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REVIEW

MicroRNA in TLR signaling and endotoxin tolerance

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Toll-like receptors (TLRs) in innate immune cells are the prime cellular sensors for microbial components. TLR activation leads to the production of proinflammatory mediators and thus TLR signaling must be properly regulated by various mechanisms to maintain homeostasis. TLR4-ligand lipopolysaccharide (LPS)-induced tolerance or cross-tolerance is one such mechanism, and it plays an important role in innate immunity. Tolerance is established and sustained by the activity of the microRNA miR-146a, which is known to target key elements of the myeloid differentiation factor 88 (MyD88) signaling pathway, including IL-1 receptor-associated kinase (IRAK1), IRAK2 and tumor-necrosis factor (TNF) receptor-associated factor 6 (TRAF6). In this review, we comprehensively examine the TLR signaling involved in innate immunity, with special focus on LPS-induced tolerance. The function of TLR ligand-induced microRNAs, including miR-146a, miR-155 and miR-132, in regulating inflammatory mediators, and their impact on the immune system and human diseases, are discussed. Modulation of these microRNAs may affect TLR pathway activation and help to develop therapeutics against inflammatory diseases.
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INTRODUCTION

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 decades, 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, 13 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

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the inflammatory response and damage. It is now known that TLR activation induces various regulatory molecules including microRNAs (miRNAs), which may participate in various mechanisms to control excessive inflammation. Thus, this review is focused on one such mechanism, endotoxin tolerance and the recent progress in the field of TLR ligand-induced miRNA in the immune system.

ENDOTOXIN 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 tumor-necrosis factor-alpha (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.

ENDOTOXIN 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). 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

Table 1 *In vitro* cytokine dysregulation in endotoxin-tolerized cells from different cell and tissue types. Adopted and modified from reviews.[19,20,55]

Cytokine	*Change in expression*	*Cells/tissues used in* in vitro *studies*
TNF-α	↓	Human PBMCs,[189,190] human monocytes,[55,189] human DC,[191] human peritoneal PMΦ,[28] rabbit PMΦ,[29] mouse PMΦ,[20,33,55] human THP-1 cells,[21,61,192] mouse RAW264.7 cells[193,194]
IL-1β	↓	Human THP-1 monocytes[21,61,192]
	↑	Human PBMCs,[195] human PMΦ,[28] mouse PMΦ[196]
IL-6	↓	Human PBMCs,[197] human monocytes,[198] human PMΦ,[28] mouse PMΦ[20,72,76]
IL-10	↓	Human PBMCs[55,197]
	↑	Human monocytes,[55,198] human MonoMac6 cell line[26]
IL-12	↓	Human monocytes,[191] human DC[191]
IL-1ra	↑	Human THP-1 cells[190]
G-CSF	↓	Human PBMCs,[197] mouse PMΦ[63]
	↑	Human PMΦ[28]
TGF-β	↑	Human PMΦ[190,199]

Abbreviations: DC, dendritic cell; G-CSF, granulocyte-colony stimulating factor; PBMCs, peripheral blood mononuclear cells; PMΦ, peritoneal macrophages; TGF, transforming growth factor; TNF, tumor-necrosis factor; ↑, increased expression; ↓, decreased expression.

associated with the decrease, increase or unchanged status of the release of IL-1, IL-6, IL-8, IL-10 and prostaglandin E_2 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 levels 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 synthase and nitric oxide production after secondary LPS challenge, reported to 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 nitric oxide 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 *et al.*,[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). 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 8 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 min 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 5 days of repetitive LPS injections at low doses.[46] Subsequently, *in vivo* endotoxin tolerance models confirm that low-dose LPS-pre-treated mouse macrophages and human monocytes lose 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 2.

Table 2 Changes in cytokine expression reported in *in vivo* endotoxin tolerance conditions. Adopted and modified from reviews[19,200]

Cytokine	*Change in expression*	In vivo *studies of endotoxin tolerance*
TNF-α	↓	Human,[46,201–203] pig,[204] rabbit,[205] guinea pig,[206] rat,[207] mouse[200,208]
IL-1β	↓	Human,[202] rabbit,[205] mouse[208]
	↑	Mouse[30]
IL-6	↓	Human,[201,202] guinea pig,[206] mouse[208,209]
	↑	Human[203]
IL-8	↓	Human[201–203]
IL-10	↓	Rat,[210] mouse[211]
IL-12	↓	Mouse[51]
G-CSF	↓	Human,[202,203] mouse[208,212]
IFN-γ	↓	Mouse[51,213]
TGF-β	↑	Mouse[46]

Abbreviations: G-CSF, granulocyte-colony stimulating factor; IFN, interferon; TGF, transforming growth factor; TNF, tumor-necrosis factor; ↑, increased expression; ↓, decreased expression.

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 interferon (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 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. Besides 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, myeloid differentiation factor 88 (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 *Escherichia 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 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, which 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

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

Cytokine	*Change in expression*	Ex vivo *studies*
TNF-α	↓	Human blood,[42,214] human PBMCs,[53,55] rabbit PBMCs,[205] mouse PMΦ,[52] rat PMΦ,[50] rat Kupffer cells[215]
IL-1β	↓	Human blood,[214] human PBMCs,[53,55] rabbit PBMCs,[205] mouse PMΦ[216]
	↑	Human PBMCs[203]
IL-6	↓	Human blood,[214] human PBMCs,[53,55] rat PMΦ,[217] rat Kupffer cells[218]
	↑	Human PBMCs[203]
IL-12, IFN-γ	↓	Mouse spleen cells[51]

Abbreviations: IFN, interferon; TNF, tumor-necrosis factor; PBMCs, peripheral blood mononuclear cells; PMΦ, peritoneal macrophages; ↑, increased expression; ↓, decreased expression.

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] which is demonstrated by our unpublished data. 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 and 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]

miRNAS 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. 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 200 miRNAs are expressed in lower metazoans and plants, but approximately 1000 are predicted in humans. Although miRNAs are similar in structure to small interfering RNAs, 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, small interfering RNAs 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] small interfering RNAs 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 are located within intronic regions or noncoding transcription units and are 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

Table 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	219
MyD88s	Unable to induce IRAK phosphorylation	Increased	220
sTREM-1	Anti-inflammatory role in mice	Increased	221
TRAF4	Interacts and counteracts TRAF6 and TRIF	Increased	222
Tripartite-motif protein,TRIM30α	Prevents downstream activation of NF-κB and cytokine induction by degrading TAK1	Increased	223

Abbreviations: IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide; MyD88s, a splice variant of MyD88; NF-κB, nuclear factor-kappa B; SOCS1, suppressor of cytokine signaling 1; sTREM, soluble triggering receptor expressed on myeloid cells; TLR, Toll-like receptor; TRAF6, tumor-necrosis factor receptor-associated factor 6.

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 miRNAS

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- to 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 *Drosophila 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. The RNA-induced silencing complex-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, and thus may be important in autoimmunity.

CHANGES IN miRNA EXPRESSION BY TLR 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 24–48 h.[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 as well as p300 transcriptional co-activator in Kaposi's sarcoma-associated herpesvirus (KSHV)-infected endothelial cells.[119] PGN-induced cyclic AMP response element-binding protein also upregulated miR-132.[118] 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 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 5). Similar to other TLR-responsive genes, miRNAs can be classified as early or late response genes; miR-146a[82] and miR-132 (unpublished data) are highly induced 2–4 h 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

Table 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 and 5	THP-1 cells,[116] MΦ,[139] BMDMs,[139] T cells[224]	EBV,[225] VSV,[122] RIG-I,[122] TNF-α,[116] IL-1β[121]
miR-132	TLR4 and 9	THP-1 cells,[116] human monocytes and MΦ,[119] BMDMs and splenocytes[151]	KSHV[119]
miR-212	Unknown	Unknown	KSHV,[119] EtOH[226]
miR-155	TLR2, 3, 4 and 9	BMDMs,[126] THP-1 cells,[116] monocytes,[227] MΦ,[228] DCs,[229] B cells,[230] Treg cells[231]	*Helicobacter pylori*,[232] KSHV,[168] EBV,[233] TNF-α and IFN-γ[234]
miR-21	TLR4	Inflamed lung tissue,[235] RAW264.7 cells and BMDMs,[125] B cells,[236] H69 cholangiocytes[237]	*Cryptosporidium parvum*,[237] EBV LMP1[238]
miR-223	TLR4	Inflamed lung tissue,[124] DCs[130]	ND
miR-147	TLR2, 3 and 4	BMDMs, RAW264.7 cells, THP-1 cells and alveolar MΦ[239]	ND
miR-9	TLR2, 4, 7 and 8	Human monocytes and granulocytes[240]	IL-1β[241]
miR-125b	TLR4	H69 cholangiocytes,[237] RA synovial fibroblasts,[135] LPS-tolerized THP-1 cells[242]	*C. parvum*[237]
let-7e	TLR4	Peritoneal MΦ[153]	ND
miR-27b	TLR4	Human MΦ[243]	ND
Downregulated			
miR-125b	TLR4	Splenocytes,[243] BMDMs,[153] DCs[130]	ND
let-7i	TLR4	H69 cholangiocytes[127]	*C. parvum*[127]
miR-98	TLR4	H69 cholangiocytes[244]	*C. parvum*[244]

Abbreviations: BMDM, bone marrow derived macrophages; DC, dendritic cell; EBV, Epstein–Barr virus; EtOH, ethyl alcohol; IFN, interferon; KSHV, Kaposi's sarcoma herpesvirus; LDL, low density lipoprotein; LMP1, latent membrane protein 1; LPS, lipopolysaccharide; microRNA, miRNA; ND, not determined; RA, rheumatoid arthritis; RIG-I, retinoic acid inducible gene I; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cells; VSV, vesicular stomatitis virus.

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. In the following section, the consequence of changes in miRNAs expression will be discussed in the context of TLR ligand-induced miRNAs with implications in innate immunity and specifically in controlling TLR signaling.

MOLECULAR INTERACTION BETWEEN miRNAS 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.[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, 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 Bruton's tyrosine kinase 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. Because most pathogens can engage many TLRs, miRNAs could help to limit robust proinflammatory 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 proinflammatory response.

TLR LIGAND-INDUCED miRNAS

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[81,82] (Figure 1). In human Langerhans cells, 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 transforming growth factor-β1, a key signal for epidermal Langerhans cell 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 Langerhans cells 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

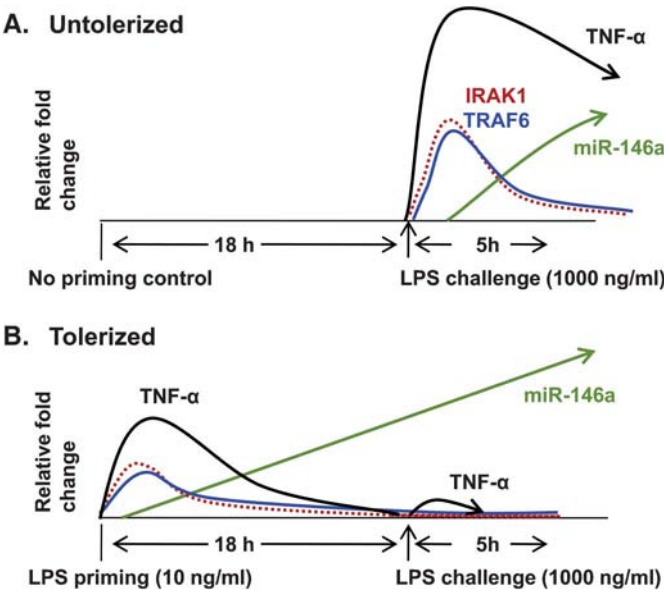

Figure 1 A schematic summary of the response to LPS stimulation in THP-1 cells. (**a**) Unprimed cells challenged with high-dose LPS at 1000 ng/ml produce a prominent TNF-α response. There are respective increases in both IRAK1 and TRAF6, which peaked at 2–4 h. The increase in miR-146a expression starts at 2 h and continues to increase in the presence of LPS. (**b**) Tolerized cells are generated by priming with a low dose LPS at 10 ng/ml leading to a rapid and transient TNF-α, IRAK1 and TRAF6 responses. TNF-α production decreases as miR-146a expression 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] IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide; TNF, tumor necrosis factor; TRAF6, TNF receptor-associated factor 6.

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 regulatory T cells (Treg) and is critical for Treg functions.[138] Indeed, deficiency of miR-146a has resulted in increased numbers but impaired function of Treg 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. 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 post-transcriptional regulation. For example, miR-132 is highly induced in response to phorbol myristate acetate, whereas no response is observed for the level of mature miR-146a. MicroRNA-132/-212 has been reported to be a cyclic AMP response element-binding protein-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 has been described in response to innate immune ligands and no known target for TLR signaling molecules have been found to date.

let-7i, 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 are 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 is also known to influence T-cell development and function,[92] supported by the expression of miR-181a which augments T-cell receptor 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 T-cell receptor 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 T-cell receptor 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 twofold 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, and 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 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 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 primate foamy virus type 1.[177] The downregulation of these viral genes results in slower primate foamy virus type 1 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.

MICRORNA 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 anti-apoptotic 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 the initiation of malignant transformation. In normal conditions, miR-155 is highly expressed both in activated B and T cells and in monocytes, 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, acute myeloid leukemia, 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 acute myeloid leukemia.[143] 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, vscular endothelial growth factor 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 silencing of miR-21 inhibits cell growth, and activates caspases-induced apoptosis by targeting tumor suppressor genes such as *PTEN*, programmed cell death 4 and tropomyosin 1.[188]

CONCLUSION

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). 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.

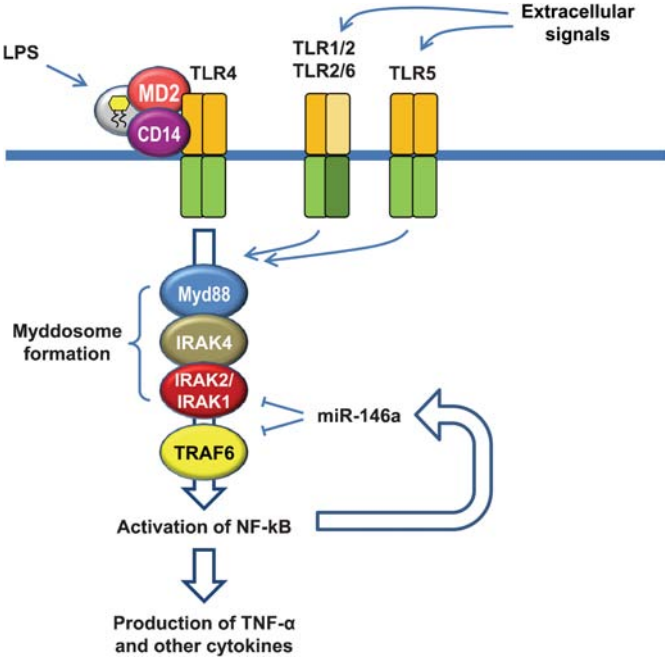

Figure 2 A model of the role of miR-146a in LPS–TLR4-mediated signal transduction contributing to endotoxin tolerance and cross-tolerance. LPS binds to the LPS-binding protein, which in turn is coupled to CD14 on the cell surface of monocytes. Subsequently, LPS-CD14 interacts with TLR4 and forms a complex 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,[245] involving six MyD88, four IRAK4 and four IRAK2/1 molecules, which in turn recruits TRAF6. This chain of events triggers the 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 that are also induced by LPS. IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide; MD2, myeloid differentiation protein-2; microRNA, miRNA; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF6, TNF receptor-associated factor 6.

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