Regulation of Endotoxin Tolerance and Compensatory Anti-inflammatory Response Syndrome by Non-coding RNAs

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The onset and the termination of innate immune response must be tightly regulated to maintain homeostasis and prevent excessive inflammation, which can be detrimental to the organism, particularly in the context of sepsis. Endotoxin tolerance and compensatory anti-inflammatory response syndrome (CARS) describe a state of hypo-responsiveness characterized by reduced capacity of myeloid cells to respond to inflammatory stimuli, particularly those initiated by bacterial lipopolysaccharide (LPS). To achieve endotoxin tolerance, extensive reprogramming otherwise termed as "innate immune training", is required that leads to both modifications of the intracellular components of TLR signaling and also to alterations in extracellular soluble mediators. Non-coding RNAs (ncRNAs) have been recognized as critical regulators of TLR signaling. Specifically, several microRNAs (miR-146, miR-125b, miR-98, miR-579, miR-132, let-7e and others) are induced upon TLR activation and reciprocally promote endotoxin tolerance and/or cross tolerance. Many other miRNAs have been also shown to negatively regulate TLR signaling. The long non-coding (lnc)RNAs (Mirt2, THRIL, MALAT1, lincRNA-21 and others) are also altered upon TLR activation and negatively regulate TLR signaling. Furthermore, the promotion or termination of myeloid cell tolerance is not only regulated by intracellular mediators but is also affected by other TLR-independent soluble signals that often achieve their effect via modulation of intracellular ncRNAs. In this article, we review recent evidence on the role of different ncRNAs in the context of innate immune cell tolerance and trained immunity, and evaluate their impact on immune system homeostasis.

Keywords: endotoxin tolerance, sepsis, immune suppression, microRNAs, non-coding RNAs, soluble mediators, lncRNAs

INTRODUCTION

The onset and termination of the host immune responses have to be tightly controlled; the initial burst of pro-inflammatory cytokines should be timely blunted to avoid overwhelming inflammatory responses causing tissue damage and secure homeostasis. Endotoxin tolerance is a crucial homeostatic mechanism that prevents from the excessive activation of innate immune responses upon sustained toll-like receptor (TLR) stimulation.
Endotoxin tolerance is defined as the reduced capacity of a cell to respond to gram(-) bacterial lipopolysaccharide (LPS) after an initial exposure to this stimulus (1, 2). Endotoxin tolerance is considered a type of “innate immune memory” (3), a condition describing tolerance to pathogens, characterized by innate immune hypo—responsiveness or “immune-paralysis”. It occurs as a result of persistent TLR stimulation not only from LPS but from other TLR agonists and even TLR-independent inflammatory mediators (1). The mechanism by which exposure to a particular TLR ligand or other inflammatory mediators such as cytokines reduces the inflammatory response to different TLR ligands is known as cross—tolerance and cytokine-induced tolerance, respectively (4–7), both being part of the innate immune system training (8, 9).

The phenotype of endotoxin tolerance and cross-tolerance has been extensively studied in monocytes and macrophages, even though the majority of innate immune cells develop tolerance to secondary TLR stimuli. These include dendritic cells, neutrophils, mast cells as well as endothelial and epithelial cells (10–14).

Endotoxin tolerance results to a shift of the cell phenotype from pro-inflammatory to anti-inflammatory (15). Endotoxin tolerant macrophages are reprogrammed to produce less tumor necrosis factor alpha (TNFα), interleukin (IL)-12 and IL-6 upon secondary stimulation and more anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGFβ), compared to the levels produced from naive cells (16, 17). Furthermore, tolerant macrophages and dendritic cells downregulate human leukocyte antigen (HLA-DR) receptors thus have impaired capability for antigen presentation (16, 18). Similar phenotype is also described in cross-tolerance, but to a lesser extent (19). Endotoxin tolerant phenotype is long lasting but reversible in nature.

The clinical manifestation of endotoxin tolerance is recognized as Compensatory Anti-inflammatory Response Syndrome (CARS) (20). CARS represents the phase of immune “exhaustion” otherwise termed “immune paralysis”, that is observed in a subset of septic patients usually following the first phase of sepsis, known as Systemic Inflammatory Response Syndrome (SIRS) (21). Endotoxin tolerance explains CARS immunosuppression state in sepsis since blood leukocytes from septic patients exhibit similar phenotype to endotoxin tolerant cells; neutrophils and monocytes from septic patients are refractory to production of inflammatory mediators while they upregulate anti-inflammatory molecules when exposed to secondary TLR stimuli (1, 17, 22, 23). As a result, patients with CARS exhibit increased susceptibility to secondary infections (24).

The mechanism of innate immune cell tolerance and CARS are tightly regulated by complex molecular signatures in macrophages and other innate immune cells. These molecular pathways are controlled not only by modulation of intracellular signaling proteins and histone modifications but also by non-coding (ncRNAs), mostly microRNAs (miRNAs) and long ncRNAs (lncRNAs). In this article, we review recent evidence on the role of ncRNAs, regulated by TLR ligands or other TLR independent soluble signals, in the regulation of endotoxin tolerance and discuss their impact in the context of sepsis.

### TLR—DEPENDENT REGULATION OF ENDOTOXIN TOLERANCE VIA ncRNAs

Upon stimulation by pathogen- or danger-associated patterns, TLR mediate signals through two distinct adapters pathways, myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). The MyD88 pathway employs interleukin-1 receptor-associated kinase (IRAK)1 and 4 kinases and TNF receptor-associated factor (TRAF)6 to activate nuclear factor κB (NFκB) and mitogen activated protein kinase (MAPK)/activator protein 1 (AP-1) signaling, promoting transcription of pro-inflammatory cytokines. Activation of TRIF pathway leads to janus kinase (JAK)/signal transducer and activator of transcription (STAT) and type I interferon activation and increases the expression of interferon-inducible genes (25, 26). In TLR4 tolerance, defects in TLR4 signaling have been observed at all levels, including receptor adaptors, signaling molecules, transcription factors, as well as, chromatin marks as histone modifications (1, 27).

The molecular signature of endotoxin tolerance involves downregulation of TLR4 expression, decreased recruitment of MyD88 or TRIF to TLR4, decreased activation of IRAK1/4 and diminished NFκB signaling via formation of the inactive p50 homodimers (1, 28). Additionally, negative regulatory molecules such as IRAK-M, A20, SH2 domain-containing inositol phosphatase 1 (SHIP1), Pellino-3, suppression of tumorigenicity 2 (ST2), suppression of cytokine signaling (SOCS)3 and SOCS1 are upregulated in endotoxin tolerant cells and inhibit the activation of TLR signaling (1, 28–33). However, during last two decades, an additional level of regulation through non-coding regulatory RNAs has been introduced.

### TLR Dependent miRNAs That Regulate Endotoxin Tolerance

miRNAs (miRNAs) are a large family of small noncoding RNAs (about 22 nucleotides in length) that regulate gene expression post-transcriptionally, by binding to the 3′-untranslated regions (UTRs) of target mRNAs (34). MiRNAs are recognized as key players in the regulation of endotoxin tolerance since multiple levels of the TLR signaling cascade are controlled by miRNAs (35, 36). At the stage of endotoxin tolerance, two LPS inducible miRNAs, miR-155 and miR-146α have been shown to be coordinately regulated via gene colocalization and transcription factor binding, contributing to the regulation of endotoxin tolerance (37). Indeed, miR-146α was the first miRNA described to promote tolerance (38, 39). MiR-146α is induced upon TLR activation in macrophages and its expression is further upregulated with LPS restimulation (17, 37). MiR-146α then targets IRAK1 and TRAF6, critical components downstream TLR signaling and its prolonged expression has been linked to endotoxin tolerance and cross—tolerance (19, 39–41). On the other hand, miR-155 inhibits the expression of the negative regulators SHIP1 and SOCS1 enhancing TLR signals, promotes TNFα translation and establishes a proinflammatory phenotype in macrophages (42–46). However, other studies show that miR-155 may exert negative regulation of pro-inflammatory
mediators (47) (Table 1). Suppression of miR-155 in Akt1−/− macrophages restored sensitivity and tolerance to LPS in vitro and in vivo, supporting its role in the regulation of endotoxin tolerance (43).

MiR-98 targets IL-10 in macrophages, a key cytokine for development of endotoxin tolerance; miR-98 is decreased by LPS in macrophages, thus failing to suppress IL-10 (68). The miRNAs miR-221, miR-579 and miR-125b are also significantly induced in endotoxin tolerant macrophages and lead to TNFα inhibition; miR-221 promotes TNFα degradation, whereas miR-579 and miR-125b block its translation (52). MiR-132 and miR-212 are also induced upon TLR2 stimulation and their sustained expression promotes cross tolerance (54). In a recent report, miR-221 and miR-222 were identified as regulators of the functional reprogramming of macrophages during LPS tolerance (3). MiR-221 and miR-222 were induced after prolonged LPS stimulation in mice and both promoted transcriptional silencing of a subset of pro-inflammatory genes via regulation of chromatin remodeling mediated by SWI/SNF (switch/sucrose non-fermentable) and STAT transcription factors (3).

However, there is a significant number of other miRNAs that have been shown to negatively regulate TLR signaling (Table 1). Among the aforementioned miRNAs, miR-146, miR-155, miR-221 and miR-222 have been extensively studied and appear to have a central role in the regulation of innate immune tolerance. In the context of sepsis, the levels of miR-146, miR-150, miR-221 and miR-222 among other miRNAs, are dysregulated in the peripheral blood leukocytes in sepsis patients and correlate with immunoparalysis and severity of the disease (3, 69–71), thus providing potential prognostic/diagnostic biomarkers.

### LncRNAs That Contribute to Endotoxin Tolerance

Long noncoding RNAs (lncRNA) are regulatory RNAs that are over 200 nucleotides in length and do not encode proteins (72–74). LncRNAs are classified based on their site of action into

| miRNA  | Response to TLR signal | Target | Mechanism of action | References |
|--------|------------------------|--------|---------------------|------------|
| miR-146a | Induced | TRAF6, IRAK1, TLR2/4, Notch1 | Targets TRAF6, IRAK1 in macrophages critical components downstream TLR signaling | (58, 48) |
| miR-146b | Induced | TRAF6, IRAK1 TLR4 | Targets TRAF6, IRAK1 critical components downstream TLR signaling | (39, 49) |
| miR-155 | Induced | SHIP, SOCS1, CEBP, FADD, Ripk1, MyD88, TAB2, IKKe | Inhibits the expression of the negative regulators of TLR signaling, SHIP1 and SOCS1, Promotes TNFα translation, Abrogates expression of anti-inflammatory genes in macrophages | (42–44) |
| miR-221 | Induced | TNFα, STAT1, STAT2 | Negative regulation of inflammatory cytokine production in macrophages and DCs | (50, 51) |
| miR-222 | Induced | TNFα, STAT1, STAT2 | Negative regulation via chromatin remodeling mediated by SWI/SNF (switch/sucrose non-fermentable) and STAT1/2 in macrophages | (3, 52) |
| miR-132 | Induced | IRAK4, p300 | Responsible for inducing cross tolerance in monocytes/macrophages. Negative effect on the expression of interferon-stimulated genes and antiviral immunity in endothelial cells | (53, 54) |
| miR-21 | Induced | PDCD4, MyD88, IRAK1 IL-12p35 | Negative regulation of TLR4 signaling in monocytes. Inhibits the expression of MyD88 and IRAK1 during viral infection. | (55–58) |
| miR-579 | Induced | TNFα | Negative regulation of TNFα translation in monocytes. | (52) |
| miR-125b | Induced | TNFα | Negative regulation of TNFα translation, Negatively regulate viral responses by targeting TLR2/MyD88 signaling in monocytes. | (42, 59) |
| miR-212 | Induced | IRAK4 | Sustained expression is responsible for inducing cross tolerance in monocytes/macrophages | (54) |
| let-7e | Induced | TLR4 | Negative regulation of TLR4 signaling in macrophages | (43) |
| let-7i | Suppressed | TLR4 | Post-transcription regulation of TLR4 in epithelial cells | (60) |
| mir-124 | Induced | TLR6, MyD88, TRAF6, TNFα | Negatively regulates TLR signaling in BCG infection in macrophages | (61) |
| miR-149 | Suppressed | MyD88 | Represses MyD88 translation in macrophages | (62) |
| miR-203 | Induced | MyD88 | Represses MyD88 translation in macrophages | (63) |
| miR-92a | Suppressed | MAPK4 | Inhibits TLR4 responses in macrophages | (64) |
| miR-210 | Induced | NFκB1 | Targets NFκB1 upon stimulation in macrophages | (65) |
| miR-9 | Induced | NFκB1 | Negative control of NFκB in monocytes | (66) |
| miR-718 | Induced | PTEN | Down regulates TLR4, IRAK1, and NFκB in a negative feedback loop in macrophages | (67) |
| miR-98 | Suppressed | IL-10 | Targets IL-10 in macrophages | (68) |
cis-lncRNAs and trans-lncRNAs (nearby or remote to genes) and based on their relative position to target mRNAs, being exonic sense, intronic sense, antisense, bidirectional, and intergenic (75, 76). In contrast to miRNAs that have a clear role in promoting post-transcriptional regulation of gene expression, lncRNAs exhibit plethora of actions via transcriptional, post-transcriptional and translational regulation of gene expression as well as via controlling mRNA stability and promoting epigenetic changes (72, 75, 77–79).

LncRNAs have emerged as important regulators of innate immune responses and TLR signaling (74, 79–83). In response to LPS or other TLR stimuli, the lncRNAs expression pattern is altered and lcnRNAs have been shown to either promote or suppress pro-inflammatory responses (80, 84–86).

Several TLR-inducible lncRNAs limit excessive inflammatory responses by negatively regulating TLR signaling. The LPS-responsive lncRNAs Mirt2, THRIL, MALAT1, NKILA, lincRNA-21, and SeT have been shown to suppress expression of pro-inflammatory mediators including TNFα, the central cytokine for tolerance and CARS (Table 2). Mirt2 is expressed in macrophages and induced by LPS, negatively regulating TLR4 signaling; Mirt2 inhibits TRAF6 ubiquitination thus blocking NFκB and MARK activation and subsequent TNFα production (87). THRIL is another immuno-regulatory lncRNA that was found to interact with hnRNPL at the promoter region of the TNFα gene inducing TNFα expression (88). However, THRIL is downregulated upon TLR2 triggering indicating that THRIL suppression may be a protective feedback loop controlling TNFα levels and promoting cross-tolerance (88). The lncRNA MALAT1 has been found to negatively regulate TLR response via inhibition of NFxB; MALAT1 is upregulated in LPS-activated macrophages and interacts with NFxB in the nucleus, inhibiting LPS-induced expression of TNFα and IL-6 (89). Importantly, MALAT1 was found to be dysregulated in granulocytes from septic patients indicating its clinical importance in sepsis and CARS (104). Similar to MALAT1, NKILA is another lncRNA that regulates TLR4 signaling and restrains NFxB activation; NKILA is induced by LPS in tumor cells and interacts with the NFxB/κκB complex, preventing its phosphorylation by IKKs and subsequent NFxB activation (90). lincRNA-p21 is induced by LPS in fibroblasts and regulates NFxB activity in monocytes; lincRNA-p21 physically binds to RelA/p65 mRNA blocking translation of p65, resulting in inhibition of NFxB (94, 97, 98). Finally, the lncRNA SeT is expressed in macrophages in response to LPS and its homologous deletion results in biallelic TNFα expression and increase in TNFα levels (91). This finding suggests that lncRNA SeT suppresses expression of one of the two TNFα alleles early upon LPS stimulation (91).

Additional lncRNAs have been shown to suppress pro-inflammatory mediators such as IL-6 but their effect on TNFα expression has not been evaluated (Table 2). Lnc-IL-17R is upregulated significantly in response to TLR2 and TLR4 agonists, promoting H3K27 trimethylation and inhibiting LPS-inducible inflammatory response genes, such as IL-6, adhesion molecules, and chemokines (84). Similarly, the lncRNA IL7-AS (antisense) is induced by LPS in macrophages; knockdown of IL7-AS results in upregulation of IL-6 (92). Finally, lincRNA-EPS is expressed in macrophages and dendritic cells and was downregulated upon microbial infection, while gain-of-function experiments revealed that lincRNA-EPS binds to chromatin, regulates the nuclear ribonucleoprotein L (hnRNPL), thus suppressing LPS-induced pro-inflammatory genes (93). In addition to the lncRNAs outlined, lincRNA-Cox2 is another LPS inducible lncRNA that regulates hundreds of genes, but it appears to act both as an enhancer and as a suppressor of inflammation (80, 94, 95, 105). Finally, in a recent report, TLR4 tolerisation reversed LPS-induced suppression of PCGEM1 and HOTTIP lncRNAs and upregulated snaR lncRNA, but further investigation is required to define the function of these lncRNAs in the context of tolerance (79).

It appears that the changes in the outlined lncRNAs significantly regulate TLR signaling toward TLR reprogramming. However, the majority of the above lncRNAs were not evaluated in endotoxin tolerant experimental setting per se since their expression and function was not evaluated upon secondary TLR stimulation. Also, their relative contribution to tolerant state in conjunction with several miRNAs, that were mentioned above and have an established central role in endotoxin tolerance, has not been studied yet. Further research is required to address the importance and the level of contribution of these lncRNAs in endotoxin tolerance and/or cross tolerance.

**TLR-INDEPENDENT REGULATION OF ENDOTOXIN TOLERANCE VIA ncRNAs**

Establishment of endotoxin tolerance and cross-tolerance is not strictly a result of excessive TLR signaling and subsequent induction of intracellular regulators. The magnitude and duration of the innate cell tolerance is also controlled by a plethora of TLR—indepedent soluble mediators.

**Soluble Mediators in Innate Immune Cell Tolerance and Their Impact in ncRNAs**

Cytokines such as IL-1β, IL-10, TGFβ, and TNFα are capable to induce cross-tolerance or cytokine-mediated tolerance initiating intracellular signals similar to those of TLR ligands (17, 106). Indeed, IL-10 and TGFβ are part of a negative feedback loop produced from activated macrophages acting in an autocrine and paracrine manner to promote tolerance and suppress secondary TLR responses. However, LPS priming provokes more sustained tolerance than IL-10 priming, since IL-10-primed monocytes rapidly recover and produce TNFα (107). Also, endogenous hormones, such as adiponectin and glucocorticoids blunt LPS-induced inflammation and promote anti-inflammatory responses (108, 109). In contrast, interferons such as interferon gamma (INF-γ) and α2-interferon, are known to abrogate endotoxin tolerance and restore induction of pro-inflammatory cytokines (110, 111).

The aforementioned soluble mediators have been reported to achieve their effect via modulation of intracellular ncRNAs. The capability of IL-1β priming to induce tolerance and cross-tolerance in monocytes and epithelial cells is mediated via the increase of miR-146α (6). IL-10 has been shown to promote miR-146b upregulation in human monocytes and its transcription
Akt/glycogen synthase kinase 3 signaling and IL-10 production

is driven by STAT3, a transcription factor induced by IL-10 signals (49, 112). Similarly, TGFβ also promotes tolerance in human monocytes via upregulation of miR-146b driven by the transcription factor RUNX3 (112). Glucocorticoids and TGFβ have been shown to downregulate TLR4 signaling via induction of miR-511-5p, which targets TLR4 (113).

Stimulation with TNFα promotes TNFα–induced tolerance via regulation of ncRNAs. The lncRNA implicated in suppression of NFκB inflammatory response in fibroblasts upon TNFα stimulation is Lethe; Lethe binds and inactivates RelA/p65 and decreases p65 binding at NFκB sites (102). Moreover, upon TNFα stimulation, lincRNA-Cox2 is induced and promotes recruitment of the Mi-2/nucleosome remodeling and deacetylase (Mi-2/NuRD) repressor complex to the IL-12β promoter suppressing IL-12β expression (96).

IFN-γ is another mediator that enhances macrophage activation and reverses tolerance via regulation of ncRNAs. IFN-γ is known to inhibit miR-146b expression, a miRNA that contributes to endotoxin tolerance (112). Also, IFN-γ induces phosphatase and tensin homolog (PTEN) via downregulation of miR-3473b; MiR-3473b targets PTEN and promotes Akt/glycogen synthase kinase 3 signaling and IL-10 production (114). Furthermore, NeST, also known as Tmevpg1 or IFNgAS1, is an lncRNA located near the IFN-γ locus in both humans and mice and positively regulates expression of IFN-γ in T cells via histone modifications in IFN-γ locus (101).

Soluble ncRNAs as Modulators of Endotoxin Tolerance

Tissue injury leads to release of extracellular vehicles (EVs) that frequently include miRNAs (115–117). EVs are present in the circulation acting in a paracrine and endocrine manner and can modulate pro-inflammatory cytokine production contributing to a tolerogenic response (116). In addition freely circulating extracellular miRNAs may function as TLR agonists inducing tolerance (55, 118). EVs also promote tolerance in distant cells. For example, Treg derived exosomes deliver miR-150-5p and miR-142-5p to dendritic cells leading to the induction of LPS-induced IL-10 and suppression of LPS-induced IL-6, thus promoting tolerance (119).

CONCLUSIONS

To conclude, it appears that a variety of TLR ligands, cytokines, and soluble mediators control endotoxin tolerance and cross-tolerance via the regulation of ncRNAs. However, there is a significant number of ncRNAs that are implicated in endotoxin tolerance and their relative importance and contribution in this process remains unknown. It is also unclear whether a level of interdependency among these ncRNAs exists and how their function may converge toward common pathways or potentially contradict each other. Further research is required to take into account the levels of contribution of each ncRNA in the context...
of innate immune tolerance and to highlight the ones that have the potential to develop into therapeutic tools for CARS, the clinical syndrome associated with innate immune tolerance.

**AUTHOR CONTRIBUTIONS**

EV, KV, and CT reviewed the literature and drafted the manuscript.

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