled inflammatory reaction caused by overactivation of TLR signaling. Modulation of miR-146a level can have an opposite effect, notably on the levels of adaptor kinases, leading to the attenuation of TLRs signaling. This finding highlights the importance of further investigations on whether miR-146a can be used as a therapeutic intervention for boosting or limiting TLR activation to maintain a controlled immune response.

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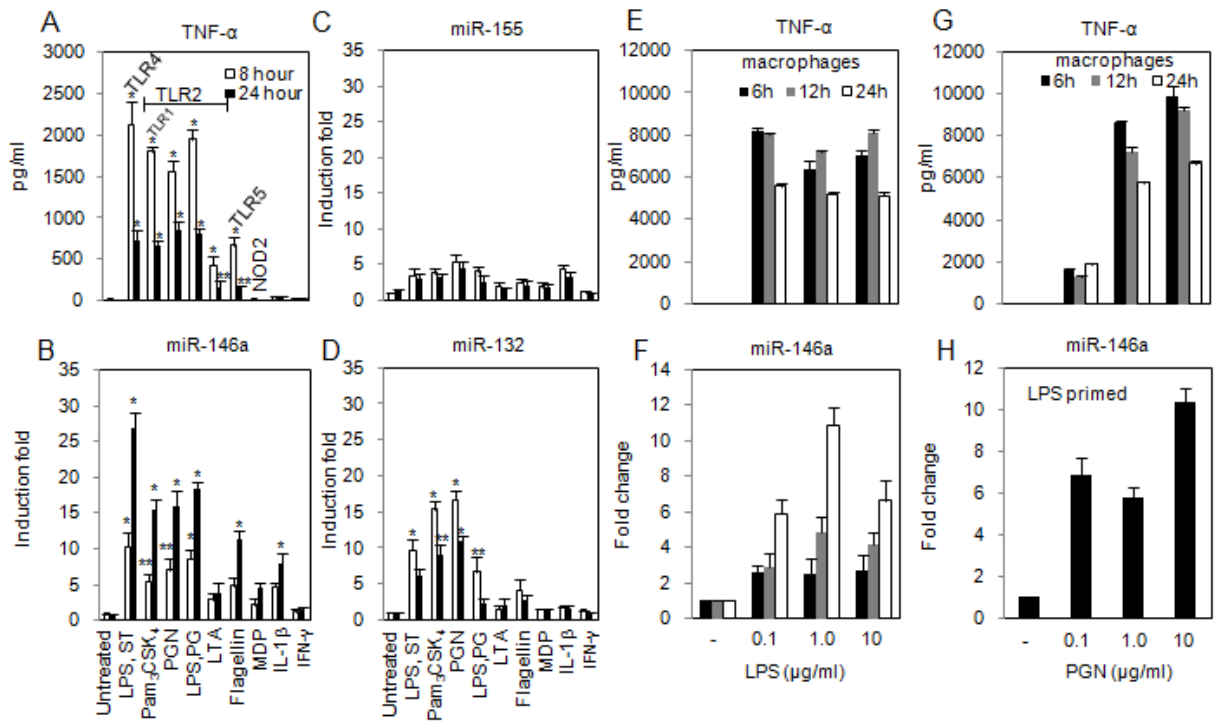

Figure 3-1. TNF-α protein secretion and miRNA expression kinetics in response to a panel of innate immune ligands in THP-1 monocytes. THP-1 cells were incubated with various ligands for 8 or 24 h, as indicated at the foot of the graphs. TNF-α in culture supernatants was measured by ELISA (A). Total RNAs were purified from the respective cell pellets and the expressions of miR-146a (B), miR-155 (C), miR-132 (D), and miR-16 (E) were analyzed by qRT-PCR. miRNA expressions were normalized with RNU44. All results are expressed as mean ± SD from three independent experiments. *= $p < 0.05$, **= $p < 0.01$ compared with untreated cells.

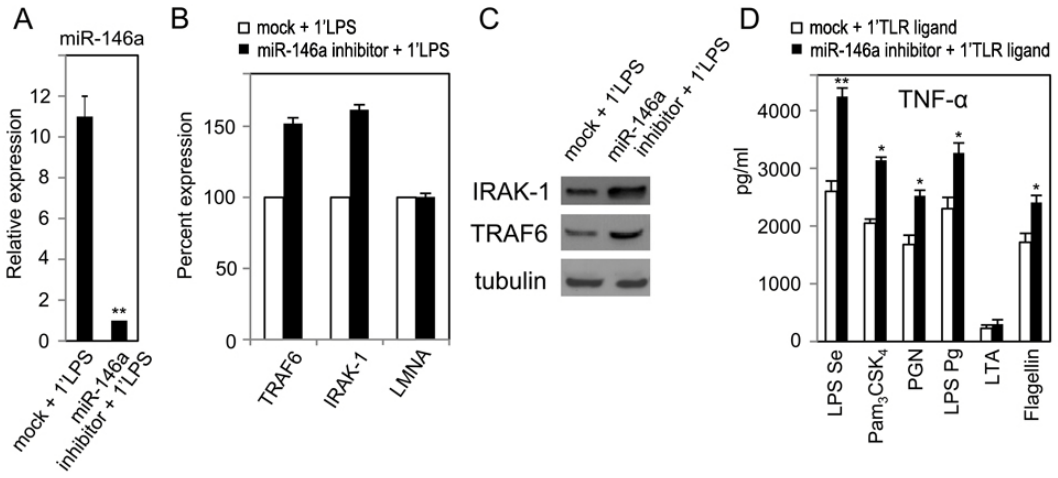

Figure 3-2. Inhibition of miR-146a expression increases TNF-α response to TLR ligands. THP-1 cells transfected with 40 nM of miR-146a inhibitor for 24 h, along with mock-transfected controls, were washed with complete growth medium. Cells were then challenged with the various ligands for 5 h. A. Total RNA from cell pellets of mock- and miR-146a inhibitor-transfected cells were analyzed by qRT-PCR for miR-146a expression. B. Total RNAs were analyzed for TRAF6, IRAK1, and unrelated lamin A/C (LMNA, negative control) mRNA expression in both mock- and miR-146a inhibitor-transfected cells by qRT-PCR. C. Western blot analysis for IRAK1, TRAF6, and tubulin in the cell lysates collected 24 h after with or without (mock) miR-146a inhibitor transfection. D. TNF-α protein in supernatants of mock-transfected and miR-146a inhibitor-transfected cells and challenged with the various ligands shown at the foot of the graph were measured using ELISA. Data are expressed as mean ± SD of three independent experiments. *= $p < 0.05$, **= $p < 0.01$ compared with mock-transfected cells.

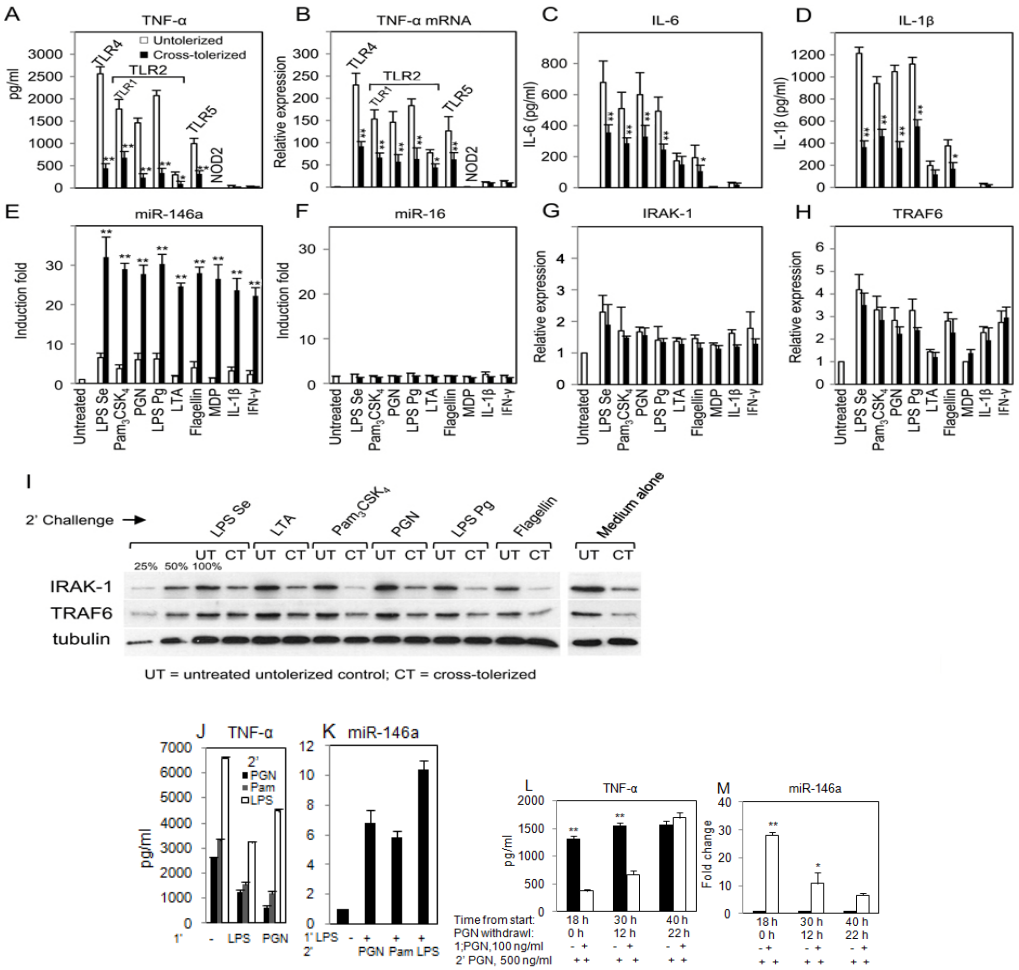

Figure 3-3. High levels of LPS-induced miR-146a may account for cross-tolerance to non-LPS agonists in the THP-1 cell model. THP-1 cells primed with 10 ng/ml LPS for 18 h (cross-tolerized, filled bars) and untreated controls incubated for the same time period (untolerized, open bars) were washed twice with PBS, then challenged with various ligands or medium alone for 5 h. Culture supernatants and total RNA were analyzed for cytokines using ELISA (A, C, D), or miRNA (E, F, J) and mRNA expression (B, G, H) by qRT-PCR, as described in Materials and Methods. Changes in IRAK1 and TRAF6 protein levels in LPS-primed and unprimed THP-1 cells challenged with various TLR ligands or medium alone for 2 h were analyzed by Western blot with tubulin expression shown as loading controls (I). Serial dilutions of untreated THP-1 cell lysates (100, 50, 25%) were included in the Western blot to document the semiquantitative measurement for IRAK1 and TRAF6 expression. THP-1 monocytes were cultured with or without 100 ng/ml PGN continuously for 18 h followed by washing and cultured in complete medium for another 0, 12, or 22 h (PGN withdrawal). At each time point, 6 × 105 cells were challenged with 500 ng/ml PGN for 3 h prior to analysis of TNF-α production by ELISA (J) and miR-146a expression by qRT-PCR analysis (K). All results are expressed as mean ± SD from three independent experiments. *$p < 0.05$; **$p < 0.01$ compared with untolerized THP-1 cells.

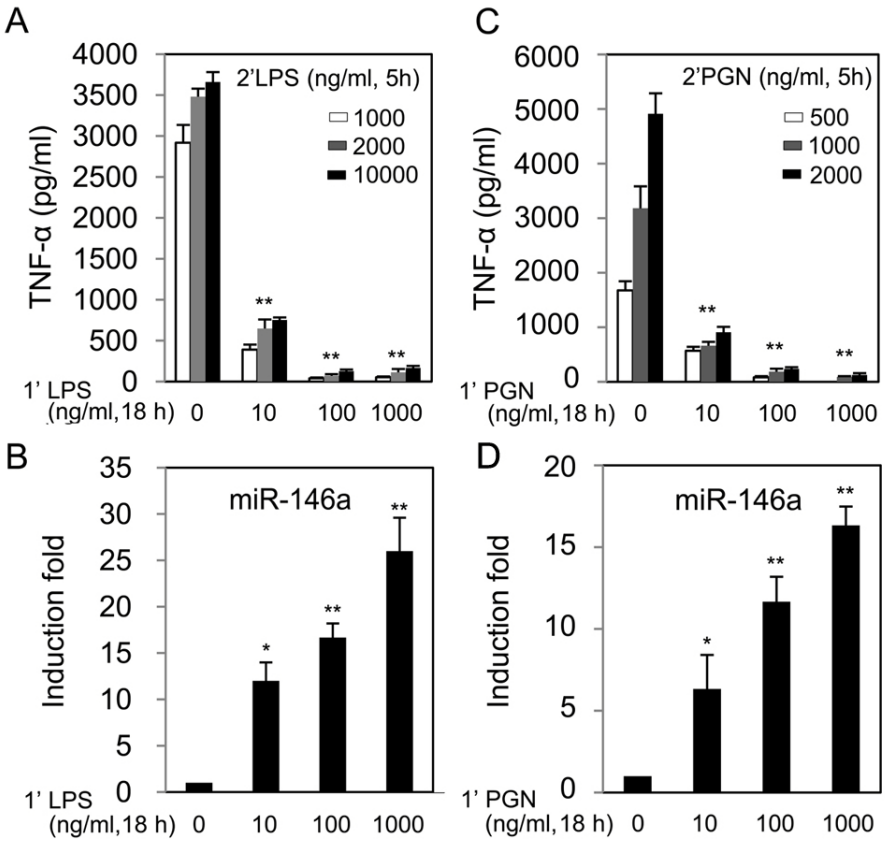

Figure 3-4. LPS- and PGN-tolerized THP-1 cells show efficient tolerance to high doses of respective ligands with dependency on priming dose and are inversely correlated to induced levels of miR-146a. THP-1 cells primed with 0, 10, 100, or 1000 ng/ml LPS Se (1'LPS) or PGN (1'PGN) continuously for 18 h were washed twice with PBS, then challenged with high doses of LPS Se up to 10,000 ng/ml (2'LPS) or PGN up to 2,000 ng/ml (2'PGN). Supernatants and cell pellets were collected 5 h after start of challenge to measure TNF-α protein by ELISA (A, C) and miR-146a expression analysis by qRT-PCR (B, D). Data points and error bars represent mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$ compared with unprimed THP-1 cells.

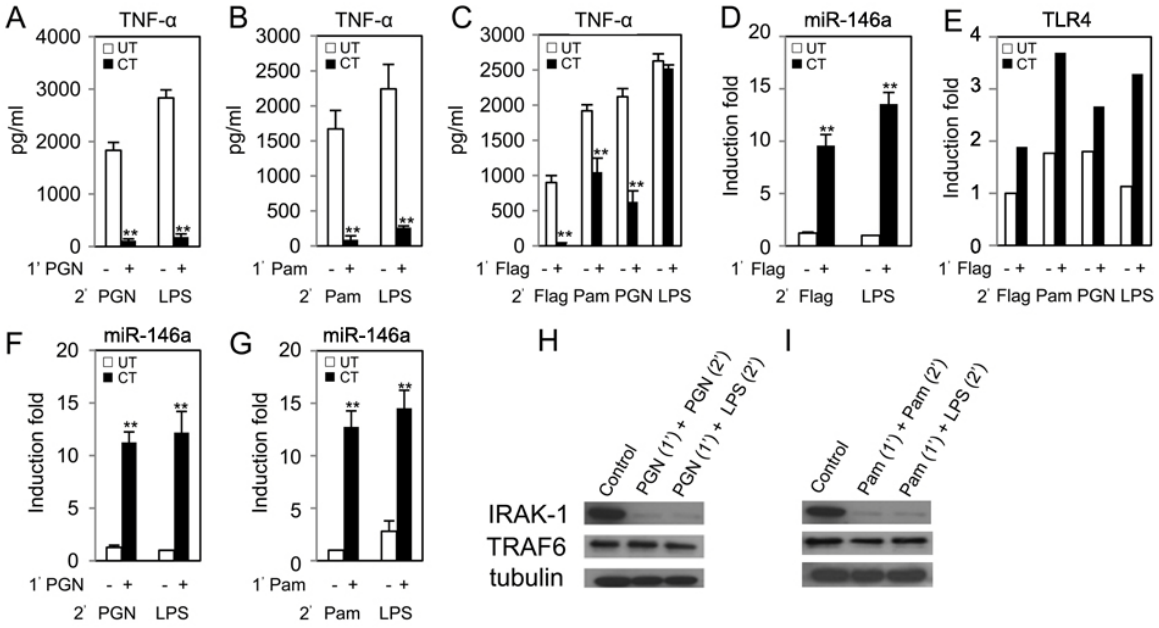

Figure 3-5. Peptidoglycan (PGN)-, Pam3CSK4 (Pam)-, and flagellin (Flag)-induced miR-146a contributes to cross-tolerance in THP-1 cells. THP-1 cells were primed with 100 ng/ml PGN, Pam3CSK4, or flagellin for 18 h, washed twice with PBS, then challenged with LPS Se (100 ng/ml), PGN (500 ng/ml), Pam3CSK4 (100 ng/ml), or flagellin (100 ng/ml). Supernatants and cell pellets were collected 5 h later for analysis of TNF-α protein by ELISA (A–C), miR-146a (D, F, G), and TLR4 mRNA expression in total RNA by qRT-PCR (E). Data points and error bars represent mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$ compared with untolerized THP-1 cells. PGN- and Pam3CSK4-primed THP-1 cells were challenged with various homologous or LPS for 2 h, and then cell lysates were analyzed for IRAK1, TRAF6, and tubulin expression compared with control by Western blot (H, I).

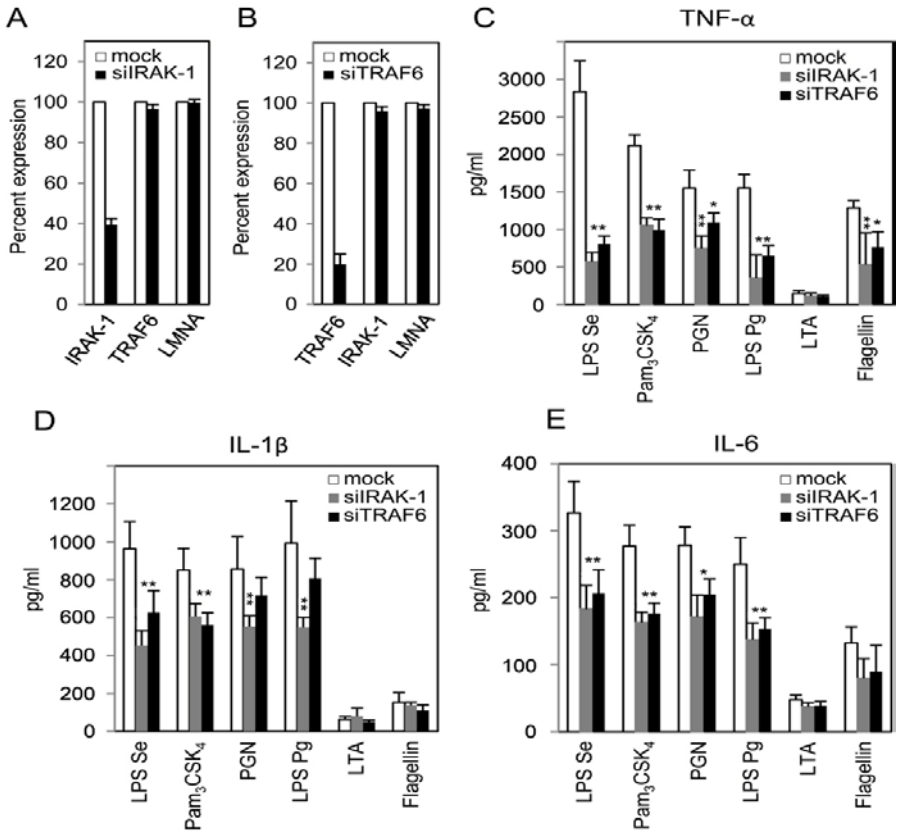

Figure 3-6. IRAK1 and TRAF6 knockdown results in decreased inflammatory response to microbial ligands. siRNA targeting IRAK1 (A) or TRAF6 (B) were transfected into THP-1 cells, and knockdown efficiency was determined using qRT-PCR; lamin A/C (LMNA) was used as an unrelated mRNA control. Twenty-four hours after transfection, mock-transfected, IRAK1-deficient, or TRAF6-deficient THP-1 cells were stimulated for 24 h with the indicated ligands shown at the foot of the graph (C–E). Cytokines released in the culture supernatants were determined by ELISA. Data are representative of three independent experiments and expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$ compared with mock-transfected cell.

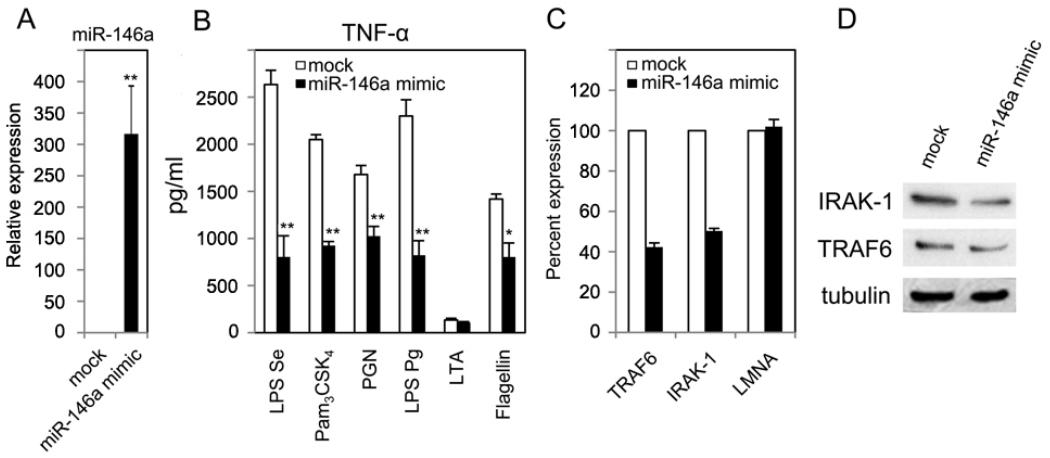

Figure 3-7. Transfected miR-146a alone mimics LPS-induced cross-tolerance to various innate immune TLR ligands. THP-1 cells were transfected with 20 nM miR-146a mimic. They and mock-transfected cells were incubated for 24 h, washed with complete growth medium, and then challenged with indicated ligands for another 3 h. A, RNA isolates were prepared from cell pellets of mock and transfected cells followed by qRT-PCR for miR-146a expression normalized to RNU44. B, TNF-α protein measured using ELISA in supernatants from mock- and miR-146a-mimic-transfected cells. C, The RNA samples were also analyzed by qRT-PCR for mRNA expression of miR-146a targets TRAF6, IRAK1, and unrelated lamin A/C (LMNA, negative control). Data are representative of three independent experiments and expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$ compared with mock-transfected cells. Western blot analysis for IRAK1, TRAF6, and tubulin in the cell lysates collected 24 h after with miR-146a-mimic transfection (D) or without (mock).

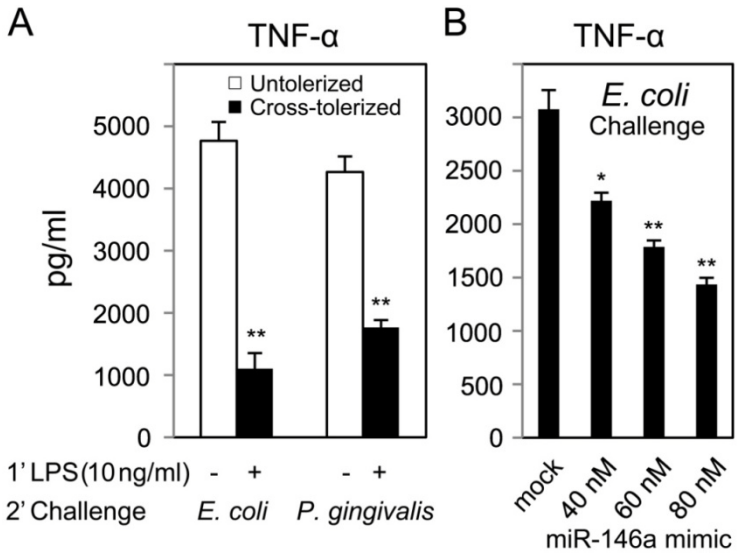

Figure 3-8. Elevated levels of LPS-induced miR-146a contribute to resistance to heat-killed bacteria in the THP-1 cell model. A, THP-1 cells primed with 10 ng/ml LPS Se for 18 h (cross-tolerized, filled bars) and untreated controls incubated for the same time period (untolerized, open bars) were washed twice with PBS, then challenged with heat-killed E. coli or P. gingivalis at 1 x 10^6 CFU/ml for 3 h. B, THP-1 cells transfected with miR-146a mimic (40, 60, or 80 nM), followed by incubation for 24 h, and mock-transfected cells were washed with complete growth medium and challenged with heat-killed E. coli (1 x 106 CFU/ml). TNF-α protein in supernatants from mock-transfected and miR-146a mimic-transfected cells was measured using ELISA. Data are representative of three independent experiments and expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$ compared with untolerized or mock-transfected cells.

CHAPTER 4
POLYMICROBIAL INFECTION WITH PERIODONTAL PATHOGENS SPECIFICALLY ENHANCES MICRORNA MIR-146A IN APOE–/– MICE DURING EXPERIMENTAL PERIODONTAL DISEASE

Background

Periodontitis is an oral disease driven by bacterial pathogens, with an associated aggressive immune and inflammatory activation affecting the connective tissue surrounding the teeth, leading to tooth loss, and is a globally distributed chronic disease in humans. Periodontal diseases affect up to 20% of the population worldwide, and no single bacterial species has been identified as an initiating factor in either the destruction of the periodontium or the associated destructive immune and inflammatory responses. However, periodontal diseases are polymicrobial in nature, with more than 450 species colonizing diseased subgingival cavities (305, 306). Socransky and colleagues (307) have reported seminal data on the complexity of the oral subgingival plaque and suggested five major bacterial complexes and several microbial species. One complex, consisting of *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* as specific bacterial components, was more frequently identified, and these species were found in higher numbers in cases of adult periodontitis. This bacterial complex was also positively correlated with gingival pocket depth and bleeding upon probing, which are measures of periodontal tissue destruction (307-309). Several previously reported studies suggested that periodontal-disease-associated bacteria can penetrate gingival tissues, enter the bloodstream, and potentially induce transient bacteremia (310, 311). In addition, periodontal lesions are recognized as being continually renewing reservoirs for the systemic spread of bacterial antigens, bacteria, cytokines, and proinflammatory mediators. Several previously reported observational studies investigated the associations between periodontal disease and cardiovascular disease in human populations (312-314). Recently, many studies demonstrated the presence of genomic DNAs from several

periodontal bacteria, including *P. gingivalis*, *T. forsythia*, and *T. denticola*, in atherosclerotic lesions (197-199). Among periodontal pathogens, *P. gingivalis* genomic DNA is the most frequently detected, followed by *T. denticola* and *T. forsythia*.

Furthermore, these pathogens elaborate an array of potential virulence determinants such as potent extracellular proteinases and other enzymes, including *P. gingivalis* RgpA and RgpB and Kgp gingipain cysteine proteinases; *T. denticola* chymotrypsin-like protease, phospholipase C, oligopeptidase, and endopeptidase; and *T. forsythia* cysteine protease, BspA, and sialidase, which contribute to their colonization in diseased sites and induce periodontal soft and hard tissue destruction consistent with periodontitis (315-317). We have recently demonstrated that these three pathogens with and without *Fusobacterium nucleatum* not only exist as a pathogenic complex that is associated with chronic periodontitis in humans but also induce synergistic virulence, which is characteristic of polymicrobial periodontitis (318).

microRNAs (miRNAs) were first described for *Caenorhabditis elegans* (100, 319). Subsequent work demonstrated that these molecules are noncoding double-stranded RNAs consisting of RNase III-processed ~23-nucleotide sequences that mediate gene silencing via the RNA interference mechanism (102). miRNAs have since been identified in vertebrates and have been suggested to regulate a significant fraction of cellular mRNAs. Recently, miRNAs have been shown to regulate a diverse set of biological functions, including diverse actions such as the regulation of developmental timing, differentiation of cells, and development of cancers (102). Recent studies suggested that bacterial, parasite, and viral infections of mammalian cells and plant cells can modulate miRNA expression (320, 321). Importantly, miRNA miR-146a was demonstrated to play an important role in innate immunity by regulating cytokine production in response to microbial ligands by various mechanisms, such as endotoxin tolerance (82) as well as

cross-tolerance (81). Cremer et al. (195) previously demonstrated that infection with the less virulent organism *Francisella novicida* in mice strongly induces miR-155 expression in lung, liver, and spleen in a Toll-like receptor (TLR)-dependent manner, while the virulent organism *Francisella tularensis* does not. This indicates that miR-155 is a component of the host defense against *Francisella* species, and the differences in the miR-155 responses to these two subspecies may help explain the success of *F. tularensis* as an infectious agent in humans (195). Another study investigated the differential regulation of miR-155 by the bacterium *Helicobacter pylori*, which induces ulcers and cancers, mainly in T cells in a cyclic AMP (cAMP)-forkhead box P3 (Foxp3)-dependent manner. These are underlying bacterial and host mechanisms associated with their regulation during the course of an infection *in vitro* (196). Most recent studies demonstrated that tooth development is tightly controlled by discrete sets of miRNAs and that specific miRNAs regulate tooth epithelial stem cell differentiation (322). Despite several reports of the role of miRNAs in the regulation of innate and adaptive immune responses, nothing is known about the miRNA-mediated regulation of oral infection with periodontal pathogens during experimental periodontal disease.

The polymicrobial periodontal disease model has been developed with *P. gingivalis, T. denticola,* and *T. forsythia* to provide a model for clinical disease outcomes in the context of specific microbial complex colonization, synergism for colonization, and progression to periodontal disease (318) and also to examine the potential of *P. gingivalis* for bacterial dissemination, the upregulation of the inflammatory response, and/or the exacerbation of atherosclerosis (313, 323). Recent studies have summarized the role of miRNAs in arterial remodeling and atherosclerosis and putative roles of miRNAs in vascular inflammation (324) through the regulation of the differentiation and functions of immune cell subsets (325). This

report examines miRNA (miR-146a, miR-132, and miR-155) expression induced by infection with the polymicrobial periodontal pathogens *P. gingivalis, T. denticola,* and *T. forsythia* in the mouse periodontium and spleen. Our findings suggest that miR-146a is upregulated in localized as well as systemic manners during periodontal pathogen infections, where miR-146a may contribute to the control of the resulting periodontal inflammation.

Material and Methods

Bacterial Strains and Microbial Inocula

P. gingivalis FDC 381, *T. denticola* ATCC 35404, and *T. forsythia* ATCC 43037 were used in this study and were cultured anaerobically at 37°C as well as maintained for mouse infection as described previously (318). The concentration of each bacterium was determined quantitatively, and the organism was resuspended in reduced transport fluid at 1 x 10^{10} bacteria per ml (318). For oral polymicrobial infection, *P. gingivalis* was gently mixed with an equal volume of *T. denticola* and allowed to interact for 5 min; subsequently, *T. forsythia* was added to the tubes containing *P. gingivalis* and *T. denticola,* and the bacteria were mixed gently for 1 min and allowed to interact for an additional 5 min. Bacteria were then mixed with an equal volume of sterile 4% (wt/vol) low-viscosity carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) and were used for oral infection (5 x 10^9 cells per ml) in 2% CMC as described previously (318).

ApoE$^{-/-}$ Mouse Infection and Sampling

ApoE$^{-/-}$ mice are used as the model for atherosclerosis, and recently, many studies used ApoE$^{-/-}$ mice to investigate the role of *P. gingivalis* in the induction of atherosclerosis (323, 326). Eight-week-old male ApoE$^{-/-}$ B6,129P2-*Apoe*^{tm1Unc}/J mice (Jackson Laboratories, Bar Harbor, ME) were maintained in groups and housed in microisolator cages. Mice were fed standard chow and water *ad libitum* and were kept at 25°C with alternating 12-h periods of light and dark. All

mouse procedures were performed in accordance with the approved protocol (protocol number F173) guidelines set forth by the Institutional Animal Care and Use Committee of the University of Florida. In addition, adequate measures were taken to minimize pain or discomfort to mice during oral infection and sampling. Mice were administered sulfamethoxazole (0.87 mg/ml) and trimethoprim (0.17 mg/ml) daily for 10 days in the drinking water (323), and the oral cavity was swabbed with 0.12% chlorhexidine gluconate (Peridex 3 M; ESPE Dental Products, St. Paul, MN) mouth rinse (318, 327) to inhibit the endogenous organisms and to promote subsequent colonization with *P. gingivalis*, *T. denticola*, and *T. forsythia* (318). After a 3-day antibiotic washout period, mice were randomized into two groups, followed by oral infection. The polymicrobial inocula (5 x 10^9 cells per ml; 1 x 10^9 cells in 0.2 ml per mouse; 3.3 x 10^8 *P. gingivalis*, 3.3 x 10^8 *T. denticola*, and 3.3 x 10^8 *T. forsythia* bacteria) were administered orally to mice (n = 15) for 4 consecutive days per week on 8 alternate weeks for a total of 32 inoculations during 16 weeks of the experimental polymicrobial infection period. Sham-infected control mice (n = 10) received vehicle (sterile 2% CMC) only. Oral microbial samples from isoflurane-anesthetized mice were collected at postinfection time points as described previously (318). Following 16 weeks of bacterial infection, ApoE$^{-/-}$ mice were killed, blood was collected, and spleens and right maxillas (periodontium) with three molars were used for this miRNA expression study.

Cell Culture and Bacterial Stimulation

Human THP-1 cells, an undifferentiated promonocytic cell line, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were maintained by twice-weekly passage in RPMI 1640 medium containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD), 10% (v/v) fetal bovine serum (Mediatech, Manassas, VA), and 100 units/ml penicillin-streptomycin (Mediatech) at 37°C with 5% CO2. Log-phase cells

were used for all experiments and were cultured at a density of 5 x 10^5 cells per ml. In all experiments, trypan blue exclusion was performed to demonstrate cell viability where cells that were >95% viable were considered for infection studies. For examining bacterium-induced miRNA and cytokine production, *P. gingivalis*, *T. denticola*, and *T. forsythia* were grown until mid-log phase and then harvested and placed into sterile saline, followed by killing at 65°C for 30 min as described previously (281). Heat-killed (HK) bacteria were then washed three times with sterile saline and used at an MOI (multiplicity of infection) of 10. THP-1 monocytes were suspended in complete RPMI 1640 culture medium and seeded at 5 x 10*5* cells per ml in a 24-well plate. Cells were then stimulated with HK and infected with live *P. gingivalis*, *T. denticola*, and *T. forsythia* (as mono- and polymicrobial infections) at an MOI of 10. THP-1 cells were harvested at various time points over 72 h, and culture supernatants were collected and stored at –80°C until assayed for cytokines levels. The cell pellet was washed in sterile PBS and stored in RNAlater (Ambion, Austin, TX) at 4°C or frozen at –80°C for total RNA isolation for subsequent analyses.

Quantification of miRNA and mRNA Expression Levels by qRT-PCR

An aliquot of spleen (~50 mg from each mouse) and entire maxilla was homogenized by using 1.0-mm silicon carbide beads (BioSpec Products Inc., Bartlesville, OK). Total RNA from the homogenized samples was prepared by using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. Similarly, total RNA from bacterium-treated and untreated THP-1 cells was prepared by using the *mir*Vana miRNA isolation kit. The RNA yield and purity were determined by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology Inc., Wilmington, DE), and equal amounts of each RNA (A_{260}/A_{280} = ~2.0; 6.7 ng for miRNA) were used for quantitative stem-loop reverse transcription and quantitative real-time PCR (qRT-PCR) analysis. Quantification of mature miRNA expression

was performed by using the TaqMan microRNA reverse transcription kit, TaqMan Universal PCR master mix, and TaqMan microRNA assay primers of interest for human miRNAs (Applied Biosystems, Foster City, CA). For mRNA analysis, a high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA) and TaqMan mRNA assay primers (Applied Biosystems, Foster City, CA) for tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-6, IRAK1 (IL-1 receptor-associated kinase 1), and TRAF6 (TNF receptor-associated factor 6) were used with 33 ng of total RNA per reaction. The cycle threshold (C_t) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined, and miRNA expression values were calculated using abundant RNU44 (human monocytes) or snoRNU202 (mouse) as an endogenous control (Applied Biosystems) according to the $2^{-\Delta\Delta CT}$ method (259). mRNA for gene expression values was quantified in the same way after normalization to mammalian 18S rRNA. The fold changes in miRNA expression were calculated by comparing the value of treated THP-1 monocytes to that of untreated samples cultured in parallel or by comparing the values of infected versus control tissues.

Cytokine Assays

Following HK and/or live bacterium induction of THP-1 monocytes, the kinetics of cytokine secretion were examined. Log-phase THP-1 monocytes were stimulated or infected with *P. gingivalis*, *T. denticola*, and *T. forsythia* either as a mono- or polymicrobial infection at an MOI of 10 and harvested at different time points from 4 to 72 h postinfection to assess cytokine expression. Secreted TNF-α, interleukin-8 (IL-8), or IL-1β proteins in the supernatants were measured by an enzyme-linked immunosorbent assay (ELISA) using an Opt EIA kit as recommended by the manufacturer (BD Biosciences, San Diego, CA). The absorbance was measured at 405 nm using a microplate reader (model 680; Bio-Rad). The optical density at 405

nm (OD_{405}) was converted into concentrations using standard curves of the respective recombinant cytokines.

Western Blot Analysis

THP-1 cells (5 x10^5 cells per condition) were treated with or without HK bacteria (MOI of 10), incubated for 2 h and 4 h in a CO_2 incubator at 37°C followed by centrifugation (1,000 x *g* for 10 min), and lysed on ice for 10 min in 0.2 ml of lysis buffer (50 mM HEPES [pH 7.6], 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 5 mM *para*-nitrophenyl phosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture [Roche Diagnostic, Indianapolis, IN]) (82). Supernatants were collected after centrifugation at 12,000 x *g* for 20 min at 4°C. Soluble lysates were quantitated for protein concentrations by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), separated by SDS-PAGE (10% acrylamide, w/v) along with Precision Plus protein standards (Bio-Rad), and electrotransferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were then blocked for 1 h at room temperature or overnight at 4°C with 5% (w/v) nonfat milk in PBS-0.05% Tween 20 (PBS-T) and were probed with primary rabbit anti-IRAK1 or anti-TRAF6 antibody at a concentration of 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed three times with PBS-T and incubated for 1 h with goat anti-rabbit IgG-horseradish peroxidase at a concentration of 1:5,000 (Southern Biotechnology, Birmingham, AL). After washing with PBS-T, reactive protein bands were visualized with Super Signal Pico chemiluminescent reagent (Pierce, Rockford, IL). As a loading control, tubulin was blotted with mouse anti-tubulin (1:5,000; Sigma-Aldrich, St. Louis, MO).

Statistical Analysis

Data are presented in figures as means ± standard deviations (SD). For multiple-group comparisons, a one-way analysis of variance ($P < 0.05$) was performed, followed by the two-sided, unpaired Student *t* test as described previously by Shaffer (260). The unpaired, two-tailed Student *t* test was used to compare two independent groups. For all statistical analyses, Prism for Windows, version 5.0 (GraphPad Software, San Diego, CA), was used, and a *P* value of <0.05 was considered statistically significant.

Results

miR-146a Expression in Maxilla and Spleen

Previously, miRNA expression kinetics were observed *in vitro* in a cell-based model in response to Gram-positive and Gram-negative bacterial components (82, 116). This is the first report of miRNA expression observed for the maxilla and spleen from $ApoE^{-/-}$ mice infected with *P. gingivalis*, *T. denticola*, and *T. forsythia* by qRT-PCR analysis and is consistent with *in vitro* findings. As shown in Fig. 4-1, the fold changes in miRNA expression were calculated by comparing the value of the infected mouse group (n = 15) to that of the sham-infected control group (n = 10) after normalization to snoRNA202. Interestingly, miR-146a showed a significant increase ($P < 0.001$) in levels in spleens of mice infected with periodontal pathogens compared to the sham-infected control spleens (Fig. 4-1A). Similarly, Fig. 4-1B shows significant changes ($P < 0.001$) in miR-146a expression in the maxilla from mice exposed to *P. gingivalis*, *T. denticola*, and *T. forsythia*. On the other hand, levels of miR-132 and miR-155 did not change significantly in either the spleen or maxilla (Fig. 4-1C to F), but a few animals expressed over a 5-fold increase in levels of miR-132 and miR-155 in the maxilla. Notably, during this polymicrobial exposure, only miR-146a had persistent expression both locally in the maxilla and systemically in the spleen.

Cytokine mRNA Expression in Maxilla and Spleen

In periodontal inflammation, cytokines (IL-1β, TNF-α, and IL-6) are expressed by infiltrating cells such as neutrophils, monocytes/macrophages, dendritic cells, T cells, and plasma cells. The expressions of many cytokines, including TNF-α, as well as a few miRNAs, including miR-146a are NF-κB dependent. Following miR-146a expression in the periodontium and spleen, the expressions of the cytokines TNF-α, IL-1β, and IL-6 were examined in the same RNA samples, as shown in Fig. 4-2. The fold changes in mRNA expression were calculated by comparing the value for infected mice to that for sham-infected mice after normalization to 18S rRNA. As shown in Fig. 4-2A, the TNF-α mRNA expression level was significantly increased ($P < 0.01$) in the spleens isolated from infected mice compared to uninfected controls, whereas no significant changes were observed for maxilla samples (Fig. 4-2B). The level of the proinflammatory cytokine IL-1β showed a significant increase in both spleen ($P < 0.01$) and maxilla ($P < 0.05$) isolated from mice infected with *P. gingivalis*, *T. denticola*, and *T. forsythia* compared to the sham-infected controls (Fig. 4-2C and D), whereas the bone-resorbing cytokine IL-6 was minimally expressed in spleen and maxilla (Fig. 4-2E and F). Strikingly, the level of TNF-α was not significantly different in maxilla of mice infected with *P. gingivalis*, *T. denticola*, and *T. forsythia*, possibly due to higher levels of miR-146a expression found in the maxilla, as shown in Fig. 4-1B.

IRAK1 and TRAF6 mRNA Expressions in Maxilla and Spleen

IRAK1 and TRAF6 are considered to be important adaptor kinases expressed during signal transduction following bacterial ligand stimulation. These adaptor molecules are targeted by miR-146a, as predicted by TargetScan algorithms (www.targetscan.org) (95) and as experimentally documented by Taganov et al. (116). As shown in Fig. 4-3, the expressions of these molecules in the maxilla and spleen isolated during periodontal disease in mice were

analyzed by qRT-PCR. The fold changes in mRNA expression were calculated by comparing the value for infected mice to that for sham-infected mice after normalization to 18S rRNA. Neither the IRAK1 nor the TRAF6 adaptor kinase showed significant changes in spleen or maxilla of mice infected with *P. gingivalis*, *T. denticola*, and *T. forsythia* in comparison to the sham-infected controls, which indicates that miR-146a may have an effect at the posttranscriptional level.

Cytokine Expression in THP-1 Monocytes Stimulated by HK and Live Bacteria

Bacteria or cellular components are potent stimulators of monocytes and macrophages. Bacteria and bacterial components have been shown to induce a diverse array of inflammatory mediators, including TNF-α, *in vitro* as well as under *in vivo* or *ex vivo* conditions (261, 286, 287). Innate immune cells such as activated tissue macrophages are closely associated with acute inflammation and also arterial atherosclerosis development and progression to unstable thrombotic plaque. Human THP-1 monocytes have been well described in studies of TLR signaling and were used in this study for the analysis of potential effects of polymicrobial periodontal pathogen infections *in vitro*. Thus, the ability of HK (Fig. 4-4A to D) and live (Fig. 4-4E and F) *P. gingivalis*, *T. denticola*, and *T. forsythia* periodontal pathogens (mono- or polymicrobial) to induce THP-1 cells to secrete proinflammatory cytokine was evaluated. As shown in Fig. 4-4, for treatment with both HK and live bacteria, TNF-α expression appeared within 4 h and reached maximal levels by 8 h of stimulation, followed by a gradual decline starting at 12 h (Fig. 4-4A). Among the HK periodontal pathogens, *P. gingivalis* was the most potent in stimulating TNF-α secretion, with up to 7,000 pg per ml detected. This level was almost identical to the level of polymicrobial stimulation-induced secretion observed at the 4-h time point (Fig. 4-4A). In contrast, *T. denticola* was less potent for inducing TNF-α secretion but still induced up to 2,500 pg TNF-α per ml by 4 h. The progressive change in TNF-α levels was

validated at the mRNA level by qRT-PCR analysis (Fig. 4-4B) performed on RNA samples collected from the same cells, as illustrated in Fig. 4-4A. The kinetics of TNF-α mRNA expression had a similar progression compared with those of the secreted TNF-α protein except that the peak level of TNF-α mRNA was seen at 4 h (versus the peak level of the TNF-α protein observed at 8 h). Interestingly, a significant increase in the level of IL-8 secretion was detected at all time points following stimulation with all three HK bacterial species whether alone or in combination (Fig. 4-4C). Figure 4-4D shows data for IL-1β secretion by these THP-1 monocytes. Among the three HK pathogens, *T. forsythia* was the most potent inducer of IL-1β (2,500 pg per ml) by 12 h, whereas *T. denticola* only moderately induced IL-1β (1,300 pg per ml) after 72 h. In contrast, polymicrobial-infection-induced IL-1β levels were similar to *T. forsythia* levels. In comparison to HK bacterial stimulation, for infection of THP-1 cells with live bacteria, *T. forsythia* was the most potent at inducing TNF-α, and *T. denticola* was the least potent infecting bacterium (Fig. 4-4E). However, the kinetics of TNF-α progression by live monoinfection and *P. gingivalis*, *T. denticola*, and *T. forsythia* polymicrobial infection were lower than those with HK bacterial stimulation (Fig. 4-4A). Similarly to HK bacteria, significant increases in levels of IL-8 were observed with infection by all three live bacteria whether alone or in combination (Fig. 4-4F).

Kinetics of miRNA Expression in THP-1 Monocytes Stimulated by HK and Live Bacteria

In addition to cytokine analyses, THP-1 monocytes are frequently used to examine innate immune ligand-induced miRNA expression. Previously, with THP-1 monocytes, miRNA expression was observed in response to bacterial ligands such as lipopolysaccharide (LPS) from *Salmonella enterica* serotype Minnesota (82). Thus, THP-1 monocytes were used to examine the kinetics of miR-146a, miR-132, and miR-155 expression following HK bacterial stimulation and live periodontal pathogen infection (Fig. 4-5). For both mono- and polymicrobial HK bacterial

stimulation, the same RNA samples analyzed in Fig. 4-4B were analyzed for miR-146a expression, showing a significant increase of 7-fold after 8 h and gradually increasing up to 22-fold at 72 h compared to controls (Fig. 4-5A). Remarkably, there was a minimal difference in miR-146a expression levels between mono- and polymicrobial HK bacterial stimulations at each of the time points analyzed. Similarly, both mono- and polymicrobial infections induced significant (10- to 40-fold change) levels of miR-146a expression in THP-1 cells compared to controls. *T. denticola* infection induced lower (10-fold change) levels of miR-146a expression than did other infections (Fig. 4-5D). In general, the level of miR-146a expression was higher for live infections than for HK bacterial stimulation (Fig. 4-5A). miR-132 levels were the highest (17- to 18-fold) when induced by *P. gingivalis*, *T. forsythia*, or the polybacterial stimulation at the 8- to 12-h time points, and expression then declined gradually (Fig. 4-5B). Similarly, miR-132 showed the highest expression level with live *T. forsythia* infection at 8 and 12 h and with polymicrobial infection at 12 h (Fig. 4-5E). *T. denticola* did not cause significant changes in miR-132 expression, whereas polymicrobial infection induced similar or higher levels of expression than did infection with individual species. In addition, the miR-132 expression level was lower for live infection (Fig. 4-5E) than for HK bacterial stimulation (Fig. 4-5B). However, no significant changes in expression levels were observed for miR-155 with either HK or live bacterial infection (Fig. 4-5C and F).

Kinetics of Adaptor Kinase Expression in THP-1 Monocytes Stimulated by HK Bacteria

The adaptor kinases IRAK1 and TRAF6 are involved in transcriptional factor activation and are regulated by miR-146a (82, 116). Thus, Fig. 4-6A and B demonstrate the respective increases in IRAK1 and TRAF6 mRNA expression levels for both mono- and polymicrobial stimulations at 4 h and decreases by 8 h or subsequent time points, in line with the elevated expression level of miR-146a starting at 8 h, as shown in Fig. 4-4A. IRAK1 and TRAF6 were

affected not only at the mRNA level but also at the posttranscriptional level, as demonstrated by Western blot analysis (Fig. 4-6C). The IRAK1 and TRAF6 proteins are more sensitive to degradation during bacterial ligand stimulation, and thus, a moderate reduction in levels of the IRAK1 and TRAF6 proteins was observed at 2 h and subsequent time points in response to the HK bacterial treatment compared to that of the unstimulated control. Taken together, these data suggest that periodontal pathogens induce miR-146a expression, which affects cytokine expression kinetics and in turn may interfere with pathogen-induced periodontitis.

Discussion

microRNAs are a new class of regulatory RNAs which have significant roles in many physiological and pathological processes in human diseases, including cardiac arrhythmias, ischemic heart disease, cardiac hypertrophy, viral hepatitis, and diabetes (328). miRNAs such as miR-146a are implicated in inflammatory diseases and cancer and during viral infection (122, 249, 329). Notably, miRNAs negatively regulate the dynamic changes in proinflammatory gene expression during the activation of the innate immune response, particularly in the regulation of TLR4 signaling (328, 330). Furthermore, miR-146a is critical for *in vitro* monocytic cell-based endotoxin tolerance (82). Bacterial and protozoan pathogens also induce miR-155 and regulate proinflammatory cytokine responses, suggesting a specific role for miRNA in the host response to infection. In addition, previously reported studies indicated that specific miRNAs, such as miR-155, miR-21, and miR-126, contribute to vascular disease progression and inflammation (324). Bacterial components and, as determined more recently, whole bacteria such as *H. pylori* induce miR-146a expression in various cell lines (331). However, miR-146a expression and its potential link to periodontal bacterial infection have not been examined, and thus, our *in vivo* and *in vitro* finding of miR-146a expression associated with infection with *P. gingivalis*, *T. denticola*, and *T. forsythia* is novel.

P. gingivalis, *T. denticola*, and *T. forsythia* are frequently detected in periodontal sites during chronic infection (307, 308). In this report, mice chronically infected with major periodontal pathogens demonstrated consistent miR-146a expression both locally in the maxilla and systemically in the spleen. These localized and systemic expressions suggest that miR-146a expression might be due to a bacterial presence and/or inflammation in periodontium and spleen, also based upon the finding that genomic DNA from *P. gingivalis*, *T. denticola*, or *T. forsythia* was detected in oral plaque samples, liver, aorta, heart, and spleen (data not shown). We have also shown that miR-132 and miR-155 expression levels were not statistically significant but that some infected mice had higher levels detected. Thus, miR-146a is highly induced by Gram-negative anaerobic periodontal pathogens (*P. gingivalis*, *T. denticola*, and *T. forsythia*) during polymicrobial periodontal disease.

These three major periodontal pathogens possess a broad array of virulence factors, and additionally, their cell wall components, such as peptidoglycan and LPS, trigger periodontal inflammation, and the host immune/inflammatory responses lead to gingival connective tissue and alveolar bone losses. The incidence and rate of progression of periodontitis involve synergistic interactions among periodontopathic bacteria and/or between periodontopathic bacteria and host cells such as gingival fibroblasts, periodontal ligament cells, neutrophils, monocytes/macrophages, dendritic cells, T cells, and B cells. Interactions of bacteria and host cells lead to the host cell secretion of various cytokines and chemokines, including IL-1β, IL-1α, TNF-α, IL-6, IL-10, and IL-8, as well as prostaglandin E_2 (PGE_2) and matrix metalloproteinases (MMPs), which have been identified in periodontitis lesions from patients with adult periodontitis at higher levels than at healthy sites (332). However, the inflammatory response is tightly controlled by various innate immune regulators, including miRNA. Thus, to gain insight

into the biological significance of miR-146a expression associated with periodontal pathogens, cytokine expression levels were also determined in this study. The level of TNF-α in spleens of mice infected with *P. gingivalis*, *T. denticola*, and *T. forsythia* was more significantly increased than that in the periodontium. The increase in TNF-α secretion may be due to the lower levels of miR-146a in spleens than in the periodontium. However, additional studies are required to clarify the mechanism of miR-146a expression during infection. Measurements of IL-1β mRNA demonstrated significant increases in levels in both the periodontium and spleen. The expression of IL-1β did not show a negative correlation with miR-146a, suggesting that more miR-146a expression might be required. The results can be due to low levels of miR-146a causing a negative impact on IL-1β expression or the involvement of unknown factors.

Several previously reported *in vitro* studies have shown that *P. gingivalis*, *T. denticola*, or *T. forsythia* stimulates cytokine expression in gingival epithelial cells. Thus, to validate our *in vivo* findings, a detailed time course analysis was performed for the expression of cytokines in human monocytes (THP-1 cells) after HK bacterial stimulation with *P. gingivalis*, *T. denticola*, and *T. forsythia*. HK bacterium-stimulated THP-1 cells showed an increase in the level of TNF-α expression in a time-dependent manner but also had a gradual decrease over extended time points. In contrast, IL-8 and IL-1β did not show expression kinetics similar to those of TNF-α. Interestingly, the proinflammatory cytokine IL-8 was present at similar levels for all three HK bacterial stimulations and remained at higher levels for several hours. The potency of all three HK bacterial species alone or in combination with respect to cytokine excretion is consistent with data from previously reported studies (333-335). Sometimes, the nature of cytokine secretion varies in monocytes, as shown previously by Zaric et al. (335). In their study, *Escherichia coli* LPS-tolerized THP-1 monocytes had reduced levels of IL-8 excretion with repeated LPS

challenges, whereas *P. gingivalis*-tolerized monocytes did not. More importantly, IL-1β, IL-6, and IL-8 are present in diseased periodontal tissues, and their induction appears to play a role in periodontal inflammation. Markedly, the stimulation of IL-1β secretion by *P. gingivalis*, *T. denticola*, and *T. forsythia* may be an important mechanism that amplifies the inflammatory response, the induction of connective tissue-degrading enzymes, and osteoclastic alveolar bone resorption (332). Furthermore, IL-8 as well as RANTES aid neutrophil, monocyte, and TH_1 cell recruitment to the site of infection (335, 336). Previously, cytokine expression was observed for infection with HK *P. gingivalis*, *T. denticola*, and *T. forsythia*, but there has been no report on the effect of live bacterial infection on undifferentiated THP-1 monocytes (333, 334). Thus, to mimic active infection (which can also be considered a critical control in this study), undifferentiated THP-1 monocytes were infected with live *P. gingivalis*, *T. denticola*, and *T. forsythia* bacteria. The TNF-α and IL-8 production kinetics observed with the live bacterial infection(s) were not similar to those of HK bacterial stimulation. LPS from these bacteria is thus a strong inflammatory inducer. However, Sahingur et al. (334) showed previously that THP-1 monocytes induce IL-1β, IL-6, TNF-α, and IL-8 in response to DNA (TLR9 ligand) from *P. gingivalis* and *T. forsythia* by binding with TLR9 through NF-κB. Accordingly, in our study HK bacteria showed a strong inflammatory response in THP-1 monocytes, possibly through various ligand-TLR interactions. The differences in TNF-α expression levels between live and HK bacteria may be due to antigenic alterations resulting in differential interactions with THP-1 cells.

Recently, miR-146a expression in LPS (*Salmonella enterica* serotype Minnesota)-induced THP-1 monocytes was demonstrated to be negatively correlated with cytokine stimulation, including TNF-α (82). A similar negative correlation was observed in response to LPS from *P. gingivalis* (data not shown), indicating that LPS is the most potent stimulator of miR-146a

induction. However, the cell response to membrane-bound LPS can differ from that to soluble LPS due to the fact that lipid A is exposed to immune cells in the extracted form, as shown previously by Van Amersfoort et al. (337). Consistent with the LPS-induced miRNA response, a negative correlation of TNF-α secretion and miR-146a expression was observed for the HK periodontal pathogens after monocyte stimulation. However, in comparison to HK bacteria, live infection caused higher miR-146a expression levels after 24 h and reduced TNF-α expression levels. This difference may be due to the increased numbers of bacteria at the latter time points, whereas the level of HK bacteria remained unchanged. Interestingly, IL-8 remained at a higher level throughout the bacterial infection or stimulation and was not negatively correlated with miR-146a expression, as IL-8 is a vital chemotactic factor. miR-155 was shown previously to be induced by the gastric pathogen *H. pylori* (196), but not by either HK or live periodontal pathogens, indicating that the expressions of these miRNAs might be bacterium specific as well as cell specific. Thus, miR-155 could be considered a control miRNA that is regulated independently from bacterial stimulation. The miR-132 level was increased *in vitro*, and significant increases were not demonstrated for the infected mice, suggesting that the level of miR-132 might have increased at the beginning of the infection with *P. gingivalis*, *T. denticola*, and *T. forsythia* and then diminished during subsequent infections. Thus, the expression of miR-146a both *in vivo* and *in vitro* is thought to be a component of the host defense against periodontal pathogens, and further studies are needed to understand this mechanism.

Previously, LPS priming or miR-146a-mimicked transfection in monocytes was found to maintain higher levels of miR-146a expression and to lead to endotoxin tolerance by negatively affecting the IRAK1 and TRAF6 adaptor kinases (miR-146a targets) at the posttranscriptional level (82). Furthermore, it was also demonstrated that following the withdrawal of LPS, the miR-

146a expression level gradually diminished and was correlated with recovery from endotoxin tolerance. This finding suggests the importance of the presence of higher levels of miR-146a in maintaining tolerance. In this study, miR-146a showed higher levels of expression in both the periodontium and spleen, whereas the IRAK1 and TRAF6 adaptor kinase mRNA levels did not change. However, THP-1 monocytes stimulated *in vitro* by these bacteria had reduced levels of adaptor kinases at the protein level, again negatively correlating with miR-146a expression. Due to multiple polymicrobial infections (similar to LPS priming), miR-146a was maintained at a higher level in the spleen and periodontium. Thus, miR-146a may play a role in regulating cytokine secretion in experimental mice, possibly by a tolerance mechanism.

In summary, our findings reveal that *P. gingivalis, T. denticola,* and *T. forsythia* upregulate miR-146a expression. It affects cytokine excretion by targeting IRAK1 and TRAF6 during polymicrobial-infection-induced periodontal disease. Since miRNAs exert profound effects during the development of disease, it is likely that miR-146a may constitute a possible target for therapeutic interventions in periodontal disease.

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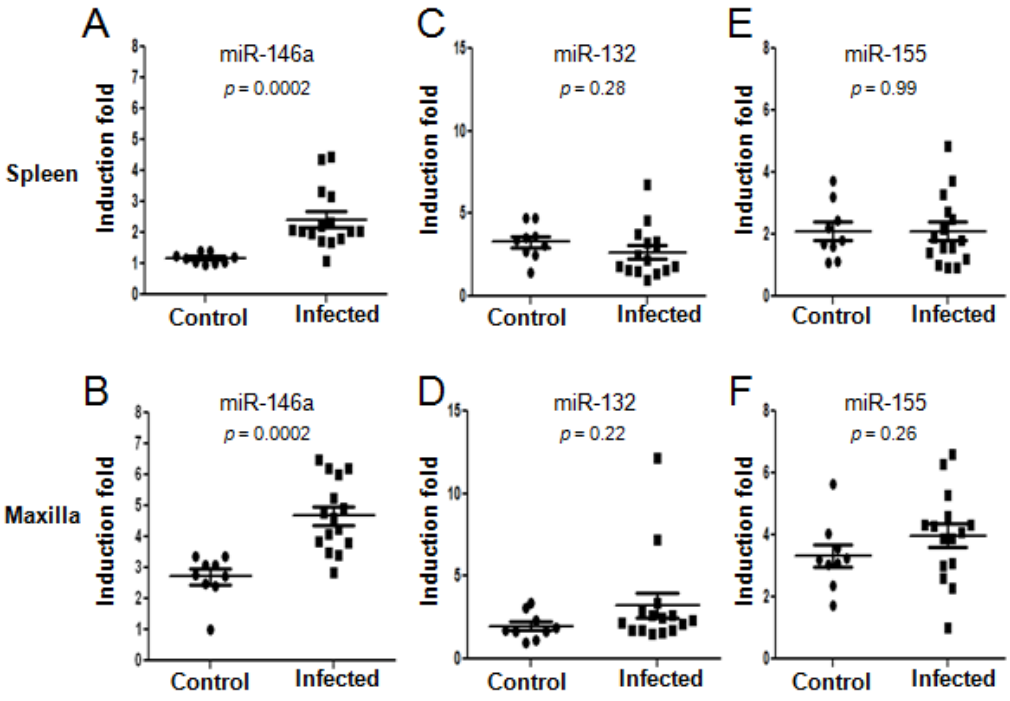

Figure 4-1. Polymicrobial infection with the periodontal pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* elicits miRNA in spleen and maxilla following 16 weeks of periodontal disease in $ApoE^{-/-}$ mice. Total RNAs from spleen and maxilla were isolated as described in Materials and Methods and were analyzed by qRT-PCR for the expression of miR-146a (A and B), miR-132 (C and D), and miR-155 (E and F). Data points and error bars represent means ± SD for infected (n = 15) and sham-infected control (n = 10) mice.

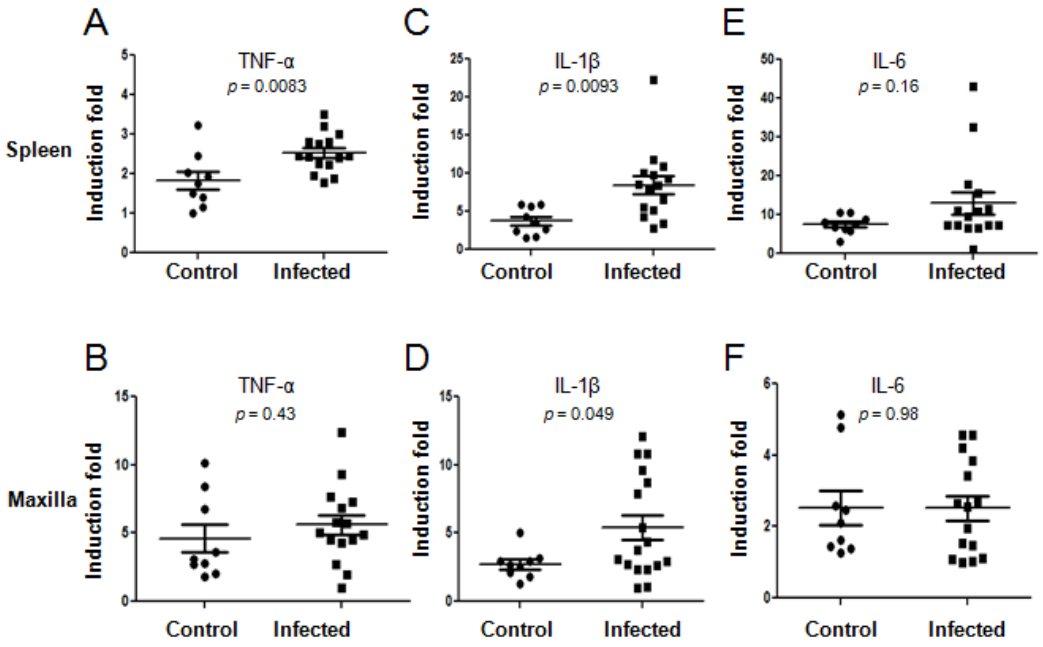

Figure 4-2. Polymicrobial infection with *P. gingivalis*, *T. denticola*, and *T. forsythia* induces proinflammatory cytokine responses in spleen and maxilla in ApoE$^{-/-}$ mice. Total RNAs from spleen and maxilla were analyzed by qRT-PCR for the expression of TNF-α (A and B), IL-1β (C and D), and IL-6 (E and F). The data are shown as means ± SD for infected (n = 15) and sham-infected control (n = 10) mice.

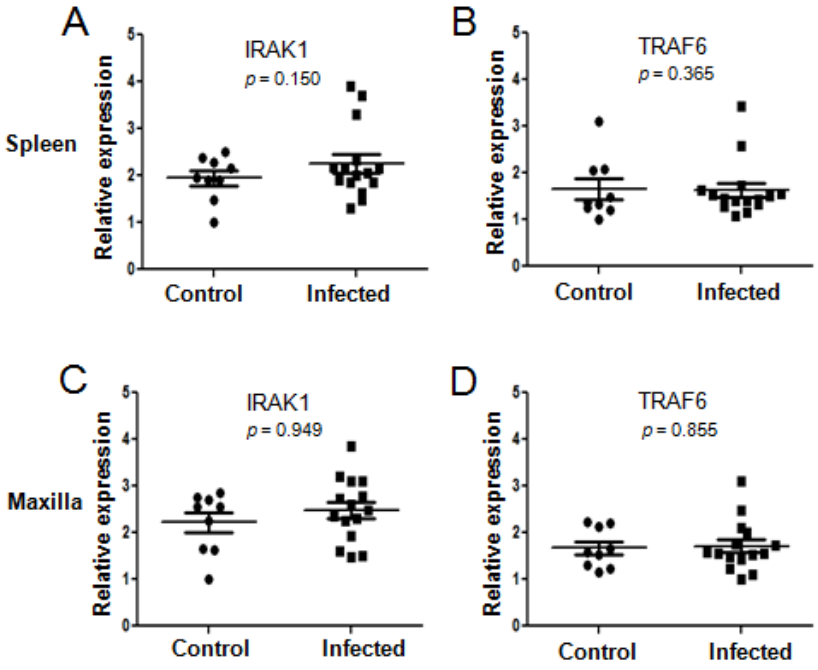

Figure 4-3. Expression of IRAK1 and TRAF6 in response to polymicrobial (*P. gingivalis*, *T. denticola*, and *T. forsythia*) infection in spleen and maxilla following 16 weeks of periodontal disease in ApoE$^{-/-}$ mice. Total RNAs from spleen and maxilla were analyzed by qRT-PCR for the expression of IRAK1 (A and C) and TRAF6 (B and D). The data are shown as means ± SD for infected (n = 15) and sham-infected control (n = 10) mice.

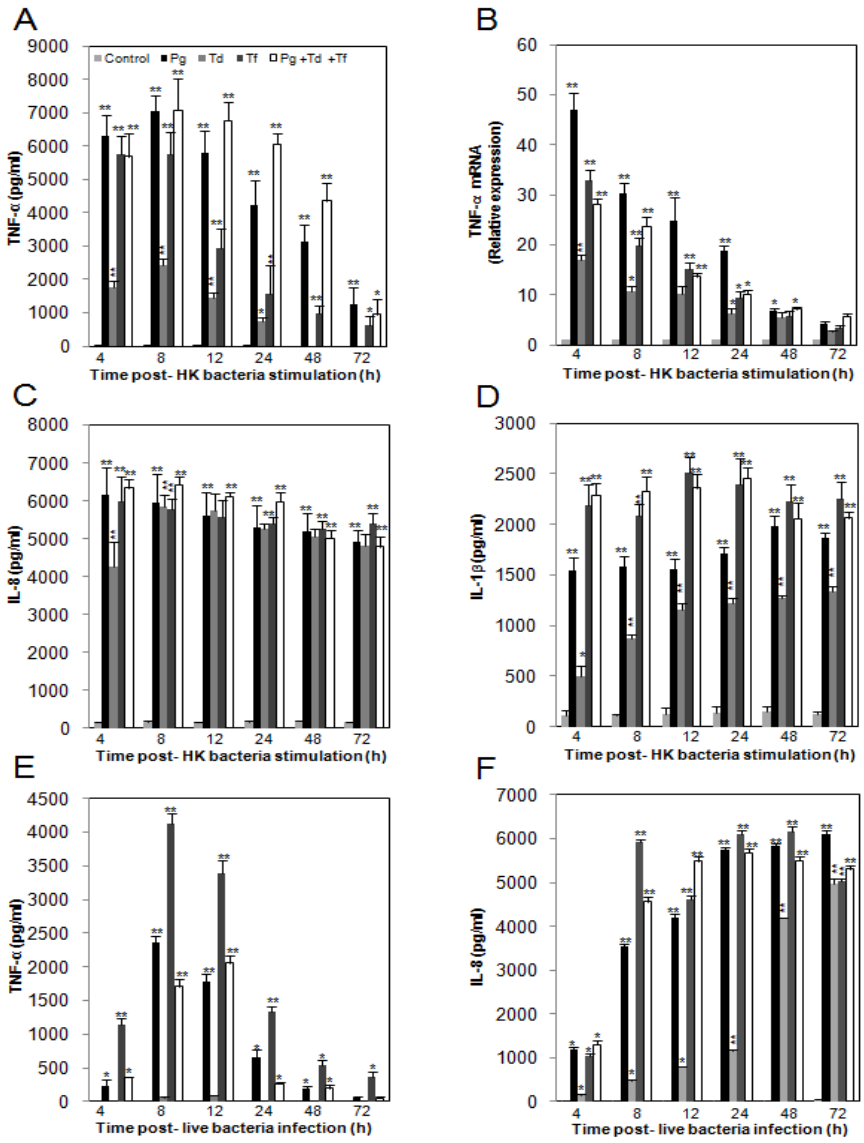

Figure 4-4. Monomicrobial (*P. gingivalis* [Pg], *T. denticola* [Td], or *T. forsythia* [Tf]) and polymicrobial (*P. gingivalis*, *T. denticola*, and *T. forsythia*) stimulation and infection of THP-1 monocytes and induction of proinflammatory cytokines or chemokines. THP-1 cells were stimulated with HK and live bacteria (MOI of 10) as mono- and polymicrobial infections for up to 72 h. (A and C to F) Supernatants were collected from THP-1 cells treated with HK and live bacteria at the indicated time points, and concentrations of TNF-α (A and E), IL-8 (C and F), and IL-1β (D) were measured by ELISA (A, C, and D). (B) Total RNAs from cell pellets of untreated and treated THP-1 monocytes were analyzed by qRT-PCR for TNF-α mRNA. All results are expressed as means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (compared with untreated THP-1 cells).

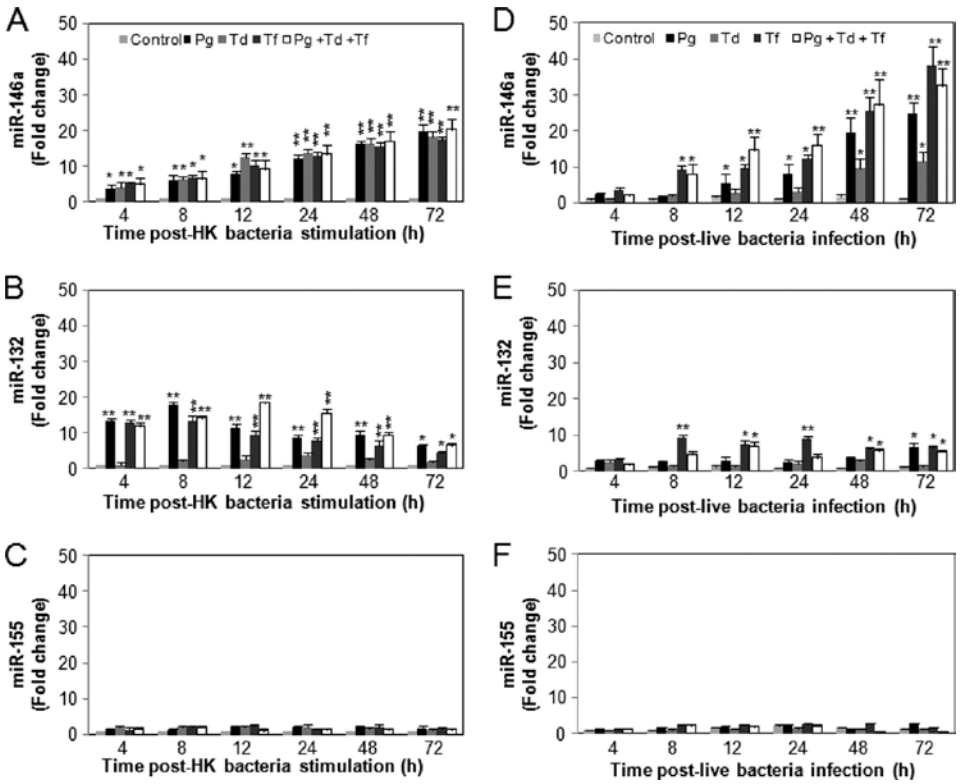

Figure 4-5. Elevated levels of expression of miR-146a and miR-132, but not miR-155, demonstrated in response to monomicrobial (*P. gingivalis*, *T. denticola*, or *T. forsythia*) and polymicrobial (*P. gingivalis*, *T. denticola*, and *T. forsythia*) stimulations (HK bacteria) and infection with live bacteria in THP-1 monocytes *in vitro*. The same RNA samples as those used for Fig. 4-4 were analyzed for miR-146a (A and D), miR-132 (B and E), and miR-155 (C and F) expressions by qRT-PCR as described in Materials and Methods. Data are expressed as means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (compared with untreated cells).

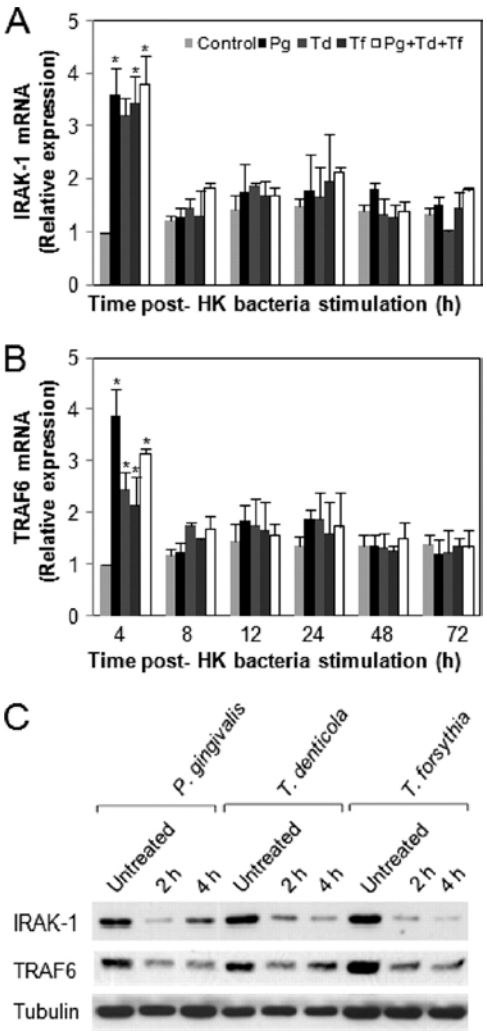

Figure 4-6. Expression of IRAK1 and TRAF6 in response to monomicrobial (*P. gingivalis*, *T. denticola*, or *T. forsythia*) and polymicrobial (*P. gingivalis*, *T. denticola*, and *T. forsythia*) HK bacterial stimulation in THP-1 monocytes *in vitro*. (A and B) The same RNA samples as those used for Fig. 4-4 were analyzed by qRT-PCR for IRAK1 and TRAF6 mRNAs. All results are expressed as means ± SD from three independent experiments. *, $P < 0.05$ compared with the control. (C) Protein levels of IRAK1 and TRAF6 in HK bacterium-stimulated and unstimulated THP-1 cells were analyzed by Western blotting, with tubulin expression shown as a loading control.

CHAPTER 5
MIR-132 AND MIR-212 ARE IMPORTANT FOR INNATE IMMUNITY THROUGH ESTABLISHING TLR2 LIGAND-INDUCED TOLERANCE VIA MODULATION OF IRAK4 EXPRESSION

Background

Innate immune system is the primary defense mechanism that is ignited shortly after pathogenic invaders are detected by various conserved pattern recognition receptors. One such class of well-documented receptors is toll-like receptors (TLRs). These receptors are efficient in detecting signature molecules including lipopeptides, peptidoglycan (PGN), lipopolycaccharide (LPS), flagellin, and nucleic acids (5). Upon detection, the subsequent signaling events elicit a core set of stereotyped responses, including cytokines, chemokines, and adhesion molecules, most notably through activation of NF-kB transcription factor, which leads the immune system to sense and react to infection. In contrast, pathological dysregulation of this process is a hallmark of inflammatory damage, autoimmune diseases, and possibly cancer (338). Therefore, innate immune response involving TLR signaling cascades must be tightly regulated by elaborate mechanisms to control its onset and termination. It is acknowledged that TLR4 signaling events have been extensively studied both *in vivo* and *in vitro* in terms of endotoxin tolerance, which limits the pathogenic effects of LPS (10, 45, 48, 71, 226). Other microbial components such as PGN (a potent TLR2 agonist) are also involved in priming of innate immune cells (88, 339). To explain this tolerance mechanism, a number of negative regulatory controllers have been proposed. These include soluble decoy receptors for TLR4, IRAKM, A20, TRIM30α, and splice variants of signal-transduction proteins such as MyD88-s (78, 86, 125, 204, 235). However, at this time, there is no consensus on the molecular mechanisms involved to resolve inflammation.

microRNAs (miRNAs), short noncoding RNA, have emerged recently as key regulators of gene expression acting at the posttranscriptional level (89). miRNAs have been shown to be

critical in many biological processes, ranging from development to differentiation and including regulation of the mammalian immune system (92, 93). A few miRNAs are induced in innate immune cells in response to cognate TLR ligands, with a consensus emerging that miR-146a, miR-155, and miR-21 are important to negatively regulate the activation of inflammatory pathways in myeloid cells (122, 125, 239). Although miR-146a regulation of IRAK1 and TRAF6 adaptor molecules has been shown to play a major role in endotoxin tolerance and cross-tolerance, cytokine response is not extinguished completely, suggesting the possible involvement of other miRNAs in this intricate process (81, 82). It is well established that recruitment of adaptor kinases are the prime factor for triggering a TLR signaling cascade. Upon TLR activation, IL-1 receptor-associated kinase 4 (IRAK4) is known to be recruited to MyD88, forming a helical assembly of the MyD88-IRAK4-IRAK2/1 complex (256) that further activates TRAF6 and eventually leads to NF-κB activation for inflammatory gene transcription. Thus, IRAK4 should be the pivotal adaptor kinase used by all TLR signaling (except TLR3). In this connection, De Nardo et al. (83) have shown that, compared to IRAK1 knockdown, the knockdown of IRAK4 renders immune cells much less responsive to TLR agonists. Suzuki et al. reported that IRAK4 knockout mice, phenotypically similar to mice lacking MyD88, show severe impairment of IL-1 and TLR signaling (84). Based on these reports, regulation of adaptor kinases might be an important molecular mechanism for maintaining cytokine response in a controlled manner. Although IRAK1 and TRAF6 are known to be regulated by miR-146a (116), no such miRNA-mediated regulation of IRAK4 has been documented. IRAK4 has been found to be a putative target of miR-132 and miR-212 by bioinformatics analysis using TargetScan (TargetScan.org), but this has not been experimentally validated. Accordingly, in a very recent

review, O'Neill et al. have substantiated that it is still unknown whether signaling molecules in TLR pathways are targeted by miR-132 and miR-212 (120).

Mature miR-132 and miR-212 are processed from a single non-coding gene transcript, regulated primarily by the cyclic AMP-response-element-binding (CREB) transcriptional factor (149, 340). Recently, miR-132 has been shown to regulate neuronal morphogenesis and the dendritic plasticity of cultured neurons (149, 150). miR-132 may also be responsible for limiting inflammation in the mouse brain by targeting acetylcholinesterase (AChE) (151). miR-212 can interfere with the craving for cocaine in mice and acts as a tumor suppressor (152). miR-132 can also modulate inflammation induced by early stage Kaposi's sarcoma-associated herpesvirus (KSHV) infection (119), although no detailed expression kinetic of miR-132 or miR-212 has been described in response to innate immune ligands associated with TLR ligand-induced tolerance.

Our study shows the first evidence that the exposure of innate immune cells to PGN, Pam, flagellin, or whole bacteria induces rapid expression of mature miR-132 and miR-212. This report highlights the importance of investigating their mechanistic role in innate immunity in the context of TLR2 ligand-induced tolerance, which can modulate innate immune system.

Methods

Reagents

Ultrapure TLR-grade lipopolysaccharde (*Salmonella enterica* serotype Minnesota Re595), lipoteichoic acid (*Staphylococcus aureus*) were from Sigma-Aldrich (St. Louis, MO).Peptidoglycan (*Escherichia coli* 0111:B4), synthetic bacterial lipoprotein Pam3CSK4CysSerLys4, LPS from Porphyromonas gingivalis ATCC 33277, poly(I:C), and recombinant flagellin (*Salmonella typhimurium*) were from InvivoGen (San Diego, CA). siGENOME SMARTpool siRNA for IRAK4 and CREB were from Dharmacon (Lafayette, CO).

miRNA mimics and inhibitors (anti-miRNA inhibitor) were from Ambion (Austin, TX). Antibodies to human IRAK1,IRAK4, CREB, and pCREB were from Santa Cruz Biotechnology (Santa Cruz, CA). Kinase inhibitors PD98059 and U0126 were from Calbiochem (San Diego, CA).

Cell Culture and Innate Immune Ligand Stimulation.

Human THP-1, HEK293, and murine RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cells were maintained in either RPMI or DMEM media containing 10% (v/v) FBS (Mediatech, Manassas, VA), and 100 U/ml penicillin-streptomycin (Mediatech). For analysis of THP-1 monocyte response to microbial ligand in vitro, log phase cells were seeded at $5x10^5$ cells/ml in a 24-well plate. Unless otherwise mentioned, cells were stimulated with the following agonists: 1000 ng/ml of *S. enterica* lipopolysaccharide (LPS Se, TLR4 ligand), Pam3CSK4CysSerLys4 (Pam, TLR2/TLR1 ligand), Peptidoglycan (PGN, TLR2 ligand), LPS from *P. gingivalis* (LPS Pg, TLR2 ligand), lipoteichoic acid (LTA, TLR2 ligand), and 300 ng/ml recombinant flagellin (TLR5 ligand). TLR ligands were reconstituted in endotoxin-free water and used at concentrations as reported before1. For analysis of human peripheral blood mononuclear cells (PBMCs) response, mononuclear cells were isolated from whole blood by a Ficoll gradient (GE Healthcare, Uppsala, Sweden) and were grown in RPMI media as described above. PBMCs (10^6 cells/ml) were then stimulated for 6-24 h with PGN (0-5 μg/ml). Mouse macrophages were from, 3-mo-old female C57BL/6 mice, which were intraperitoneally injected with 0.5 ml of 4% (w/v) sodium thioglycollate 3 d earlier. Peritoneal cells (~90% macrophages) were harvested by lavaging the peritoneal cavity and grown in DMEM as described above. After 5 h, cells were washed with growth medium and then stimulated for 6 24 h with PGN (0–10 μg/ml). Protocol was approved by the Institutional Animal Care and Use Committee of the University of Florida.

TLR Ligands-induced Tolerance

PGN-, Pam- or flagellin-induced tolerance and/or cross-tolerance experiments were performed using the THP-1 monocyte model, adapted from methods described previously (81, 88) with minor modifications. Briefly, before starting tolerance assays, THP- 1 cells were cultured until they were in log phase and reached densities of 10^6 cells/ml. In all experiments, trypan blue exclusions were performed to verify cell viability and cells that were >95% viable were considered for study. THP-1 cells were then transferred to fresh complete medium at $5x10^5$ cells/ml. Cells were incubated with a low dose of PGN (100 or 500 ng/ml) or Pam (100 ng/ml) for 18 h. In some tolerance assays, THP-1 cells were primed for 18 h with flagellin (200 ng/ml) or heat-killed (HK) *Tannerella forsythia* at multiplicity of infection (MOI) 100. After two washes with tissue culture-grade PBS, primed cells were re-stimulated with various ligands or cultured without stimulation. For bacteria-induced tolerance study, heat-killed *P. gingivalis* FDC 381, *Treponema denticola* ATCC 35404, and *T. forsythia* ATCC 43037 were prepared as described previously (281). For human PBMCs and RAW264.7 tolerance assays, cells were primed with PGN (500 ng/ml) or Pam (100 ng/ml) for 18 h, followed by challenge with PGN and LPS (1 μg/ml). For the mouse primary macrophages tolerance assay, primary cells were primed with PGN (100 ng/ml), followed by washing and challenging with PGN, Pam, or LPS. In all cases, after 3-24 h secondary challenge, supernatants were harvested and stored at -80°C until assays were performed for secreted inflammatory mediators.

RNA Extraction and Real-time RT-PCR

Total RNA from microbial ligands-treated and untreated THP-1 cells was prepared using the *mir*Vana miRNA isolation kit (Ambion). For miRNA analysis, 6.7 ng RNA of each sample was used for quantitative stem-loop reverse transcription and real-time PCR (qRT-PCR). Quantification of expression of mature miRNAs was performed using the TaqMan microRNA

RT kit, TaqMan Universal PCR Master Mix, and TaqMan miRNA assay primers of interest for human or mouse miRNAs (Applied Biosystems, Carlsbad, CA). For gene expression analysis, cDNA was prepared with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and individual mRNA was monitored with the following inventoried Taqman assays (Applied Biosystems): human IRAK1, IRAK4, and CREB with 33 ng total RNA per reaction. The cycle threshold (*C*t) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined, and miRNA expression values were calculated using human RNU44 or mouse SnoRNA202 (Applied Biosystems) as an endogenous reference following the $2^{-\Delta\Delta Ct}$ method (259). mRNA for gene expression values were quantified in the same way after normalization to mammalian 18S rRNA. Standard curves for mature miR-132 and miR-146a were prepared by qRT-PCR analysis using synthetic mature miRNA (IDT, Coralville, IA). The *C*t values were determined by qRT-PCR analysis of the PGN (2500 ng/ml) treated total RNA and then converted to miR-132 and miR-146a copy numbers using the standard curve.

Transient Transfection

miR-132, miR-212 and miR-146a functional analyses were performed by transfecting synthetic mimic or inhibitor (40 nM) in THP-1 monocytes and RAW264.7 cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described (81, 82). THP-1 cells were transfected with siRNA targeting IRAK4 and CREB using the same above protocol. For luciferase assays, HEK293 cells were plated in 24-well plate at 10^5 cells/well and transfected 24 h later by 3% lipofectamine reagent. Complete 3'UTR of IRAK4 was subcloned downstream of firefly luciferase coding sequence in pMiRTarget vector (Origene Technologies, Rockville, MD) and then this reporter (50 ng) and renilla luciferase reporter (0.1 ng) were co-transfected together with 100 nM of miR-132-mimic, miR-212-mimic, or miR-146a-mimic into HEK293 cells or 48

h. A mutated version of this construct carrying 4-bp substitutions in the putative miR-132/-212 seed sequence target site was obtained by using the site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Reporter luciferase activities were measured using the Dual-Luciferase kit (Promega, Madison, WI) 48 h after transfection.

ELISA

Cytokine concentrations in cell culture supernatants were measured by ELISA using OptEIA cytokine kits (BD Biosciences, Franklin Lakes, NJ) and the DuoSet Development system (R&D Systems, Minneapolis, MN) following the manufacturers' instructions.

Immunoblot

PGN-, Pam- or flagellin-primed and unprimed THP-1 cells (5 x106/condition) were collected 2 h after secondary challenge with ligands and then lysed with lysis buffer containing Complete Protease Inhibitors Cocktail (Roche, Indianapolis, IN) as described1. THP-1 cell lysate pre-treated with or without PD98059 (MEK1 inhibitor, 50 μM) and U0126 (MEK1/2 inhibitor, 0.5 μM), were similarly prepared. Soluble lysates were quantitated for protein concentration (Bio- Rad Bradford protein assay), separated by 10% (w/v) SDS-PAGE, and electrotransferred to a polyvinylidene difluoride membrane. The membranes were blocked for 1 h at room temperature with 5% (w/v) nonfat milk in PBS/0.05% Tween 20 (PBS-T, v/v) and were probed with rabbit polyclonal antibody anti-IRAK1 (1:300), anti-IRAK4 (1:300), and mouse monoclonal anti-tubulin (1:5000, Sigma-Aldrich). The membranes were then washed with PBS-T and incubated for 1 h with goat anti-rabbit or anti-mouse IgG-HRP (1:5000, Southern Biotech, Birmingham, AL). After washing with PBS-T, reactive protein bands were visualized by SuperSignal Pico chemiluminescent reagent (Pierce, Rockford, IL). Similarly, CREB and pCREB were visualized using specific antibodies at 1:300 dilutions. Integrated density of protein bands in the scanned image of Western blot film was analyzed using IMAGE-J software and

normalized to tubulin or CREB in each lane, and is presented relative to results obtained with the control sample (percentage of fraction), which was set as 1.0.

Statistical Analysis

Student's *t*-test (two-tailed) was used to compare data between groups, except where mentioned otherwise. Prism for Windows, version 5.0 (GraphPad Software, San Diego, CA) was used, and $P < 0.05$ was considered statistically significant.

Results

TLR Ligand-induced TNF-α Secretion and Kinetics of miR-132 and miR-212 Expression in Innate Immune Cells

Emerging data have shown that microbial ligands are recognized through specific receptors found on innate immune cells. Beside LPS, PGN is known to be a potent inducer of a diverse array of inflammatory mediators, including TNF-α both *in vitro* and *in vivo*. To monitor TNF-α *in vitro* tissue culture, the most commonly employed acute monocytic leukemia cell line THP-1 was used. TNF-α was detected in culture supernatants of PGN-treated THP-1 monocytes starting at 2 h and peaked at 8 h post-stimulation, followed by gradual decrease thereafter (Fig. 5-1a). The progressive changes of TNF-α secretion showed a dose- and time-dependent pattern, similar to LPS stimulation (82). The highest dose of PGN (5 μg/ml) induced the highest level of TNF-α, up to 12 ng/ml at 8 h. The kinetics of TNF-α mRNA expression was also measured by quantitative real-time PCR (qRT-PCR) and was consistent with the secreted TNF-α protein (data not shown).

In our recent study, LPS-stimulated THP-1 monocytes showed a continuous expression of miR-146a, consistent with its critical role in innate immunity (81, 82). Similarly, an up to 25-fold increase of miR-146a expression was noted at 48 h with the highest concentration of PGN tested in THP-1 cells (Fig. 5-1b). In sharp contrast, miR-132 and miR-212 showed more impressive

110- and 65-fold increased expression, respectively, at their peak (Fig. 5-b). PGN treatment resulted in an early increase in miR-132 and miR-212 levels at 4 h reaching 60- and 43- fold at the 5 μg/ml PGN dose, respectively, while the level for miR-146a was only 7-fold (Fig. 5-1b). Other miRNAs such as miR-155 and miR-16 (data not shown) showed little or no change under the same conditions (Fig. 5-1b). The exact copy numbers of both miR-132 and miR-146a in the PGN-treated cells (same data as in Fig. 5-1b) were determined using synthetic miR-132 and miR-146a as standards for the qRT-PCR assay (Fig. 5-2). Each unstimulated THP-1 cell showed only ~2 copies of miR-132 and ~30 copies of miR-146a. Upon PGN stimulation, each THP-1 cell could produce ~100 copies of miR-132 and ~ 600 copies of miR-146a at 12 h (Fig. 5-2). In contrast, LPS-stimulated THP-1 monocytes showed significantly lower miR-132 (10-fold) and miR-212 (3-fold) at 12 h (Fig. 5-3a,b) than when stimulated with PGN. The above results indicate that PGN is a potent inducer for both miR-132 and miR-212, which were focused for further investigation.

To understand the physiological relevance of induction of miR-132, a range of other cell types were used. Human PBMCs were treated with PGN from 6 to 24 h. PBMCs showed dose-dependent production of TNF-α up to 3 ng/ml (Fig. 5-1c) and upregulation of miR-132 up to 6-fold at 12 h (Fig. 5-1d). A similar increase of miR-132 expression (~5-fold at 24 h) was also observed in mouse macrophage cell line RAW264.7 in response to PGN, Pam_3CSK_4 (Pam), and LPS (Fig. 5-1e). Similar results were also obtained in mouse primary peritoneal macrophages, which showed high levels of TNF-α production up to 10 ng/ml peaking at 6 h (Fig. 5-1f) and similar miR-132 expression (5-fold miR-132 upregulation peaking at 6 h) in response to PGN (Fig. 5-1g). Consequently, to support the ability of PGN to induce miR-132/-212 expression, effects of a common synthetic derivative of PGN, Pam was also carefully examined in this study.

Similarly, Pam-induced TNF-α production (Fig. 5-4a) and miR-132 and miR-212 expression (Fig. 5-4c,d). The fold change for miR-132 was notably higher than for miR-146a (Fig. 5-4b) or miR-155 (Supplementary Fig. 5-4e). LPS from *P. gingivalis* (TLR2 ligand) also showed similar patterns of miR-132 and miR-212 expression (data not shown). In contrast, another TLR2 ligand LTA showed no significant induction of miR-132 (Fig. 5-4f), which indicates that miR-132 and miR-212 expression might be ligand-specific among TLR2 ligands. The capacity of TLR3 and TLR5 ligand to induce miR-132 and miR-212 were examined. TLR3 ligand Poly (I:C) (25 μg/ml) did not induce any of these miRNA (data not shown). TLR5 agonist flagellin stimulation induced a rapid production of TNF-α (Fig. 5-4g) and, at 8 h, surprisingly greater increases of miR-132 (55-fold) and miR-212 (27-fold) than miR-146a (Fig. 5-4h). Together these findings suggest the capacity of PGN, Pam, or flagellin to induce significant levels of miR-132 and miR-212 in THP-1 monocytes, human PBMCs, and mouse primary macrophages. To gain a better understanding on the dynamic nature and robust expression kinetics, their biological significance was investigated in the subsequent studies.

CREB-dependent Rapid Induction of miR-132

In the previous time-course experiment, miR-132 was rapidly induced compared to miR-146a (Fig. 5-1b). miR-132 has been shown to be regulated by a key transcription factor, CREB (149). CREB is activated in THP-1 monocytes by phosphorylation at Ser 133 (pCREB) within minutes after exposure to PGN or Pam. Notably, at 15 min, a 4.17-fold increase in pCREB compared to untreated control (UTX) was evident by Western blot analysis (Fig. 5-5a), consistent with previous reports (149, 341). In THP-1 cells with siRNA-mediated knockdown of CREB, the levels of CREB mRNA was reduced by ~50% and protein by 70% as demonstrated by qRT-PCR (Fig. 5-5b) and Western blot analysis (Fig. 5-5c), respectively. Knockdown of CREB resulted in significantly reduced ($P < 0.01$) miR-132 expression in THP-1 cells after

treatment with PGN or Pam and thus provided evidence for the involvement of CREB in the induction of miR-132 (Fig. 5-5d). CREB phosphorylation is mediated by mitogen- or stress-activated protein kinases (MAPK or SAPK), which are known to be activated by PGN in THP-1 monocytes (341). U0126 and PD98059 inhibit ERK (extracellular signal-regulated kinase) activation and result in inhibition of CREB phosphorylation after PGN treatment in THP-1 monocytes (Fig. 5-5e). Strikingly, these synthetic inhibitors actively blocked transcription of both pri-miR-132 by ~80% and pri-miR-212 (data not shown), despite a higher PGN concentration, whereas the pri-miR-146a level was unaltered (Fig. 5-5f); mature miR-132 and miR-212 were also reduced (Fig. 5-5g). Thus, U0126 and PD98059 blocked miR-132/-212 transcription, while miRNA processing activity appeared unaffected as evidenced by the unaltered expression in miR-146a. All this evidence confirming PGN- or Pam-induced higher CREB phosphorylation in THP-1 monocytes was likely attributable to substantial expression of miR-132 (and miR-212) compared to other miRNAs including miR-146a.

miR-132 and miR-212 May Account for PGN- and Pam-induced Tolerance

Previously, PGN-induced homologous and heterologous tolerance has been shown *in vitro* and *in vivo* (88, 339). In this report, to evaluate this tolerance phenomenon in greater depth, the most commonly employed THP-1 cell model was used. THP-1 monocytes were primed with PGN (500 ng/ml) or Pam (100 ng/ml) for 18 h followed by challenge exposure with various TLR agonists (1000 ng/ml). After 3 h, the TNF-α protein level was assessed by ELISA (Fig. 5-6a). As expected, TNF-α production was reduced by 80-90% in tolerized cells compared to untolerized controls, after challenge with a panel of inflammatory ligands, including LPS Se, PGN, Pam, and LPS Pg. Analysis of TNF-α mRNA by qRT-PCR showed profoundly lower expression in tolerized cell (Fig. 5-6b), consistent with the data at protein level. Beside TNF-α, production of IL-1β, IL-6, and IL-8 was also assessed in PGN- or Pam-tolerized conditions (Fig. 5-7) from the

same supernatant used in Figure 5-6a. PGN-tolerized THP-1 monocytes showed significant IL-1β reductions, by 20% against LPS Se challenge, 60% against PGN, and 50% against Pam, but not against LPS Pg (Fig. 5-7a). A similar pattern of IL-1β reduction was apparent in Pam-tolerized condition (Fig. 5-7a), where its suppression against LPS pg challenge was also significant. IL-6 was also diminished by ~50-70% in PGN- or Pam-tolerized THP-1 cells against the same ligands (Fig. 5-7b). Similarly, PGN- or Pam-tolerized THP-1 monocytes showed 20-30% reduction of chemokine IL-8 after PGN, Pam, or LPS Pg challenge, whereas no changes were observed against LPS Se challenge (Fig. 5-7c). Thus, PGN- or Pam-tolerized cells showed hyporesponsiveness to a wide range of inflammatory ligands by limiting the production of various mediators, suggesting that this tolerance plays broad role in innate immunity.

Having examined the tolerance in PGN- or Pam-primed THP-1 monocytes, we then investigated if there was any association of miR-132/-212 expression with this tolerance phenomenon. miRNA expression in the tolerized THP-1 cells was examined compared to the control (Fig. 5-6c). As expected, miR-132 and miR-212 in the PGN-tolerized sample showed significantly higher expression over 18 h of initial incubation plus 3 h of challenge compared to miR-146a, with up to a 10-fold increase, whereas little or no changes in expression of miR-155 was observed compared with untolerized controls (Fig. 5-6c). Analysis of TNF-α and miR-132 expression in the PGN-tolerized human PBMCs showed similar results to that obtained in THP-1 monocytes (Fig. 5-8d,e). Similar to PGN-primed PBMCs (226), Pam-primed PBMCs showed a reduction of TNF-α and IL-6 against PGN, Pam, and LPS challenges, negatively correlated with miR-132 expression (Fig. 5-7a-c). PGN-primed mouse primary macrophages showed a reduction of TNF-α that inversely correlated with miR-132 after challenge with PGN, Pam, and LPS (Fig. 5-6f,g). Murine macrophage RAW264.7 cells showed a similar tolerance phenomenon

(88) for which ~5-fold increase of miR-132 was observed (Fig. 5-9a,b). In addition to PGN or Pam, the capacity of flagellin to induce tolerance was also examined. Flagellin-primed THP-1 monocytes after challenge with flagellin, PGN, or Pam showed reduced TNF-α production which negatively correlated with miR-132 and miR-212 expression (Fig. 5-10a-c). Also, PGN- or Pam-primed THP-1 monocytes showed cross-tolerance to flagellin, the degree of which positively correlated with miR-132 expression (Fig. 5-10e,f).

Finding a consistent positive correlation between tolerance and miR-132/-212 expression, we investigated changes in adaptor kinase expression, which has been implicated in hyporesponsiveness. IRAK4 is an important candidate based on its indispensable role in the MyD88-dependent TLRs pathway and found to be a putative target of of miR-132/-212 (TargetScan.org). To find such potential link between IRAK4 and miR-132/-212, IRAK4 expression was analyzed at the levels of mRNA (Fig. 5-6h) and protein (Fig. 5-6i). PGN and Pam priming did not result in significant decrease of either IRAK4 or IRAK1 mRNA, the latter a known target of miR-146a, compared to control (Fig. 5-6h). This is not unexpected, as miRNA can regulate mRNA molecules via primarily translational repression. To support this, immunoblot analysis of IRAK4 protein was performed, showing a moderate reduction of ~32–76% as expected in all cases when normalized to tubulin level and compared to unprimed control, no matter the priming with PGN or Pam (Fig. 5-6i). A similar decrease of IRAK4 protein was evident in PGN-treated RAW264.7 cells (up to 64% reduced, Fig. 5-9c), in flagellin-treated THP-1 cells (up to 41% reduced, Fig. 5-10d), and in THP-1 monocytes treated with PGN or Pam for 18 h (up to 90% reduced, Fig. 5-10g). To investigate the probable cause for the variations in IRAK4 protein reduction, increasing priming concentrations of PGN or Pam were examined. The data showed that increasing priming concentrations from 0.01 to 1000 μg/ml

yielded a greater reduction in IRAK4 protein, as evidenced by immunoblot analysis (Fig. 5-11d and h). Notable tolerance was demonstrated by significant reduction of TNF-α at priming with 0.5 or 1 μg/ml PGN or Pam (Fig. 5-11a,e). With the increase of priming dose, miR-132/-212 expression was also increased as expected and priming with 1 μg/ml PGN or Pam caused a greater than 90% reduction ($P < 0.01$) of TNF-α, which correlated with the higher miR-132 or miR-212 levels that were not observed at lower PGN/Pam priming concentrations (Fig. 5-11a-c,e-g). In our recent report, elevated miR-146a expression depended on continuous exposure to LPS or PGN22. Similarly, in this report, after 12 h of PGN withdrawal, cells started to regain PGN responsiveness and were almost completely recovered from tolerance after 22 h. At this point, miR-132 and miR-212 expression was significantly lower ($P < 0.01$) compared to 18 h continuous PGN priming plus 5 h PGN challenge (Fig. 5-12a,b) indicating the importance of their presence at high levels to keep cytokine levels under control. The half life ($t_{1/2}$) of miR-132 and miR-212 estimated from this experiment was ~9-10 h and ~10-12 h, respectively. These data suggest the likely role of miR-132/-212 in PGN- or Pam-induced tolerance, based on the inverse correlation with proinflammatory cytokine production and the repressed levels of IRAK4. Another promising concept that can be introduced is that bacterial components seem to cause priming of miR-132/-212, which renders innate immune cells hyporesponsive to subsequent challenge.

Upregulation of miR-132 and miR-212 Alone Can Mimic PGN or Pam Priming to Induce Tolerance

Earlier in this report, the dramatic induction of miR-132 and miR-212 by PGN or Pam was shown (Fig. 1). To monitor the direct consequence of miR-132 and miR-212 expression in TLR2 ligand-induced tolerance, THP-1 cells were transfected with miR-132- or miR-212-mimic alone or in combination with miR-146a-mimic. miR-132- and miR-212-mimic transfection efficiency

was first confirmed ($P < 0.01$) in THP-1 cells by qRT-PCR (Fig. 5-13a). After 3 h challenge of transfected THP-1 cells with PGN, TNF-α production decreased by 58% in miR-132 mimic, 47% in miR-212 mimic, 50% in miR132 plus miR-212 mimic, 63% in miR-146a-mimic transfection (positive control), 70% in miR-132- plus miR-146a-mimic transfection, and 67% in miR-212-plus miR-146a-mimic transfection compared to mock transfected control (Fig. 5-13b left). A similar reduction of TNF-α was observed after challenge with Pam in the same transfected THP-1 monocytes (Fig. 5-13b right). However, miR-132 or miR-212 in combination with miR-146a was unable to eliminate TNF-α production, possibly because this certain level of TNF-α might be required to maintain innate immune homeostasis. The conserved nature of the effects of the miR-132 mimic was further examined by transfection of miR-132-mimic into RAW264.7 cells. Transfected cells showed ~25-30% TNF-α reduction compared to mock transfected control after challenge with PGN, Pam, and LPS (Fig. 5-13c).

As the IL-1 receptor and TLRs employ the same TIR domain to produce subsequent signaling, IL-1β signaling is supposed to be affected by miR-132 overexpression. To support this hypothesis, the inhibitory effects of miR-132-mimic in THP-1 monocytes were examined and the results showed a reduction of IL-8 (~20%) and RANTES (~55%) production after re-stimulated with IL-1β (Fig. 5-13d). To corroborate such a role of miR-132 or miR-212, a converse experiment was performed using those miRNA inhibitors. Respective inhibitors inhibited miR-132 or miR-212 expression by 90% ($P < 0.01$) compared with mock transfected THP-1 monocytes (Fig. 5-13e). In turn, TNF-α production was partially reversed (by ~35%) after 8 h post-challenge with Pam, compared to the mock transfected control (Fig. 5-13f). These data support the dominant role of PGN- or Pam-induced miR-132 and/or miR-212 to mediate tolerance.

IRAK4 is the Molecular Target of miR-132 and miR-212

In the above experiments, the levels of IRAK4 showed a negative correlation with miR-132/-212. According to the TargetScan algorithm, IRAK4 shares the same single 3'UTR binding site for both miR-132 and miR-212 (Fig. 5-14a). Following this, the interaction between IRAK4 and miR-132/-212 was validated by a reporter expression assay, where THP-1 monocytes were co-transfected with pMir-3'IRAK4 vector (reporter) and miR-132-mimic, miR-212 mimic, or mock into HEK293 cells. Luciferase expression was significantly reduced with miR-132 mimic (~50%) and miR-212-mimic (~25%), while no significant modulation was observed with miR-146a-mimic or for IRAK4-mut (Fig. 5-14b). To further verify the direct regulation of IRAK4 by miR-132/-212, the expression of IRAK4 mRNA and protein was assayed in THP-1 cells, which showed 35-40% reduction after miR-132- and miR-212-mimic transfection compared to mock or miR-146a-mimic control oligos transfection (Fig. 5-14c,d). Conversely, IRAK4 mRNA was moderately increased after blocking of mature miR-132/-212 using the antisense in THP-1 monocytes (Fig. 5-14e). Having confirmed IRAK4 as a molecular target of miR-132 and/or miR-212 using various approaches, we examined the knockdown effect of IRAK4 in terms of cytokine secretion in THP-1 monocytes. siRNA targeting IRAK4 (siIRAK4) showed ~50% reduction at the mRNA level , which was supported by data at protein levels using immunoblot (Fig. 5-14f,g). With silencing of IRAK4, TNF-α, IL-6, and IL-8 were markedly reduced (40-50%) after PGN, Pam, and LPS challenge (Fig. 5-14h-j); part of those reductions are similar to previous reports (83, 342). The overall findings suggest that IRAK4 might be targeted by miR-132 and/or miR-212, a promising mechanism to prevent excessive cytokine production.

Bacteria-induced miR-132 and miR-212 Contribute to Resistance to Bacterial Infection

In the preceding experiments, PGN- or Pam-induced tolerance or cross-tolerance was observed against purified TLR2, TLR4, and TLR5 ligands. However, in nature, hosts are

exposed to whole bacteria that usually display more than one type of TLR ligands. Thus, to extend the analysis of miR-132/miR-212 expression, induction of miR-132 and miR-212 by whole bacteria to cause tolerance in THP-1 monocytes was examined. In this study, heat-killed (HK) *P. gingivalis* and *T. forsythia* significantly induced miR-132 ($P < 0.01$) and miR-212 to a similar extent (data not shown), whereas HK *T. denticola* had no detectable effect (Fig. 5-15a). When infected with live *T. forsythia*, THP-1 cells showed a similar miR-132 expression whereas *P. gingivalis* and *T. denticola* showed relatively low levels of induction (Fig. 5-15b left). As expected, in the same infected monocytes, miR-212 showed a similar expression pattern as miR-132 (Fig. 5-15b right). In contrast, miR-146a expression was significantly lower than miR-132 or miR-212 (data not shown), similar to previous results (118). HK *T. forsythia*-primed THP-1 cells showed significant reduction ($P < 0.01$) of TNF-α after challenge with HK bacteria and various ligands (Fig. 5-15c) and a similar reduction was observed in live *T. forsythia*-infected THP-1 monocytes (data not shown). HK *T. forsythia*-tolerized THP-1 cells showed a significant increase ($P < 0.001$) of miR-132 (Fig. 5-15d) compared to untolerized controls. In line with higher miR-132 and miR-212 expression, HK and live *P. gingivalis*- and *T. forsythia*-treated THP-1 monocytes showed substantial reduction (50-60%) of IRAK4 protein expression vs control (Fig. 5-15e). These data were consistent with the above findings in PGN- and Pam-tolerized THP-1 monocytes. Of note, none of these bacteria induced miR-132 and miR-212 to a similar extent (especially *T. denticola*), suggesting their expression might be bacteria- and ligand-specific. In summary, higher expression of miR-132/-212 plays an important role in providing tolerance or cross-tolerance in the in vitro THP-1 cell model against various TLRs ligands, as well as bacterial infection.

Discussion

Emerging results indicate that TLR activation affects the expression of a few key miRNAs. Recently, an increased expression of miR-146a in response to LPS in THP-1 monocytes was described (82), while a relatively smaller amount of miR-132 induction was noted in the same condition, although no detailed expression analysis of miR-132 and miR-212 by other TLR ligands has been documented. Of note, induction of miR-212 by innate immune ligand has not been described previously.

Rapid and High Levels of miR-132/-212 Induction by PGN, Pam, or Flagellin Stimulation Controlled by CREB Activation

In this report, miR-132 and miR-212 expression kinetics following exposure to several microbial ligands were examined in THP-1 monocytes, human PBMCs and mouse primary macrophages. Unlike LPS, TLR2/TLR5 ligands (PGN, Pam, or flagellin) triggered a sharp increase in miR-132/-212 expression at earlier time-points compared to miR-146a in THP-1 monocytes (Fig. 5-16a). The level of miR-132 remained at a significantly high levels over 48 h, from which we can conclude that, compared to miR-146a, miR-132/-212 have earlier expression kinetics, which may be very important during acute infection. It is noted that although miR-132/-212 showed higher fold changes than miR-146a (~80-fold vs 15-fold, respectively, at 12 h), copy number of miR-132 (~100 copies/cell) was lower than miR-146a (~500 copies/cell). The fact is that untreated THP-1 cells have a lower basal level of miR-132 than miR-146a. Upon PGN or Pam stimulation, TNF-α production was negatively correlated with miR-132/-212 expression similar to previous observations on kinetics of miR-146a and TNF-α after LPS stimulation21. Similar to LPS-induced tolerance mediated by miR-146a (82), the PGN-induced miR-132/-212 highlights a promising avenue for study in relation to innate immunity. Higher fold change of

miR-132/-212 induced by TLR2/TLR5 ligands might represent the early response to infection, followed by the miR-146a response as reported for TLR4 stimulation (81, 82).

miR-132/-212 is located on chromosome 17p13 and transcriptionally activated by CREB in neurons. Its rapid upregulation has been observed in KSHV-infected cells through phosphoryation of CREB (119). In this report, LPS induced a lower level of miR-132/-212 compared to PGN/Pam stimulation. Although both LPS and PGN have been reported to interact with CD14 (343, 344), differences in binding affinity to different receptors (TLR4 vs TLR2) and other unknown factors may all contribute to differences in the specificity of the dominant miRNA induction (miR-146a vs miR-132/-212). Gupta et al. reported that PGN-induced phosphorylation of CREB was not due to endotoxin contamination (341); this demonstration of PGN specificity is consistent with the fact that only ultra pure ligands were used in this study. Moreover, PGN and LPS induce differential activation of MAP kinases, with LPS strongly inducing all three families of kinases (ERK, JNK, and p38), whereas PGN only induces ERK and JNK without affecting p3835. Inhibition of activation of these kinases by U0126 and PD98059, reduced both in primary and mature miR-132/-212, while miR-146a was not diminished, indicating their specificity and probably without affecting other general miRNA processing function. CREB-mediated expression of miR-132/-212 induced by PGN or Pam is a novel finding and this opens a new horizon to evaluate their kinetics in depth in innate immunity regarding the mechanism of cross-tolerance. CREB-regulated rapid miR-132 induction may serve as an anti-apoptotic response in macrophages. Thus, CREB activity is important in innate immunity against certain bacteria, such as *Salmonella spp*., *Shigella spp*., and *Yersiniae spp*., which inhibit survival signals and induce apoptosis of macrophages as a mechanism to evade the host immune response (345).

miR-132/-212 Plays Important Roles in TLR2 Ligand-induced Tolerance and Cross-tolerance

Although PGN is a potent trigger for cytokine production, tolerance induced by this gram-positive bacteria cell wall component has not been studied as extensively as endotoxin tolerance. The ability of PGN to induce heterologous tolerance shown in this study is congruent with previous findings (88, 339). Accordingly, monocytes primed with PGN or Pam showed hyporesponsiveness to TLR2 ligands (PGN, Pam, LPS from *P. gingivalis*) or TLR5 ligand flagellin. Moreover, flagellin-primed cells showed tolerance to itself, PGN, or Pam with notably higher miR-132/-212 expression. Thus, these new findings support that tolerance or cross-tolerance is linked to miR-132/-212 overexpression. To rule out other players in the complex TLR signaling pathway and to verify the dominant effect of miR-132 and miR-212 alone or in combination with miR-146a in tolerance, transfection experiments were performed with the corresponding miRNA-mimics (Fig. 5-13). These miRNA-mimics transfected cells were significantly less responsive to either PGN or Pam, a similar phenomenon observed in PGN-induced tolerance. On the other hand, knockdown of miR-132 and miR-212 expression using chemically modified miRNA inhibitors, also known as antagomirs (267), alone or combination with knockdown of miR-146a in THP-1 cells showed an increased inflammatory response to TLR2 ligand or a sometimes delayed decrease of TNF-α compared to control. Taken together, miR-132/-212 plays an important regulatory role in cytokine production.

Regulation of IRAK4 by miR-132/-212 is a Major Mechanism for PGN-induced Tolerance

miR-132 and miR-212 share an identical seed sequence and thus, would be expected to regulate a similar subset of target genes. However, miR-132 and miR-212 can be employed for similar (346) or distinct functions in different cell types (151, 152). In this report, IRAK4 was validated as a molecular target for miR-132 and miR-212 with apparently different degree of

efficiency. In experiments to evaluate the effect of IRAK4 knockdown on PGN-stimulated tolerance, cytokine response was not eliminated, but significantly reduced, suggesting that other IRAK family members may compensate in part during infection. This mechanism seems biologically relevant since IRAK4-deficient mice were viable against *S. typhimurium* infection (342). Fig. 5-16 outlines the model that demonstrates how PGN-induced miR-132/-212 play an important role in the response to microbes or its components at early stages of infection and limits the overstimulation of proinflammatory cytokines by suppressing IRAK4. Low-dose PGN-primed THP-1 cells (10 ng/ml, panel A) produce TNF-α rapidly and continue to do so for 4 to 6 h. As soon as regulatory miR-132 starts to increase, then TNF-α production decreases. At 18 h post priming, a profound difference between miR-132 expression and TNF-α secretion is established due to the negative effect on IRAK4 by upregulated miR-132, which leads to tolerance (Fig. 5-16a). miR-212 is not shown in Fig 5-16a,b, but it likely serves as an additive to miR-132. Unlike the untolerized control, tolerized cells do not respond to even high dose of PGN challenge (Fig. 5-16b). In TLR signaling, the current understanding from structural studies is that binding of TLR ligand to receptor activates the formation of myddosome which involves the helical assembly of the MyD88-IRAK4-IRAK2/IRAK1 complex (256). Thus IRAK4 is recruited to MyD88 earlier than IRAK2/IRAK1, both targets of miR-146a (116, 122). As discussed above, the miR-132/-212 response to TLR2 ligand appears earlier acting on IRAK4, while miR-146a affects IRAK1/2 and TRAF6 somewhat later. From our tentative model, it is reasonable to assume that either their sequential (first miR-132 and followed by miR-146a) or combined miRNA regulatory activity provides an efficient negative regulatory loop for TLR signaling with the apparent focus on the formation of the myddosome complex (Fig. 5-16c), which in turn obviates the driving forces behind inflammatory diseases. Our findings fully

support the dominant role of miR-132/-212 in *in vitro* PGN- and related ligand-induced tolerance. Beside purified ligands, whole heat-killed or live *P. gingivalis* and *T. forsythia* stimulation showed significant expression of miR-132/-212 in monocytes. Subsequently, *T. forsythia*-primed monocytes showed significant reduction of TNF-α after challenge with various ligands due to the reduction of IRAK4 by higher miR-132/-212 expression. It is interesting to speculate that miR-132/-212 induced by bacteria or its components may play a role in immune-inflammatory diseases, such as periodontitis, by affecting IRAK4 or other targets like MMP-9 as shown in a report on the critical role of miR-132/-212 for epithelial-stromal interactions via targeting MMP-9 (346).

Biological Significance of TLR2 Ligand-induced Tolerance Via miR-132/-212 Expression

The major milestone of this study has been highlighted using various cell types, including human and murine monocytes/macrophage cell lines, murine primary macrophages, and human PBMCs. In these cells, miR-132 and/or miR-212 were upregulated in response to purified ligands, as well as bacteria. Through upregulation of these miRNAs, cells showed tolerance to various inflammatory components. This phenomenon can be mimicked in humans when exposed to microbial components where the induced miR-132/-212 plays various roles in the host, including innate immunity. MyD88-dependent TLR pathways use IRAK4 and patients deficient in IRAK4 failed to respond to IL-1, IL-18, and six other TLRs (TLR1-5 and TLR9) as expected. Accordingly, the upregulated miR-132/-212 in PGN-mediated tolerance is likely to affect other pattern recognition receptor activity in innate immunity. As all TLRs, with the exception of TLR3, use IRAK4 and the MyD88-dependent pathway, they all are likely to be regulated by miR-132/-212 in a comparable manner. Detection and activation of immune cells in response to PGN can also occur through alternate pattern recognition receptors including NOD1 and NOD2, CD14, and a family of peptidoglycan recognition proteins and it remains possible that these

receptor signaling pathways are involved in the mechanism of PGN-induced tolerance. As a consequence, PGN-induced tolerance associated with the upregulated miR-132/-212 may have a broader role in regulating TNF-α by TLR pathways.

The innate immune response to the invading microorganism in animals may be influenced by miR-132/-212. Murphey et al. found that PGN-tolerant mice were significantly resistant to both gram-positive (*S. aureus*) and gram-negative (*Pseudomonas aeruginosa*) bacteria (339). Overexpression of miR-132/-212 associated with bacteria or PGN tolerance is likely to have important consequences in host innate immunity responding to myriad bacterial infections. More extensive studies on the expression kinetics are needed to fully explore the role of these miRNAs, especially in terms of its half-life in PGN-tolerant animals. *In vivo* investigations, such as the phenotypic analysis of mice with targeted deletion of miR-132/-212, will be necessary to fully elucidate the role of these miRNAs in innate immunity. Note that miR-132/-212 have been reported as dysregulated in cancer (152, 347, 348) and overexpression of miR-132 has been shown in such inflammatory diseases as rheumatoid arthritis and osteoarthritis (349). Therefore, miR-132/-212 expression may be associated with inflammation and tumorigenesis, and, given its role in innate immunity, might be an important link between inflammation and cancer.

In summary, a series of evidence provides mechanistic insights into the function of miR-132/-212 in TLR2/TLR5 ligand-induced tolerance, which operates as a negative regulatory feedback mechanism to prevent uncontrolled inflammatory reaction potentially comparable to that observed in sepsis. It should be noted that such key components of MyD88-pathway as IRAK1/2 and TRAF6 are regulated by miR-146a and our report shows that IRAK4 is targeted by miR-132/-212. These miRNAs have now been shown to work in what appear to be a complementary fashion in response to the various ligand stimulations or bacterial infections.

Thus, further investigations of the modulation of the levels of miR-132/-212 alone and/or in combination with miR-146a may be very important as these are interesting targets for therapeutic intervention for boosting or limiting TLR activation.

This work has been submitted for publication. Nahid MA, Yao B, Dominguez-Gutierrez PR, Satoh M, Chan EKL. miR-132 and miR-212 are important for innate immunity through establishing TLR2 ligand-induced tolerance via modulation of IRAK4 expression.

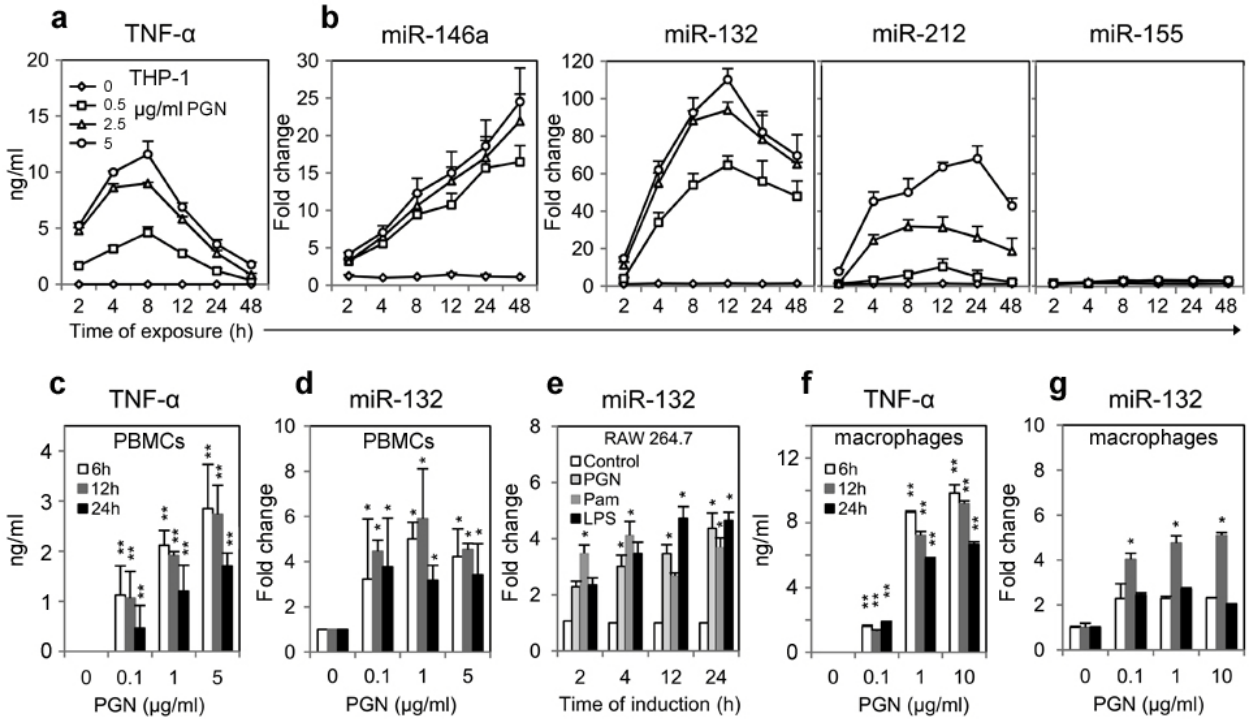

Figure 5-1. PGN induction of TNF-α and miRNA expression kinetics in monocytes/macrophages. (a) Dose-response and time-course analysis of TNF-α secretion in culture supernatant by THP-1 monocytes stimulated with 0-5 μg/ml PGN for 2-48 h (horizontal axis). TNF-α in culture supernatants at the indicated time points was measured by ELISA. (b) Quantitative RT-PCR (qRT-PCR) analysis of miR-146a, miR-132, miR-212, and miR-155 expression kinetics in respective PGN-treated THP-1 cells. (c,d) Dose- and time-dependent secretion of TNF-α and induction of miR-132 in human PBMCs stimulated with 0-5 μg/ml PGN for 6-24 h. (e) qRT-PCR analysis of miR-132 expression kinetics in mouse RAW 264.7 cells stimulated for 2-24 h with 1 μg/ml PGN, Pam, or LPS. (f) TNF-α secretion by mouse primary macrophages treated for 6-24 h with 0-10 μg/ml PGN. (g) qRT-PCR analysis of miR-132 expression. Human and mouse miRNA expression was normalized with control RNU44 and SnoRNA202, respectively. Data are from three independent experiments (a-e, mean ± s.d.), or triplicate measurement of each condition (f,g; mean ± s.d.). *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with untreated control.

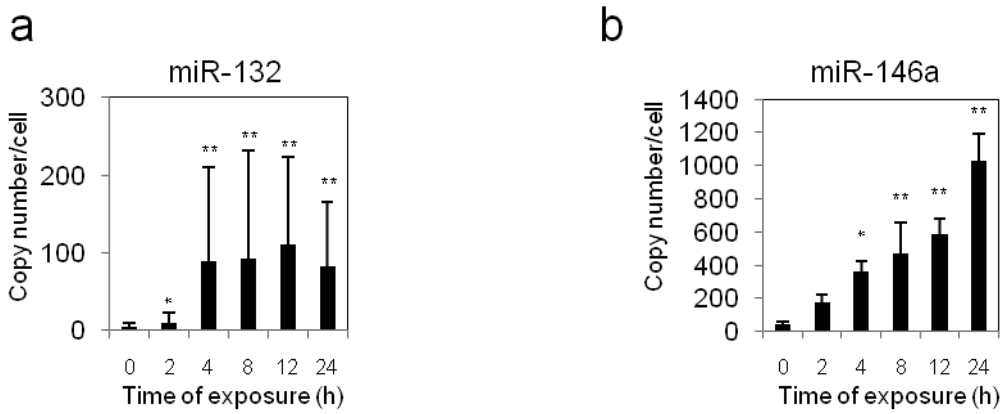

Figure 5-2. Time-course analysis of copy number of miR-132 and miR-146a in PGN-stimulated THP-1 monocytes. (a,b) Copy number of miR-132 and miR-146a in THP-1 cells stimulated for 0-24 h (horizontal axis) with PGN (2500 ng/ml). Data are expressed as mean ± s.d. from three independent experiments. *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with untreated control.

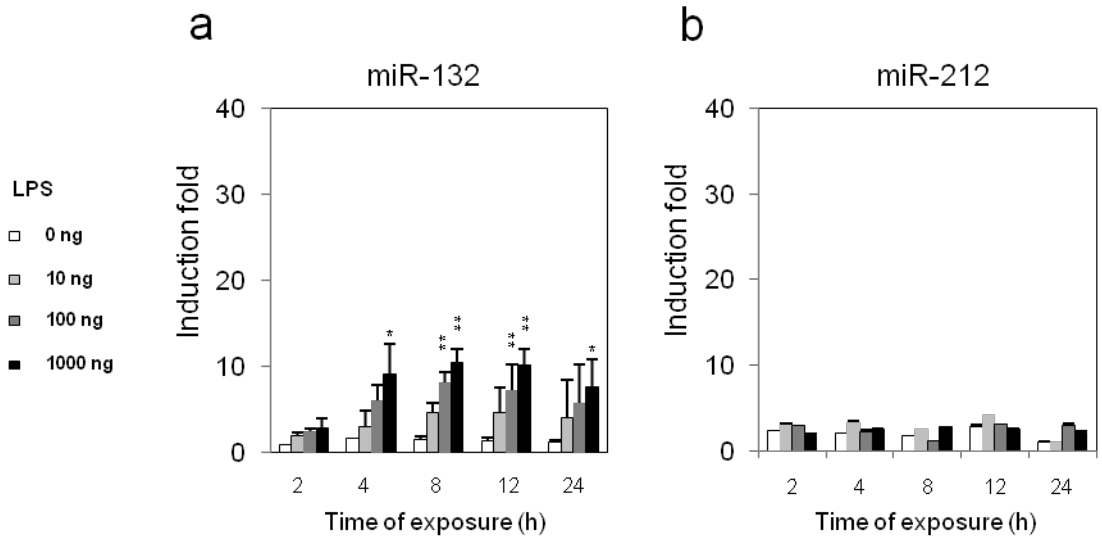

Figure 5-3. Induction of miR-132 and miR-212 by LPS treatment in THP-1 monocytes. qRT-PCR analysis of dose- and time-dependent expression of miR-132 (a) and miR-212 (b) in THP-1 monocytes stimulated for 2-24 h (horizontal axis) with 0-1000 ng/ml LPS. Data are expressed as mean ± s.d. of three independent experiments. *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with untreated control.

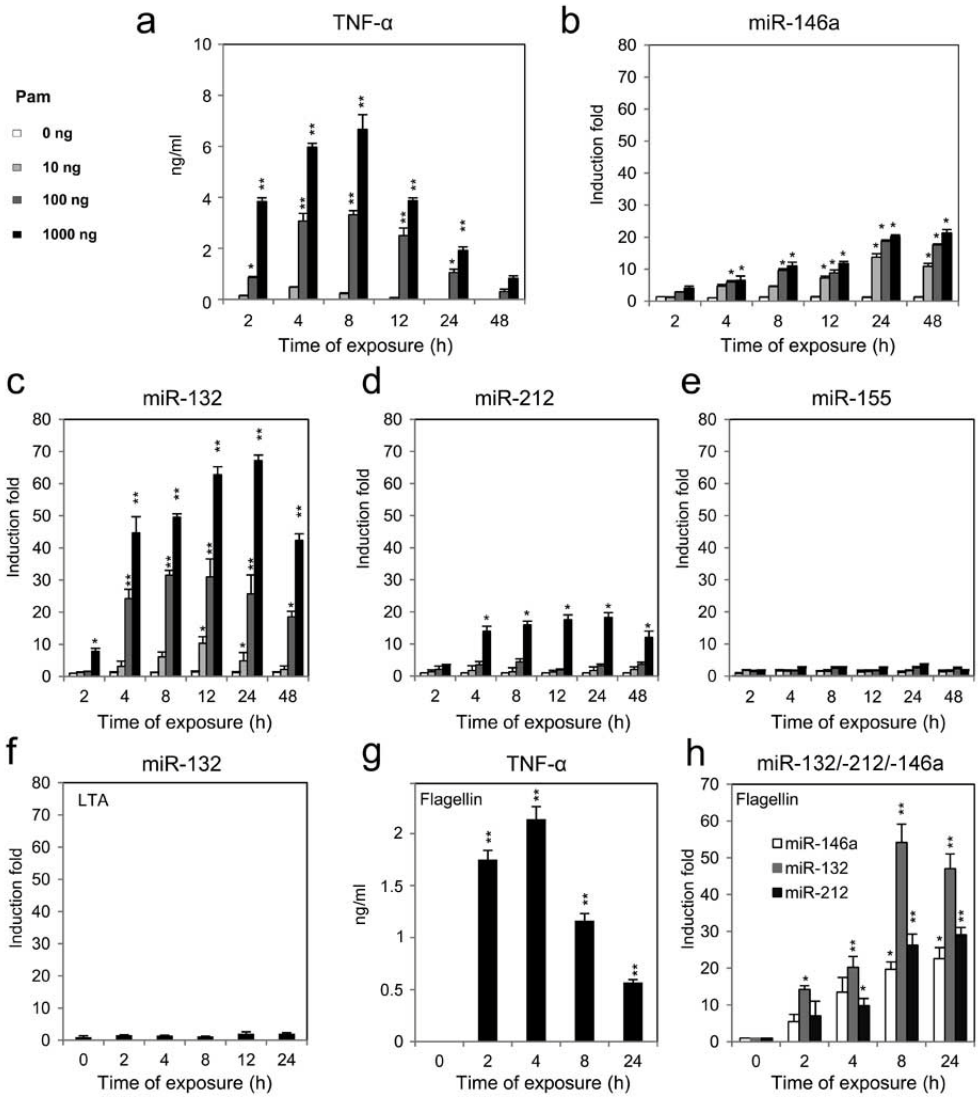

Figure 5-4. TNF-α protein secretion and miRNA expression kinetics in PGN, Pam3CSK4 (Pam), and lipoteichoic acid (LTA)-stimulated THP-1 monocytes. (a) TNF-α production by THP-1 cells stimulated with Pam. THP-1 monocytes were incubated for 2-48 h with 0-1000 ng/ml Pam and TNF-α in culture supernatant was measured by ELISA. (b-e) qRT-PCR analysis of miR-146a, miR-132, miR-212, and miR-155 expression kinetics in Pam-treated THP-1 cell. (f) No significant expression of miR-132 in THP-1 monocytes stimulated with LTA (1000 ng/ml) for 2-48 h. (g) TNF-α production by THP-1 monocytes stimulated for 0-24 h with 300 ng/ml flagellin. (h) qRT-PCR analysis of miR-146a, miR-132, and miR-212 expression in the same THP-1 cells stimulated with 300 ng/ml flagellin. All miRNA expressions were normalized to RNU44. Data are from three independent experiments (mean ± s.d.). *$P < 0.05$; **$P < 0.01$ (two-tailed unpaired t-test) compared with untreated control.

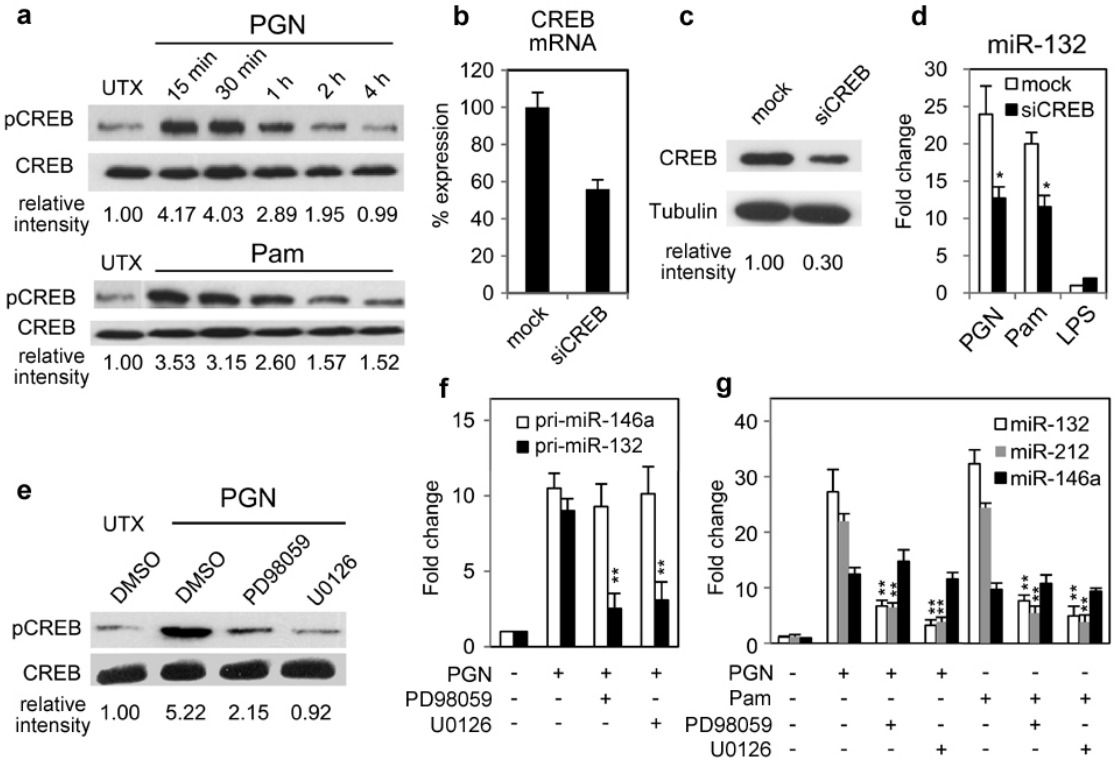

Figure 5-5. Rapid induction of miR-132 and miR-212 in PGN-/Pam-stimulated THP-1 monocytes is mediated through CREB-dependent machinery. (a) Immunoblot analysis demonstrating phosphorylation of CREB (Ser 133, pCREB) within 15 min after PGN (top) or Pam (bottom) stimulation of THP-1 cells. Relative intensity is calculated as ratios of pCREB to total CREB in cell lysates. (b) qRT-PCR analysis of CREB mRNA expression in THP-1 cells transfected with (100 nM) or without (mock) CREB-specific siRNA (siCREB). (c) Immunoblot analysis showing 70% reduction of CREB expression in THP-1 cells transfected with 100 nM siCREB using tubulin as a loading control. (d) miR-132 expression in siCREB-knockdown THP-1 cells stimulated for 5 h with PGN, Pam, and LPS. (e) Two-hour pre-treatment of synthetic kinase inhibitors PD98059 and U0126 blocked phosphorylation of CREB in THP-1 monocytes stimulated with 1 μg/ml PGN for 30 min. Immunoblot analysis showed 5.22-fold increase in relative intensity of pCREB in PGN-treated versus untreated control (UTX) with addition of DMSO alone and then stimulated for 30 min with 1 μg/ml PGN. (f,g) qRT-PCR analysis of primary- and mature-miR-132, miR-212, and miR-146a expression in THP-1 monocytes pre-treated for 2 h with inhibitors PD98059 and U0126 and then stimulated for 8 h with PGN or Pam. Data points and error bars represent three independent experiments (b,d,f,g, mean ± s.d.). *$P < 0.05$; **$P < 0.01$ (two-tailed unpaired t-test) compared with untreated THP-1 monocytes.

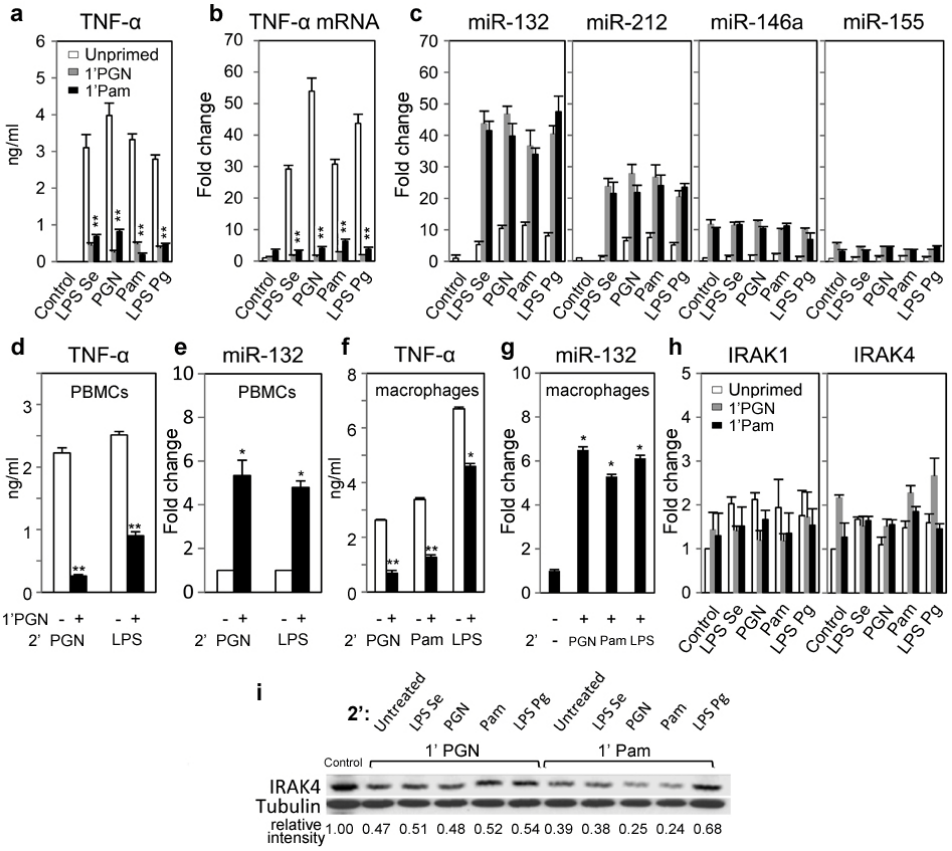

Figure 5-6. High levels of miR-132 and miR-212 may account for TLR2 ligand-induced tolerance. (a) TNF-α production by THP-1 monocytes primed with (tolerized) or without (untolerized) PGN or Pam (500 ng/ml) for 18 h and then challenged with ligands (1000 ng/ml) for 3 h. (b) qRT-PCR analysis of TNF-α mRNA in the PGN and Pam primed THP-1 cells as in a. (c) qRT-PCR analysis of miR-132, miR-212, miR-146a, and miR-155 expression in the same tolerized THP-1 cells as in a. (d) TNF-α secretion by human PBMC primed with or without PGN (1000 ng/ml) for 18 h and then challenged with PGN or LPS for 5 h. (e) qRT-PCR analysis of miR-132 in the PGN-tolerized and untolerized PBMC as in d. (f) TNF-α production by mouse primary macrophages primed with or without PGN (1000 ng/ml) for 18 h and then challenged with PGN, Pam, or LPS for 5 h. (g) qRT-PCR analysis of miR-132 in the same macrophages as in f. (h) IRAK1 and IRAK4 expression in the THP-1 monocytes as in a, assessed by qRT-PCR. (i) IRAK4 protein levels in PGN- or Pam-primed and unprimed THP-1 cells challenged with various TLR ligands for 2 h, analyzed by Immunoblot. Tubulin serves as a loading control. Data are expressed as mean ± s.d. of three independent experiments (a-c and h), triplicate samples of each condition (d,e), and two experiments with three individual (f,g). *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with control.

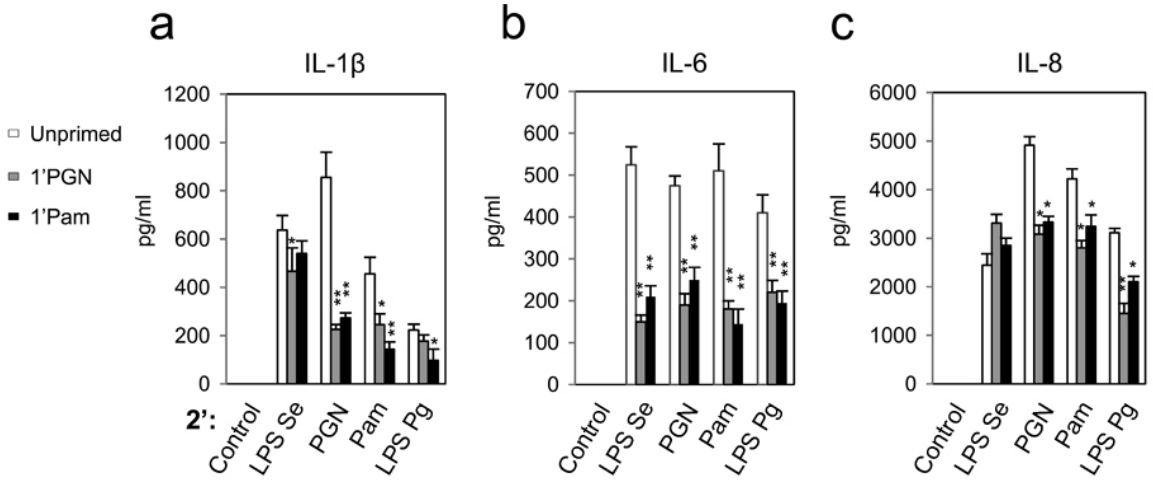

Figure 5-7. Diminished proinflammatory cytokine secretions by PGN- and Pam-primed THP-1 monocytes. ELISA of IL-1β (a), IL-6 (b), and IL-8 (c) production by THP-1 monocytes primed with or without PGN or Pam (500 ng/ml) for 18 h and then challenged with various ligands (horizontal axis) for 3 h (IL-1β) or 24 h (IL-6 and IL-8). Data are expressed as mean ± s.d. of two independent experiments. *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with unprimed control.

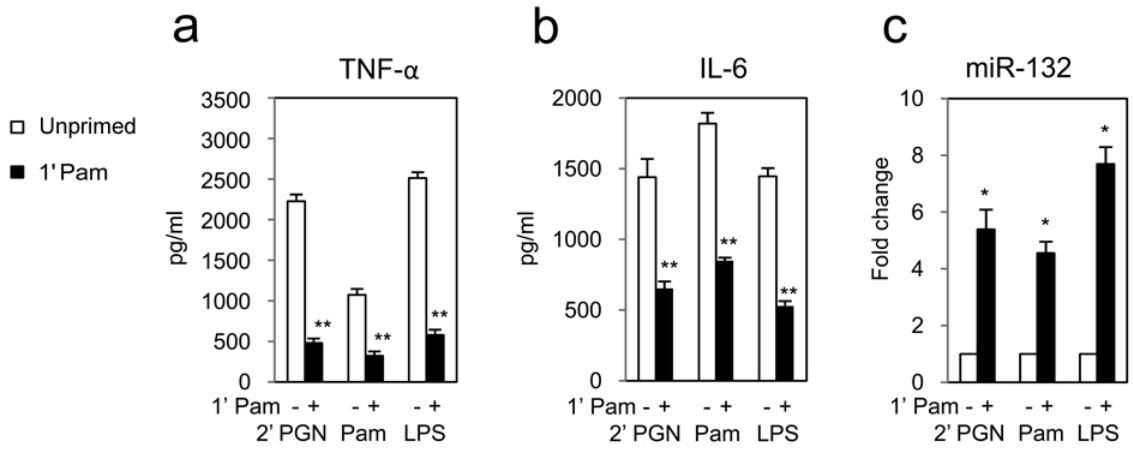

Figure 5-8. High levels of miR-132 may account for Pam-induced tolerance in human PBMCs. TNF-α (a) and IL-6 (b) production by THP-1 monocytes primed with or without Pam (1000 ng/ml) for 18 h and then challenged with various ligands (horizontal axis) for 3 h (TNF-α) or 24 h (IL-6). (c) qRT-PCR analysis of miR-132 in the PBMC treated as in a. Data are representative of three independent experiments (mean ± s.d.). *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with unprimed control.

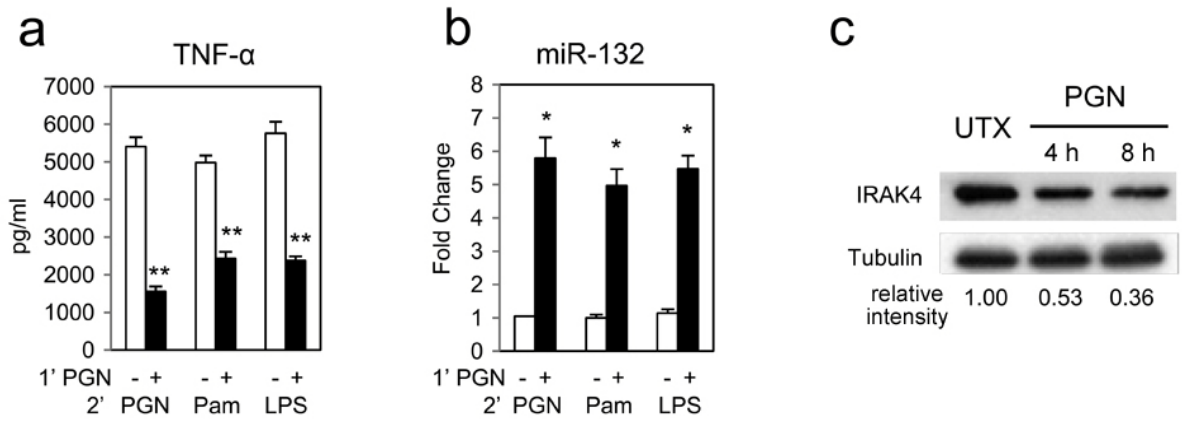

Figure 5-9. High levels of miR-132 may promote PGN-induced tolerance in the mouse RAW264.7 cells. (a) TNF-α production by RAW264.7 cells primed with or without PGN (1000 ng/ml) for 18 h and then challenged with various ligands (horizontal axis) for 3 h. (b) qRT-PCR analysis of miR-132 in the purified total RNA obtained from cells described in a. (c) Immunoblot analysis of IRAK4 in RAW264.7 cells stimulated for 4 and 8 h with PGN (2000 ng/ml). Tubulin serves as a loading control. Data and error bars are from two independent experiments (a,b, mean ± s.d.). *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with unprimed control.

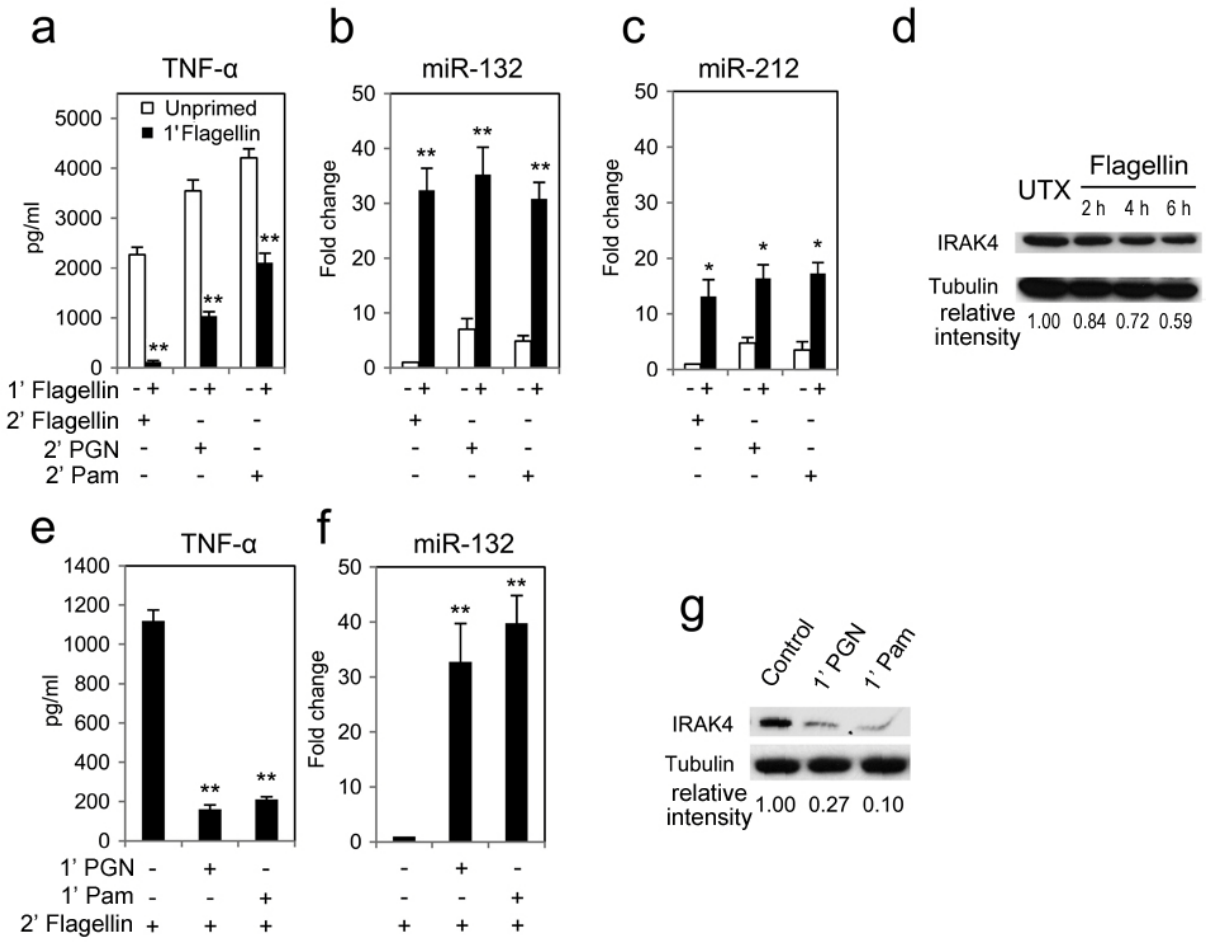

Figure 5-10. Flagellin-induced tolerance contributed by high level of miR-132 and miR-212 expression. (a) TNF-α production by THP-1 cells primed with or without flagellin (200 ng/ml) for 18 h and then challenged with flagellin (300 ng/ml), PGN (1000 ng/ml), or Pam (1000 ng/ml) for 3 h. (b,c) qRT-PCR analysis of miR-132 and miR-212 expression in the same THP-1 cells as in a. (d) Immunoblot analysis of IRAK4 in THP-1 monocytes stimulated for 2, 4, or 6 h with flagellin (200 ng/ml). Tubulin serves as a loading control. (e) TNF-α production by THP-1 cells primed with or without PGN or Pam (1000 ng/ml) for 12 h and then challenged with 300 ng/ml flagellin for 3 h. (f) qRT-PCR for miR-132 expression in the PGN or Pam primed THP-1 monocytes as described in e. (g) Immunoblot analysis of IRAK4 and tubulin (loading control) in THP-1 monocytes stimulated for 3 h with PGN and Pam (1000 ng/ml). Data are representative of three independent experiments (a-c,e,f, mean ± s.d.) and two experiments (d,g). *$P < 0.05$; **$P < 0.01$ (two-tailed unpaired t-test) compared with untreated control.

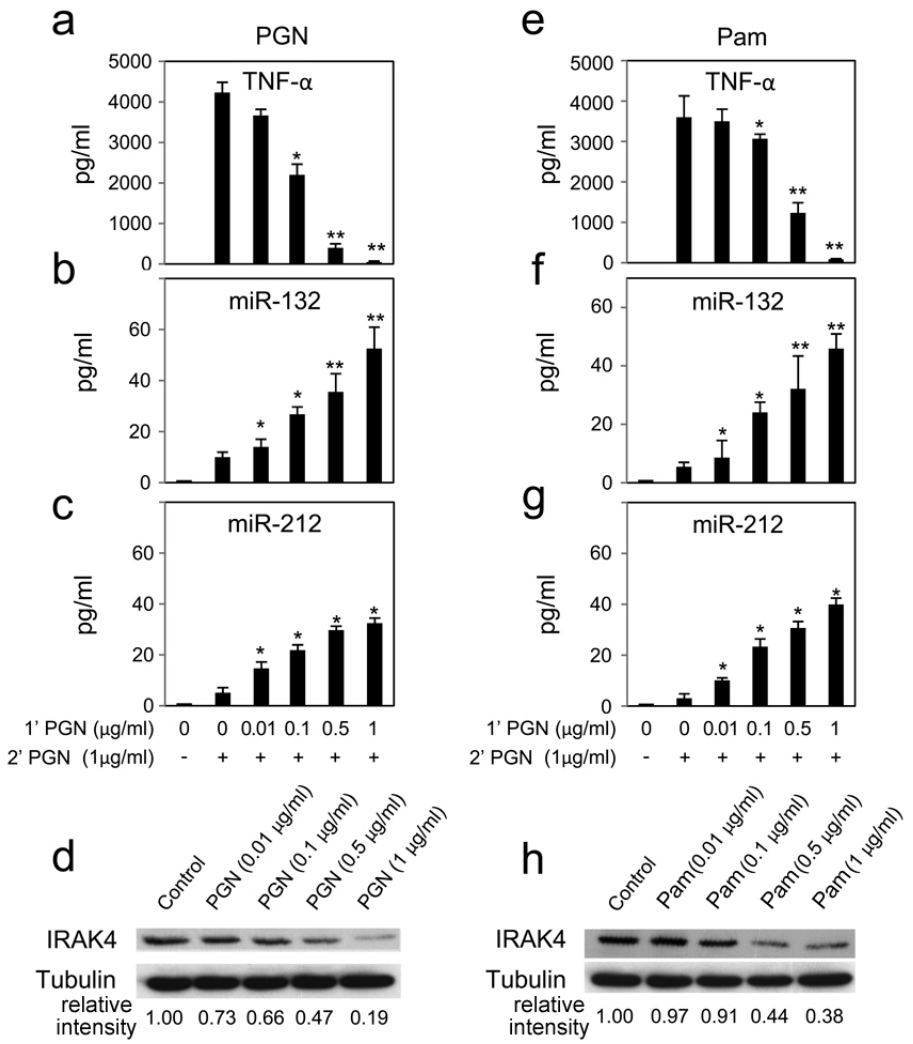

Figure 5-11. Dose-dependent priming effect of PGN and Pam to induce efficient tolerance are inversely correlated to the levels of miR-132 and miR-212 expression. (a,e) TNF-α levels by THP-1 monocytes primed with 0-1 μg/ml PGN (1'PGN) or Pam (1'Pam) for 18 h and then challenged with high doses of PGN or Pam (~1 μg/ml, 2' challenge) for 3 h. (b,f) miR-132 expression in the same PGN-treated THP-1 monocytes as in a and e. (c,g) miR-212 expression in the THP-1 monocytes as mentioned in a and e. (d,h) Immunoblot analysis for IRAK4, and tubulin (loading control) in the THP-1 cell lysates collected 2 h after PGN or Pam challenge (0-1 μg/ml) Data points and error bars represent of three independent experiments (a-c,e-g, mean ± s.d.) and two experiments (d,h). *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with untreated THP-1 cells.

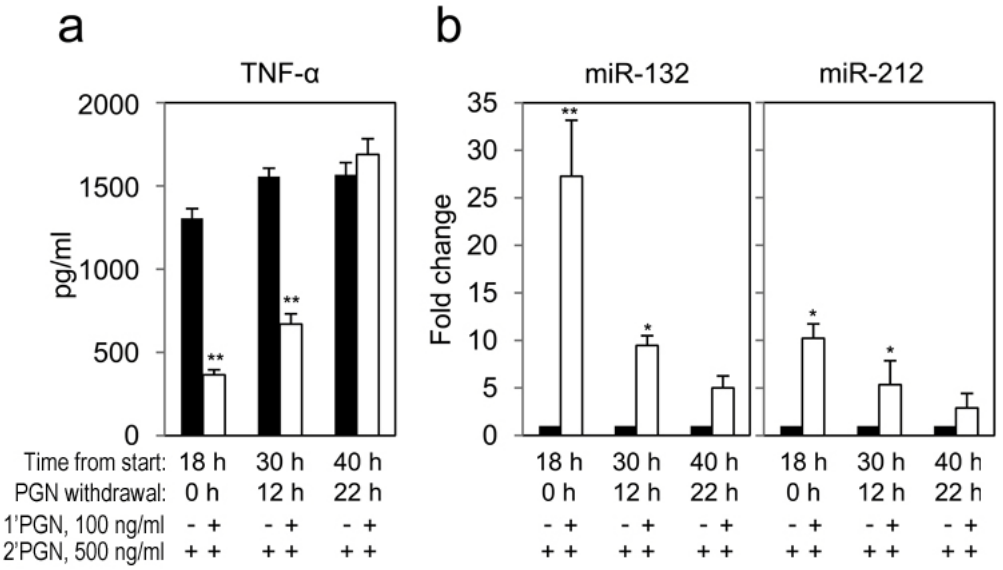

Figure 5-12. Attenuation of miR-132 and miR-212 expression in PGN-tolerized THP-1 cells inversely correlated with TNF-α production. (a) TNF-α production by THP-1 cells cultured with (tolerized) or without (untolerized) PGN (100 ng/ml) for 18 h and then cells were washed with PBS and cultured in complete growth medium for an additional 0, 12, or 22 h (PGN withdrawal). At each time point, 8x105 cells were challenged for 5 h with PGN (500 ng/ml) prior to analysis of TNF-α in culture supernatant. (b) qRT-PCR analysis of miR-132 and miR-212 in the same THP-1 cells as described in a. Values are mean ± s.d. from three independent experiments. *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with control THP-1 cells.

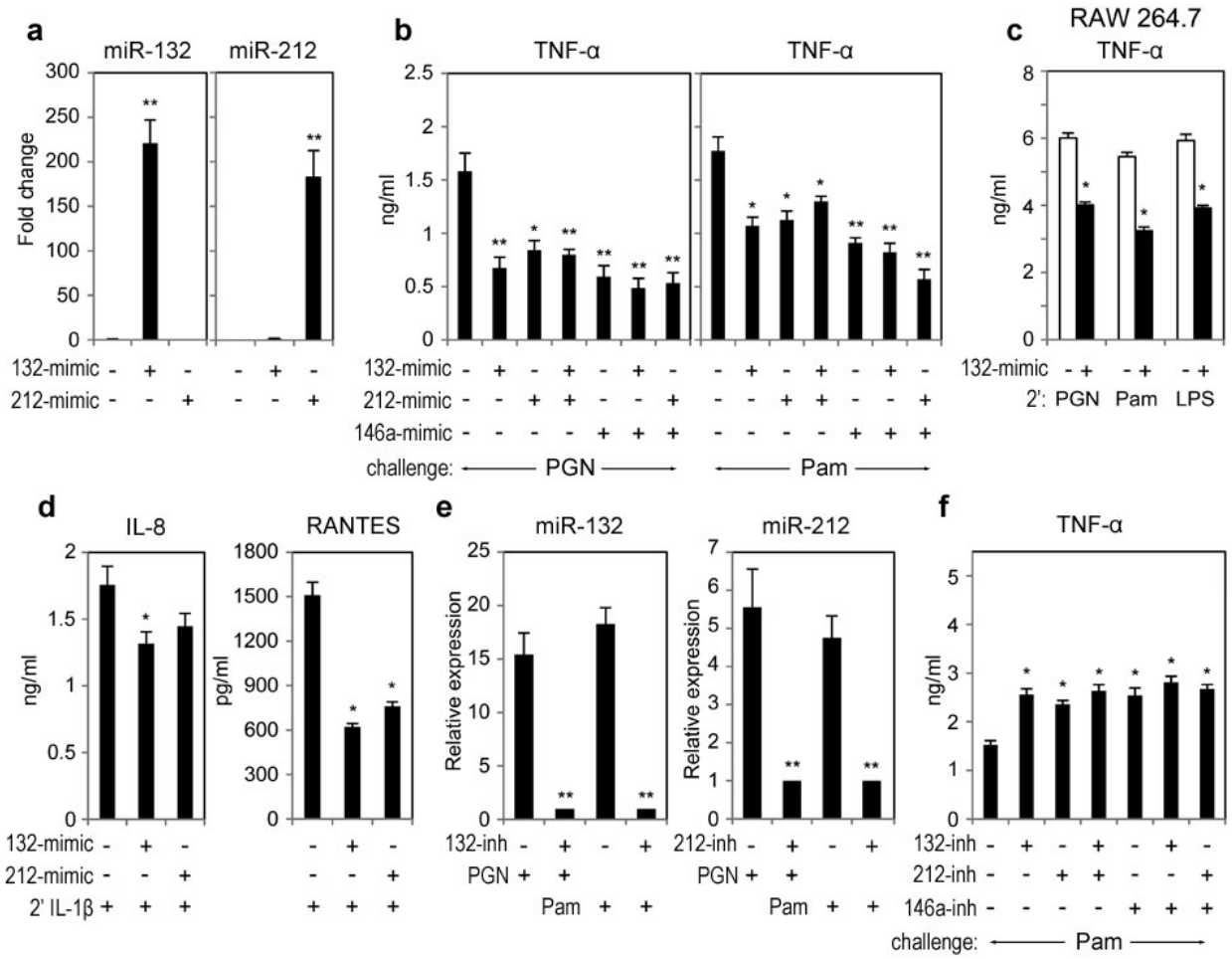

Figure 5-13. Overexpression of miR-132 or miR-212 alone can mimic TLR2 ligand priming while specific miRNA inhibitors block PGN-/Pam-induced tolerance. (a) qRT-PCR analysis demonstrating effective and specific expression of miR-132 and/or miR-212 in THP-1 cells transfected for 24 h with 40 nM respective mimics (132-mimic and 212-mimic). (b) TNF-α production by THP-1 cells transfected for 24 h with miRNA mimics and then challenged for 5 h with 500 ng/ml PGN or Pam. (c) Conserved function of miR-132 shown in TNF-α production by mouse RAW264.7 cells transfected with 100 nM miR-132-mimic for 48 h and then challenged with PGN, Pam, or LPS. (d) Inhibition of IL-8 and RANTES production by miR-132- or miR-212-mimic in transfected THP-1 monocytes challenged for 24 h with 100 ng/ml IL-1β. (e) Effective inhibition of miR-132 and miR-212 expression in THP-1 cells transfected with 40 nM miRNA respective inhibitors (132-inh, 212-inh) for 24 h and then stimulated for 16 h with 100 ng/ml PGN or Pam. (f) Enhanced TNF-α production by THP-1 monocytes transfected with 40 nM of each miRNA inhibitor and then challenged with 500 ng/ml Pam for 8 h. Data represent of three independent experiments (a-i, mean ± s.d.) or two experiments (f, mean ± s.d.). *$P < 0.05$; **$P < 0.01$ (two-tailed unpaired t-test) compared with mock transfected control.

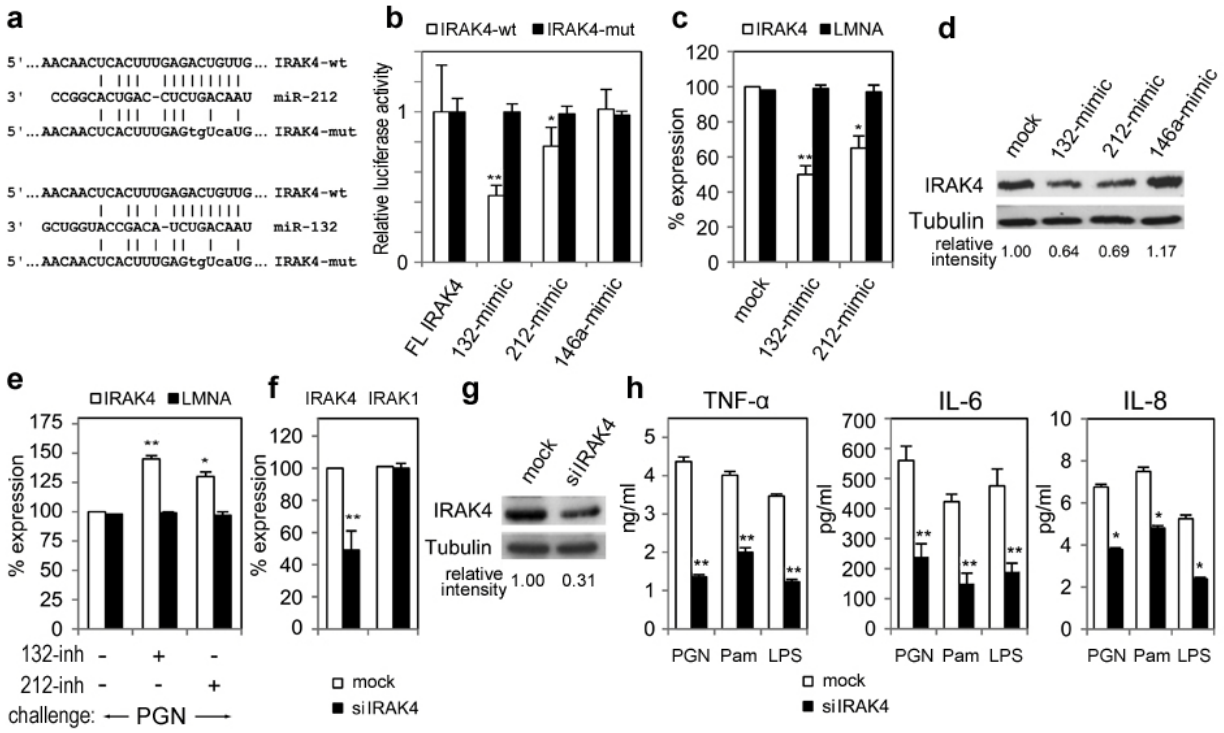

Figure 5-14. IRAK4 is a molecular target of miR-132 and miR-212 post-transcriptional silencing in TLR signaling. (a) Sequence alignment of miR-212 (top) and miR-132 (bottom) with putative target site in 3'UTR of IRAK4 (IRAK4-wt) and mutant construct (IRAK4-mut). (b) Luciferase activity in HEK293 cells cotransfected for 48 h with either wild type or mutant 3'UTR IRAK4-luciferase reporter (FL IRAK4) and 100 nM miR-132-, miR-212-, or miR-146a-mimic. (c) qRT-PCR analysis of IRAK4 and lamin A/C (LMNA, a negative control) in THP-1 monocytes transfected for 48 h with 40 nM miR-132- and miR-212-mimic. (d) Immunoblot analysis of IRAK4 and tubulin (loading control) in THP-1 monocytes transfected with 40 nM miR-132- and miR-212-mimic for 48 h. (e). qRT-PCR analysis showing significant and specific elevated level of IRAK4 in THP-1 monocytes transfected with 40 nM miR-132 or miR-212 inhibitor for 48 h. Lamin A/C (LMNA) was monitored as an unrelated negative control. (f) qRT-PCR analysis showing specific reduction of IRAK4 mRNA in THP-1 monocytes transfected with 100 nM IRAK4-specific siRNA (siIRAK4) for 48 h; IRAK1 mRNA served as a negative control. (g) Immunoblot analysis of IRAK4 and tubulin (loading control) in THP-1 monocytes transfected with 100 nM siIRAK4 for 48 h. (h) TNF-α, IL-6, and IL-8 production by THP-1 monocytes transfected with 100 nM siIRAK4 for 48 h and then treated for 3 to 24 h with PGN, Pam, or LPS (1000 ng/ml). Data points and error bars represent three independent experiments (b,c,e,f,h, mean ± s.d.) or two experiments (d,g) . *$P < 0.05$; **$P < 0.01$ (two-tailed unpaired t-test) compared with untreated or mock transfected control.

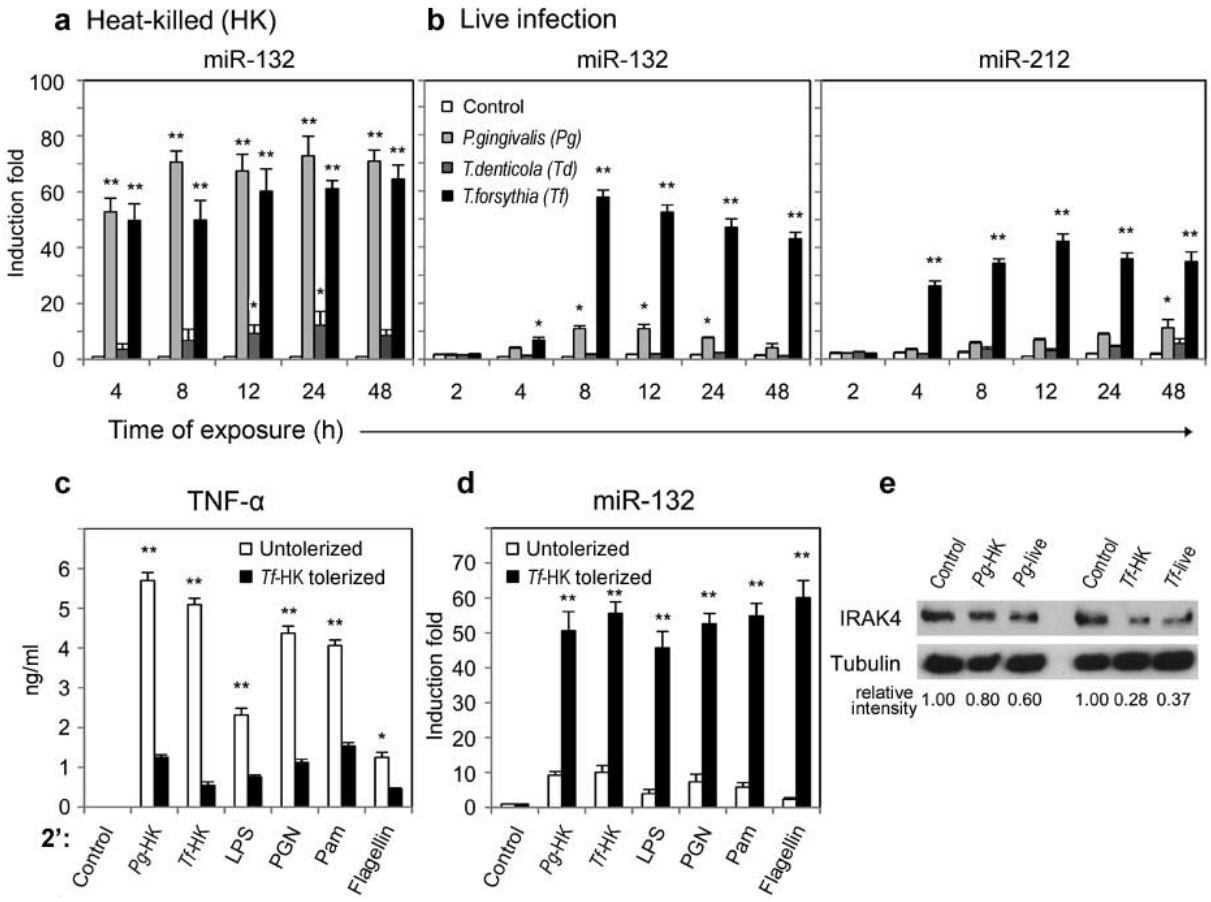

Figure 5-15. Heat-killed bacterial stimulation- or live bacterial infection-induced miR-132 and miR-212 contribute to resistance to recurrent bacterial challenge. (a,b) qRT-PCR analysis of miR-132 and miR-212 expression in THP-1 monocytes stimulated for 2-48 h with heat-killed (HK) and live P. gingivalis, T. denticola, and T. forsythia at MOI 100. (c) TNF-α production by THP-1 cells primed with or without HK T. forsythia (MOI 10) for 18 h and then challenged for 2 h with HK bacteria (MOI 100), 1000 ng/ml of LPS, PGN, and Pam and 300 ng/ml flagellin. (d) qRT-PCR of miR-132 expression in the same THP-1 monocytes as described in c. (e) Immunoblot analysis of IRAK4 and tubulin (loading control) in THP-1 monocytes treated for 4 h with HK and live P. gingivalis or T. forsythia (MOI 100). Data are representative of three independent experiments (a-d, mean ± s.d.) or two experiments (e). *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with untreated control.

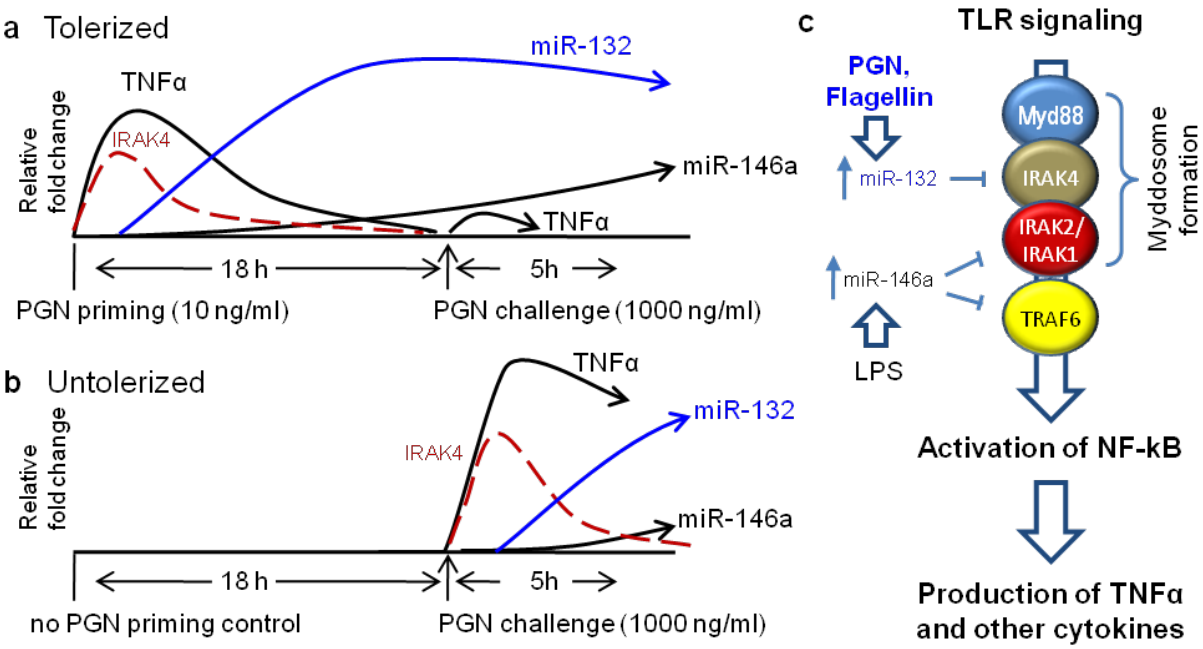

Figure 5-16. A model for the role of miR-132 in PGN-mediated tolerance in THP-1 monocytes targeting IRAK4, which is the first protein in the complex with the MyD88 in the formation of myddosome critical for the activation of NF-κB, and cytokine production. See text under “Discussion.”

CHAPTER 6
CONCLUSION

Innate immunity provides protection primarily against a range of microbial attack including bacteria and virus. Pathogenic conserved molecules are recognized by innate immune receptors mostly by toll-like receptors (TLRs). The molecular interaction between those ligands and TLRs is being extensively studied, providing a greater insight of innate immune mechanism. During infection, the moderate activation of innate immunity is mandatory to fight against invaders. In contrast, over activation of innate immunity leads to systemic inflammation and thus homeostasis should be maintained by various mechanisms. One such mechanism, endotoxin tolerance has been elaborately examined in order to infer its critical role in innate immunity. Endotoxin tolerance is known to control the intensity and duration of innate immune cell activation through affecting TLR pathways. The signaling molecules that comprise each TLR signaling pathway are regulated by various factors (350) which are responsible to diminish cytokine production. However, a more energy-efficient way to regulate the activity of TLR signaling molecules could be to destabilize the mRNA molecules that encode them. miRNAs are such important factors that may render hyporesponsiveness via affecting on adaptor kinase mRNAs and this is the focus in this study.

miRNAs are known to be induced by microbial components. The upregulation of miRNAs such as miR-146a was first described in LPS-induced THP-1 monocytes by Taganov et al. (116). They demonstrated that IRAK1 and TRAF6 kinases for TLR signaling were targeted by miR-146a and thus postulated that miR-146a could be important for innate immunity. Later in our laboratory Pauley et al. (189), showed that LPS treatment caused upregulation of miR-146a up to 100-fold. Consequently, to find the relevance of miR-146a expression in innate immunity, we analyzed its detailed kinetics in response to LPS. Interestingly, at LPS induction miR-146a

showed a continuous expression over 24 hour. Remarkably, this expression pattern was unique compared to other miRNAs such as miR-132 and miR-155 under the same condition. The biological significance of miR-146a expression was evaluated regarding endotoxin tolerance in THP-1 monocytes. LPS primed monocytes showed elevated level of miR-146a and positively correlated with endotoxin tolerance, suggesting its role in LPS tolerance. Recently, Jurkin et al. observed a constitutive expression of miR-146a at higher levels in human LCs compared with interstitial dendritic cells (intDCs) and very low level in monocytes (136). Their results suggest that high constitutive miR-146a levels were induced by microenvironmental signals in the epidermis and might render LCs less susceptible to inappropriate activation by commensal bacterial TLR2 triggers at body surfaces. This observation is relevant to our study where LPS-induced higher miR-146a causes THP-1 monocytes less susceptible to LPS challenge

To understand the mechanism how miR-146a plays a role, targets IRAK1 and TRAF6 expression were examined and demonstrated to be downregulated in LPS tolerized cells. miR-146a-mediated control of the expression of IRAK1 and TRAF6 might not be as rapid as control through proteasomal degradation. Thus, LPS-induced miR-146a expression might be an advantage during infection, as miRNA-mediated control of mRNA levels allows for a strong initial immune response, needed for defending the pathogens and then it is gradually dampened down. Functionally, a higher level of miR-146a prevents cytokine production in immune cells and critical for endotoxin tolerance as mature miR-146a alone can mimic LPS-induced tolerance. Of note, cytokines such as TNF-α are not completely abolished by miR-146a alone in comparison to LPS treatment. A higher dose of LPS-priming was shown to cause >95% decrease of cytokine response. This observation indicates that LPS-priming may induce other inhibitory molecules including miRNAs, which might act together to abolish cytokine response and this

prompted further investigation. Endotoxin tolerance has been established in animal models in order to observe its true biological significance. LPS-primed animals were survived to lethal dose of LPS or pathogenic microbial challenge (201). It is worth noting that LPS-induced miR-146a has been observed in primary cells including PBMCs. Accordingly, LPS-induced miR-146a expression might have an important role in host immunity responding to a wide range of bacterial infections and subsequently might contribute toward their survival. However, a detailed *in vivo* investigation to comprehend its significant role in host innate immunity is still required.

In addition to homologous tolerance, LPS-primed monocytes show hyporesponsiveness to other microbial components. To understand the broad role of miR-146a in innate immunity, its association with LPS-induced cross-tolerance was investigated. LPS-primed THP-1 monocytes showed a consistent miR-146a expression, affecting IRAK1 and TRAF6 which are required for activation in other TLR pathways. Therefore, overexpression of miR-146a can cause resist not only LPS challenge but also to a range of other innate immune ligands. The level of LPS-induced cross-tolerance differs among various ligands and thus it is not clear whether miR-146a affects on all TLR pathway to the same extent. A continuous expression of miR-146a expression is not only observed by LPS but also by TLR2 and TLR5 ligands, which show differential cross-regulation. Thus, once miR-146a is induced by any microbial components including LPS, it is likely to affect all other MyD88-dependent TLR signaling. miR-146a-mimic transfection mediated cross-tolerance level was not similar to LPS-induced condition and this indicates that other components may be involved in this intricate process.

In nature hosts are infected with bacteria, which liberate various pathogenic components rather than an individual one. LPS-induced miR-146a-mediated cross-tolerance causes hyporesponsiveness to a majority of the bacterial components. miR-146a expression has not

reported to be induced by nucleic acid (TLR3,7 or 9 ligand) and NOD2 ligand such as MDP. However, vesicular stomatitis virus (VSV) induces miR-146a (122) and thus further studies are needed to conclude that miR-146a can contribute tolerance against all of these ligands. Knockdown of IRAK1 and TRAF6 reduces cytokine response in THP-1 monocytes, and also IRAK1 knockout mice show hyporesponsiveness against bacterial infection (85). This can be relevant to miR-146a-mediated cross-tolerance which affects on the translational inhibition of IRAK1 and TRAF6 and could protect animal against microbial component. In our study miR-146a associated LPS-tolerized monocytes show hyporesponsiveness to heat-killed and live bacterial infection, in part consistent with previous study (201). miR-146a mediated cross-tolerance has also been observed in primary mouse macrophages. Accordingly, it can be speculated that LPS-induced miR-146a can help animal to survive against bacterial infection. Recently, Curtale et al. (236) showed that miR-146a is overexpressed in human memory and activated T cell, consistently,miR-146a is induced in human primary T lymphocytes upon TCR stimulation. In that study, upregulated miR-146a-mediated impairedness of both activator protein 1 (AP-1) activity and IL-2 production was also observed, suggesting a role of this miRNA in the modulation of adaptive immunity. In another study, miR-146a has been shown to be prevalently expressed in $Foxp3^+$ regulatory T (Treg) cells and its deficiency resulted in increased numbers but impaired function of Treg cells (138). Consequently, a breakdown of immunological tolerance was manifested in fatal IFNγ-dependent immune-mediated lesions in a variety of organs in miR-146a-deficient mice. Accordingly, endotoxin tolerance associated miR-146a expression might influence adaptive immunity.

Chronic inflammation contributes to cancer initiation and progression. miR-146a is involved in many inflammatory diseases and viral infection (145). Recently, Zhao et al. (140)

showed that the knockout of the miR-146a gene in C57BL/6 mice leads to histologically and immunophenotypically defined myeloid sarcomas and some lymphomas due to NF-kB dysregulation. In order to find such an effect of miR-146a in inflammatory periodontal diseases, its expression was observed in chronically infected animal model with *P.gingivalis, T. denticola*, and *T. forsythia*. Infected mice showed a consistent expression of miR-146a. A similar miRNA expression pattern was observed in THP-1 monocytes in response to the same mono- and poly-microbial infection. Thus, miR-146a might be implicated in periodontal disease, due to the fact that proinflammatory cytokines are diminished. miR-132 expression was observed in infected THP-1 monocytes but not in infected mice, indicated that miR-132 expressed at the early stage of infection was not persistent as like as miR-146a.The presence of higher levels of miR-146a in maintaining tolerance is critical as shown in our *in vitro* studies (82). Similarly, this study suggests that periodontal bacteria-induced miR-146a might cause tolerance in the infected animal. Due to involvement of miRNA in diseases, this study suggests a possible target for therapeutic intervention in periodontal diseases.

Peptidoglycan (PGN) is another strong proinflammatory component as observed in our study and others (88). Thus, control of PGN-induced cytokine production is equally important to LPS induction. miR-146a expression is not restricted to LPS but also induced by other pathogenic components. A similar occurrence can be observed for other miRNAs such as miR-132/-212, although the kinetics may vary. In contrast to LPS, PGN stimulation causes a sharp increase at early stage of stimulation and rises about four times higher than miR-146a at 12 hour time point, which remains at a significant level until 48 hours. miR-212 shows a similar kinetics in response to PGN. Thus, depending on the ligand, miRNA expression kinetics can differ and play specific role during innate immune activation. In response to PGN, miR-132/-212 starts to

increase within two hours which is earlier than miR-146a. Thus, miR-132/212 can be considered as a very early responsive gene compared to miR-146a. LPS-induced other miRNAs such as miR-21 and miR-147 as reported by others are considered as late responsive gene. A sharp increase of miR-132 alone or in combination with miR-146a might prevent acute infection to control cytokine due to the fact that diminished cytokine was observed.

PGN-primed monocytes showed higher miR-132/-212 expression which was positively correlated to tolerance and cross-tolerance to various ligand, indicating its association. Although, miR-132 or miR-212 alone can mimic PGN-induce tolerance, TNF-α production was not abolished compared to higher dose of PGN-priming condition in THP-1 monocytes. This indicates that PGN-induced other inhibitory components might be involved in this process and needs to be further determined. Flagellin also showed similar tolerance mediated by miR-132/-212 indicating that these miRNAs are induced by broad range of microbial components and participate in innate immune regulation. Interestingly, primary macrophages and human PBMCs show miR-132 expression in response to PGN. However, the induction fold in these cells is lower than that observed in THP-1 monocytes. It might be due to fact that PBMCs contain a mixed cells population which is not equally efficient to respond to PGN. A similar low level expression was observed for miR-146a in PBMCs (125). However, PGN-primed primary macrophages or PBMCs show tolerance or cross-tolerance mediated by miR-132, suggesting its possible role in animal. Surprisingly, in response to whole bacterial infection, miR-132/-212 is also expressed at higher level. However, all types of bacteria did not induce miR-132/-212 to a similar extent indicating its microbial specificity. PGN-primed animal survived to some bacterial infection (88) where miR-132/-212 might play role and needs further investigation.

miR-132 and miR-212 targeting IRAK4 was found to be the novel mechanism by which PGN or flagellin priming cause tolerance. IRAK4 is the first adaptor kinase recruited to MyD88 and affected by the rapid expression of miR-132 during PGN or whole bacterial infection. Subsequently, IRAK1/2 and TRAF6 are recruited, which are controlled by miR-146a. Our findings show that miR-146a and miR-132/-212 are expressed by the similar microbial components or bacterial infection, although their kinetics differs. Thus, during innate immune activation, they are thought to play role alone in an oscillatory fashion or one-two punch to control MyD88-dependent TLR pathways. Modulation of miR-146a and miR-132/-212 level may become attractive targets for intervention against inflammatory diseases.

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BIOGRAPHICAL SKETCH

Md Abu Nahid was born in Bangladesh. He earned his Bachelor of Science in microbiology in 1999 from University of Dhaka. At this time, Nahid developed an interest in research and decided to pursue a Master of Science degree in microbiology in the laboratory of Dr. Majibur Rahman. With Dr. Rahman's guidance, Nahid completed his research project in 2001 focused on the role of biodegradation of halogenated pesticides by *Pseudomonas spp*. During this time, Nahid decided to continue his research career and went to Osaka Prefecture University, Japan. In Japan, Nahid worked with Dr. Sunji Sugii for more than three years. With Dr. Sugii's guidance, he completed his research project on purification and characterization of porcine ficolin-α and to study its immunological significance. Then, Nahid migrated to United States of America in 2006 and now he is United States citizen. In the meantime he joined the United States army reserve to serve the nation. In 2007, Nahid was accepted in the Interdisciplinary Program in Biomedical Sciences at the University of Florida. Nahid decided to join the immunology and microbiology concentration, and ended up joining the laboratory of Dr. Edward K.L. Chan. Here, his research focused on microRNAs as major players in Toll-like receptor (TLR) ligand-induced tolerance, cross-tolerance and experimental periodontal diseases. He received his Ph.D. from the University of Florida in the summer of 2011. After his doctorate work, Nahid plans on joining a postdoctoral position in the lab of Dr. Joseph Wu at Stanford Medical School where he will work on stem cell biology focusing on understanding immunobiology of human induced pluripotent stem cells. He has been married to Shamima Rahman.