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Regulation of TLR2-Mediated Tolerance and Cross-Tolerance through IRAK4 Modulation by miR-132 and miR-212

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Innate immune response is the first defense against pathogens via recognition by various conserved pattern recognition receptors, such as TLRs, to initiate a rapid and strong cytokine alarm. TLR signaling–mediated cytokine production must be properly regulated to prevent pathological conditions deriving from overproduction of cytokines. In this study, the role of specific microRNAs in TLR-signaling pathway was investigated to reveal the cross-interaction and -regulation in the MyD88 pathway. In peptidoglycan (PGN)/TLR2–stimulated THP-1 monocytes, PBMCs, and primary macrophages showed rapid and dramatic miR-132 and miR-212 (miR-132/-212) upregulation. This newly identified response appeared earlier in time than the characteristic miR-146a response in LPS/TLR4 stimulation. The rapid induction of miR-132/-212 was transcription factor CREB dependent, and the sustained expression of miR-132/-212 was responsible for inducing tolerance to subsequent PGN challenge. Cross-tolerance was observed by TLR5 ligand flagellin and heat-killed or live bacteria resulting from miR-132/-212 upregulation. Mechanistically, IRAK4 was identified and validated as a target of miR-132/-212 by luciferase reporter assay and seed-sequence mutagenesis of the reporter. Transfection of miR-132 or miR-212 alone mimicked PGN tolerance in monocytes, whereas transfected specific miRNA inhibitors tampered the tolerance effect. During bacterial infection, PGN-mediated TLR2 signaling induces miR-132/-212 to downregulate IRAK4, an early component in the MyD88-dependent pathway, whereas LPS/TLR4-induced miR-146a downregulates downstream components of the same MyD88-dependent pathway. The identification of miR-132/212 and miR-146a together to prevent damaging consequences from the overproduction of proinflammatory cytokines by targeting a common signaling pathway is significant and will provide insights into future design and development of therapeutics.

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Innate immune system is the primary defense mechanism that is ignited shortly after pathogenic invaders are detected by various conserved pattern recognition receptors. One such class of well-documented receptors is TLRs. These receptors are efficient in detecting signature molecules including lipopeptides, peptidoglycan (PGN), LPS, flagellin, and nucleic acids (1). Upon detection, the subsequent signaling events elicit a core set of stereotyped responses, including cytokines, chemokines, and adhesion molecules, most notably through activation of NF-κB transcription factor, which leads the immune system to sense and react to infection. In contrast, pathological dysregulation of this process is a hallmark of inflammatory damage, autoimmune diseases, and possibly cancer (2). Therefore, innate immune response involving TLR signaling cascades must be tightly regulated by elaborate mechanisms to control its onset and termination. It is acknowledged that TLR4 signaling events have been extensively studied both in vivo and in vitro in terms of endotoxin tolerance, which limits the pathogenic effects of LPS (3–7). Other microbial components such as PGN (a potent TLR2 agonist) are also involved in priming of innate immune cells (8, 9).

To explain this tolerance mechanism, a number of negative regulators have been proposed (10). These include soluble decoy receptors for TLR4, IL-1R–associated kinase (IRAK)M, TNF-α–induced protein 3 or TNFAIP3 (A20), tripartite motif-containing protein 30A (TRIM30a), and splice variants of signal-transduction proteins such as MyD88s (11–15). However, at this time, there is no consensus on the molecular mechanisms involved to resolve inflammation.

MicroRNAs (miRNAs), short noncoding RNA, have emerged recently as key regulators of gene expression acting at the post-transcriptional level (16). miRNAs have been shown to be critical in many biological processes, ranging from development to differentiation and including regulation of the mammalian immune system (17, 18). A few miRNAs are induced in innate immune response. A number of inhibitors have been described to block the activity of miRNA–mRNA interactions and are currently being explored as potential therapeutic targets.

These findings may provide insights into future design and development of therapeutics.
cells in response to cognate TLR ligands, with a consensus emerging that miR-146a, miR-155, and miR-21 are important to negatively regulate the activation of inflammatory pathways in myeloid cells (15, 18, 19). Although miR-146a regulation of IRAK1 and TNFR–associated factor 6 (TRAF6) adaptor molecules has been shown to play a major role in endotoxin tolerance and cross-tolerance, cytokine response is not extinguished completely, suggesting the possible involvement of other miRNAs in this intricate process (20, 21). It is well established that recruitment of adaptor kinases are the prime factor for triggering a TLR signaling cascade. Upon TLRs activation, IRAK4 is known to be recruited to MyD88, forming a helical assembly of the MyD88-IRAK4-IRAK2/1 complex that further activates TRAF6 and eventually leads to NF-κB activation for inflammatory gene transcription (22). Thus, IRAK4 should be the pivotal adaptor kinase used by all TLR signaling (except TLR3). In this connection, compared with IRAK1 knockdown, the knockdown of IRAK4 renders immune cells much less responsive to TLR agonists (23). Phenotypically similar to mice lacking MyD88, IRAK4 knockout mice show severe impairment of IL-1 and TLR signaling (24). On the basis of these reports, regulation of adaptor kinases might be an important molecular mechanism for maintaining cytokine response in a controlled manner. Although IRAK1 and TRAF6 are known to be regulated by miR-146a (25), no such miRNA-mediated regulation of IRAK4 has been documented. IRAK4 has been found to be a putative target of miR-132 and miR-212 (miR-132/-212) by bioinformatics analysis using TargetScan (TargetScan.org), but this has not been experimentally validated. Accordingly, in a very recent review, it is still unknown whether signaling molecules in TLR pathways are targeted by miR-132/-212 (26).

Mature miR-132/-212, sharing the same seed sequence, are processed from a single noncoding gene transcript regulated primarily by the CREB transcriptional factor (27, 28). The function for these miRNAs has been described in a few studies. miR-132 has been shown to regulate neuronal morphogenesis and the dendritic plasticity of cultured neurons (27, 29), miR-132 may also be responsible for limiting inflammation in the mouse brain by targeting acetylcholinesterase (30), miR-132 can also modulate inflammation induced by early-stage Kaposi’s sarcoma–associated herpesvirus (KSHV) infection (31). miR-212 can interfere with the craving for cocaine in mice (32) and acts as a tumor suppressor (33). To date, no detailed expression kinetic of miR-132 or miR-212 has been described in response to innate immune ligands associated with TLR ligand–induced tolerance.

To our knowledge, our study shows the first evidence that the exposure of innate immune cells to PGN, PamC5K,CysSerLys4 (Pam), flagellin, or whole bacteria induces rapid expression of mature miR-132/-212. This paper highlights the importance of investigating their mechanistic role in innate immunity in the context of TLR2 ligand–induced tolerance, which can modulate innate immune system.

Materials and Methods

Reagents

Ultrapure TLR-grade LPS (Salmonella enterica serotype Minnesota Re595), lipoteichoic acid (Staphylococcus aureus) were from Sigma-Aldrich (St. Louis, MO). PGN (Escherichia coli 0111:B4), synthetic bacterial lipopeptide LPS, and synthetic TLR ligands (Pam, LPS) were from InvivoGen (San Diego, CA). siGENOME SMARTpool small interfering RNA (siRNA) for IRAK4, CREB, and lamin A/C (LMNA) were from Dharmacon (Lafayette, CO). All miRNA-mimics, nonspecific (NS) miRNA-mimic negative control, and inhibitors (anti-miRNA inhibitor) were from Ambion (Austin, TX). Abs to human IRAK1, IRAK4, CREB, p-CREB, NF-κB p65 (sc-372), and ERK 1/2 (sc-15900) were from Santa Cruz Biotechnology (Santa Cruz, CA). Abs to p300 were from Abcam (ab14984; Cambridge, MA). Kinase inhibitors PD98059 and U0126 were from Calbiochem (San Diego, CA).

Cell culture and innate immune ligand stimulation

Human THP-1, HEK293, and murine RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). All cells were maintained in either RPMI 1640 medium or DMEM containing 10% FBS (v/v) (Mediatech, Manassas, VA) and 100 U/ml penicillin–streptomycin (Mediatech). For analysis of TLR-1 monocyte response to microbial ligand in vitro, log-phase cells were seeded at 5 × 10^5 cells/ml in a 24-well plate. Unless otherwise mentioned, cells were stimulated with the following agonists: 1000 ng/ml LPS from S. enterica (LPS Se; TLR4 ligand), Pam (TLR2/TLR1 ligand), PGN (TLR2 ligand), LPS from P. gingivalis (LPS Pg; TLR2 ligand), lipoteichoic acid (TLR2 ligand), and 300 ng/ml recombinant flagellin (TLR5 ligand). TLR ligands were reconstituted in endothoxin-free water and used at concentrations as reported previously (20).

For analysis of human PBMCs response, mononuclear cells were isolated from whole blood by a Ficol gradient (GE Healthcare, Uppsala, Sweden) and were grown in complete RPMI 1640 medium as described above. PBMCs (10^6 cells/ml) were then stimulated for 6–24 h with PGN (0–5 μg/ml).

TLR ligand–induced tolerance

PGN-, Pam-, or flagellin-induced tolerance and/or cross-tolerance experiments were performed using a TLR-1 monocyte model, adapted from methods previously described (9, 20) with minor modifications. Briefly, before starting tolerance assays, THP-1 cells were cultured until they were in log phase and reached a density of 10^6 cells/ml. THP-1 cells were washed with fresh medium and then stimulated for 6–24 h with PGN (0–10 μg/ml).

Mouse macrophages were from 3-mo-old female C57BL/6 mice, which were i.p. injected with 0.5 ml 4% sodium thio glycollate 3 d earlier. Peritoneal cells (~90% macrophages) were harvested by lavaging the peritoneal cavity and grown in DMEM as described above. After 5 h, cells were washed with growth medium and then stimulated for 6–24 h with PGN (0–10 μg/ml).

RNA extraction and real-time RT-PCR

Total RNA from microbial ligand–treated and untreated THP-1 cells were prepared using the miRNeasy mini RNA isolation kit (QIAGEN). For RNA analysis, 6.7 ng RNA of each sample was used for quantitative stem-loop reverse transcription and real-time PCR (qRT-PCR). Quantification of expression of mature miRNAs was performed using the TaqMan microRNA RT kit, TaqMan Universal PCR Master Mix, and TaqMan miRNA assay primers of interest for human or mouse miRNAs (Applied Biosystems, Carlsbad, CA). For gene expression analysis, cDNA was prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), and individual mRNA was monitored with the following inventoried TaqMan assays (Applied Biosystems): human IRAK1, IRAK4, CREB, and p300 with 33 ng total RNA per reaction. The cycle threshold (Ct) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined, and miRNA expression values were calculated using human RNU44 or mouse SnoRNA202 (Applied Biosystems) as an endogenous reference following the 2^−ΔΔCt (method 35). miRNA for gene expression values were quantified in the same way after normalization to mammalian 18S rRNA.
PCR analysis using synthetic mature miRNA (IDT, Coralville, IA). The Ct values were determined by qRT-PCR analysis of the total RNA from PGN (2500 ng/ml)-treated cells and then converted to miR-132 and miR-146a copy numbers using the standard curve.

**Transient transfection**

miR-132, miR-212, and miR-146a functional analyses were performed by transfecting synthetic mimic or inhibitor (40 nM) in THP-1 monocytic cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously (20, 21). THP-1 cells were transfected with siRNA targeting IRAK4, CREB, or LMNA using the same protocol. For luciferase assays, HEK293 cells were cultured in 24-well plate at 10^4 cells/well and transfected 24 h later by 3% Lipofectamine reagent.

**Luciferase reporter assay**

Complete 3′-untranslated region (3′-UTR) of IRAK4 was subcloned downstream of firefly luciferase coding sequence in pmiRTarget vector (wild-type version of IL-1R-associated kinase 4 [IRAK4-wt]; Origene Technologies, Rockville, MD), and then, this reporter (50 ng) and renilla luciferase reporter (0.1 ng) were cotransfected together with 100 nM miR-132-mimic, miR-212-mimic, or miR-146a-mimic into HEK293 cells for 48 h. A mutated version of this construct (IRAK4-mut) carrying 4-bp substitutions in the putative miR-132/-212 seed sequence target site was obtained by using the site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Reporter luciferase activities were measured using the Dual-Luciferase kit (Promega, Madison, WI) 48 h after transfection. To determine the functional regulation of IRAK4 3′-UTR during PGN stimulation, IRAK4-wt or IRAK4-mut together with the renilla luciferase reporter were cotransfected into THP-1 cells for 6 h. Cell lysates were harvested 12 h after PGN stimulation (2 μg/ml) for the measurement of luciferase activities.

**ELISA**

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

**Western blot analysis**

PGN, Pam- or flagellin-primed and unprimed THP-1 cells (5 × 10^6 condition) were collected 2 h after secondary challenge with ligands and then lysed with lysis buffer containing Complete Protease Inhibitors Cocktail (Roche, Indianapolis, IN) as described previously (20). THP-1 cell lysate preincubated with or without PD98059 (50 μM MEK1 inhibitor) and U0126 (0.5 μM MEK1/2 inhibitor), were similarly prepared. Soluble lysates were quantitated for protein concentration (Bio-Rad Bradford protein assay), separated by 10% SDS-PAGE, and electrotransferred to a polyvinylidene difluoride membrane. The membranes were blocked for 1 h at room temperature with 5% nonfat milk in PBS/0.05% Tween 20 (PBS-T) and were probed with rabbit polyclonal Ab anti-IRAK1 (1:300), anti-IRAK4 (1:300), and mouse monoclonal anti-tubulin (1:5000; Sigma-Aldrich). The membranes were then washed with PBS-T and incubated for 1 h with goat anti-rabbit or anti-mouse IgG-HRP (1:5000; Southern Biotechnology Associates, Birmingham, AL). After washing with PBS-T, reactive protein bands were visualized by SuperSignal Pico chemiluminescent reagent (Pierce, Rockford, IL). Similarly, CREB and p-CREB were visualized using specific Abs at 1:300 dilutions. Integrated density of protein bands in each lane was determined using ImageJ software and normalized to tubulin or CREB in each lane and is presented relative to results obtained with the control sample (percentage of fraction), which was set as 1.0.

**Ethics statement**

Experiments involving mice were approved by the institutional animal care and use committee of the University of Florida. The protocol for blood collection from healthy controls was approved by the Institutional Review Board; this study meets and is in compliance with all ethical standards in medicine, and informed consent was obtained from all patients, according to the Declaration of Helsinki.

**Statistical analysis**

Student t test (two-tailed) was used to compare data between groups, except where mentioned otherwise. Prism for Windows, version 5.0 (GraphPad Software, San Diego, CA), was used, and p < 0.05 was considered statistically significant.

**Results**

**TLR ligand–induced TNF-α secretion and kinetics of miR-132/-212 expression in innate immune cells**

Emerging data have shown that microbial ligands are recognized through specific receptors found on innate immune cells. Beside LPS, PGN is known to be a potent inducer of a diverse array of inflammatory mediators, including TNF-α both in vitro and in vivo. To monitor TNF-α production in vitro, supernatants of PGN-stimulated THP-1 monocytes were collected at 6 and 24 h. A similar lower TNF-α production was noted with the highest concentration of PGN tested in THP-1 cells (Fig. 1B); for comparison, LPS stimulation of THP-1 cells showed increased TNF-α production in a dose-dependent pattern, similar to LPS stimulation (21). The highest dose of PGN (5 μg/ml) induced the highest level of TNF-α, up to 12 ng/ml at 8 h (Supplemental Fig. 1A). The progressive changes of TNF-α secretion showed a dose- and time-dependent pattern, similar to LPS stimulation (21). The highest dose of PGN (5 μg/ml) induced the highest level of TNF-α, up to 12 ng/ml at 8 h. The kinetics of TNF-α mRNA expression was also measured by qRT-PCR and was consistent with the kinetics of the secreted TNF-α protein (data not shown).

In our recent studies, LPS-stimulated THP-1 monocytes showed a continuous expression of miR-146a, which was demonstrated as critical in LPS tolerance and cross-tolerance (20, 21). Similarly, to induction by LPS, an up to 25-fold increase of miR-146a expression was noted at 48 h with the highest concentration of PGN tested in THP-1 cells (Fig. 1C); for comparison, LPS stimulation of THP-1 cells often showed >100-fold increase of miR-146a (21). LPS-stimulated THP-1 monocytes are known to produce other miRNAs, including miR-132 and miR-155 (25). Interestingly and in sharp contrast, miR-132/-212 showed more impressive 110- and 65-fold increased expression in PGN-treated cells, respectively, at their peak (Fig. 1B). PGN treatment resulted in an early increase in miR-132/-212 levels at 4 h reaching 60- and 43-fold at the 5 μg/ml PGN dose, respectively, whereas the change in levels for miR-146a was only 7-fold (Fig. 1B). Although miR-132/-212 are derived from the same primary transcript, the reason for the difference in the lower fold induction in miR-212 compared with miR-132 is not clear. However, a similar lower expression in miR-212 compared with miR-132 has also been observed in KSHV-stimulated endothelial cells (31). Other miRNAs miR-155 (Fig. 1B) and miR-16 (data not shown) showed little or no change under the same conditions, although both miRNAs are expressed in relatively abundant levels in THP-1 cells. The exact copy numbers of both miR-132 and miR-146a in the PGN-treated cells (same data as in Fig. 1B) were determined using synthetic miR-132 and miR-146a as standards for the qRT-PCR assay (Supplemental Fig. 1A). Each unstimulated THP-1 cell showed only ~2 copies of miR-132 and ~30 copies of miR-146a. Upon PGN stimulation, each THP-1 cell could produce ~100 copies of miR-132 and ~600 copies of miR-146a at 12 h (Supplemental Fig. 1A). In contrast, LPS-stimulated THP-1 monocytes showed significantly lower miR-132 (10-fold) and miR-212 (3-fold) at 12 h (Supplemental Fig. 1B) than when stimulated with PGN. The above results indicate that PGN is a potent inducer for both miR-132/-212, which were focused for further investigation.

To understand the physiological relevance of induction of miR-132, a range of other cell types were used. Human PBMCs were treated with PGN from 6 to 24 h. PBMCs showed dose-dependent production of TNF-α up to 3 ng/ml (Fig. 1C) and upregulation of miR-132 up to 6-fold at 12 h (Fig. 1D). A similar increase of miR-132 expression (~5-fold at 24 h) was also observed in the mouse macrophage cell line RAW264.7 in response to PGN, Pam, and LPS (Fig. 1E). Similar results were also obtained in mouse primary peritoneal macrophages, which showed high levels of TNF-α production up to 10 ng/ml at 6 h (Fig. 1F) and
miR-132/-212 IN TLR2-MEDIATED CROSS-TOLERANCE

similar miR-132 expression (5-fold miR-132 upregulation peaking at 6 h) in response to PGN (Fig. 1G). It was noted that the expression fold change of miR-132 in these cells varied and were lower than in THP-1 monocytes. A similar variation of miR-146a expression has been observed in various cells (15). The variation may be attributed to the difference in cell types (monocytes versus macrophages) and cell population (homogeneous versus mixed cell). To further support the ability of PGN to induce miR-132/212 expression, effects of a common synthetic PGN-derivative Pam was also carefully examined in this study. Similar to PGN, Pam induced TNF-α production (Supplemental Fig. 1C) and miR-132/-212 expression (Supplemental Fig. 1D, 1E). The fold change for miR-132 was notably higher than that of miR-146a (Supplemental Fig. 1D) or miR-155 (Supplemental Fig. 1E). LPS from Pg (TLR2 ligand) also showed similar patterns of miR-132/-212 expression (data not shown). The capacity of TLR3 and TLR5 ligand to induce miR-132/212 were also examined. TLR3 ligand poly(I:C) (25 μg/ml) did not induce any of these miRNAs (data not shown), indicating that innate immune responses that involve these miRNA expression are associated with the MyD88-dependent pathway rather than the TLR3–TRIF pathway. TLR5 agonist flagellin stimulation induced a rapid production of TNF-α (Supplemental Fig. 1F) and, at 8 h, also showed greater increases of miR-132 (55-fold) and miR-212 (27-fold) than miR-146a (Supplemental Fig. 1G). Taken together, these findings suggest the capacity of PGN, Pam, and flagellin to induce significant levels of miR-132/212 in THP-1 monocytes, human PBMCs, and mouse primary macrophages. To gain a better understanding on the dynamic nature and robust expression kinetics, their biological significance was investigated in the subsequent studies.

CREB-dependent rapid induction of miR-132

In the previous time-course experiment, miR-132 was rapidly induced compared with miR-146a (Fig. 1B). miR-132 has been shown to be regulated by a key transcription factor, CREB (27). CREB is activated in THP-1 monocytes by phosphorylation at Ser133 (p-CREB) within minutes after exposure to PGN or Pam. Notably, at 15 min, PGN induced a 4.17-fold increase in p-CREB compared with untreated control by Western blot analysis (Fig. 2A), consistent with previous reports (27, 36). Pam also showed similar induction in p-CREB (Fig. 2A). In contrast, TLR3 ligand poly(I:C) stimulation of THP-1 cells did not induce p-CREB (data not shown), and little or no miR-132 induction was observed as mentioned above. It was noted that although the timing of PGN-induced p-CREB was correlated with the initiation of miR-132/-212 expression, at 4 h, the level of p-CREB was back to baseline untreated level, whereas miR-132/-212 expression continued to increase until 12 h (Fig. 1B). Although the reason is not clear, p-CREB might be only required to initiate the transcription of miR-132/-212. A similar observation was reported in KSHV-infected endothelial cells; the initiation of p-CREB correlated with the expression of miR-132 maintained at similar levels (31).

The requirement of CREB in the PGN-induced miR-132/-212 expression was further investigated. In THP-1 cells with siRNA-mediated knockdown of CREB, the levels of CREB mRNA were reduced by ~50% and protein by 70% as demonstrated by qRT-PCR (Fig. 2B) and Western blot analysis (Fig. 2C), respectively. Transfection of unrelated control siRNA for LMNA did not affect CREB mRNA level (Fig. 2B). Knockdown of CREB resulted in significantly reduced (p < 0.01) miR-132 expression in

**FIGURE 1.** PGN induction of TNF-α and miRNA expression kinetics in monocytes/macrophages. (A) Dose-response and time-course analysis of TNF-α secretion in culture supernatant by THP-1 monocytes stimulated with 0–5 μg/ml PGN for 2–48 h (horizontal axis). TNF-α in culture supernatants at the indicated time points was measured by ELISA. (B) qRT-PCR analysis of miR-146a, miR-132, miR-212, and miR-155 expression kinetics in respective PGN-treated THP-1 cells. (C and D) Dose- and time-dependent secretion of TNF-α and induction of miR-132 in human PBMCs stimulated with 0–5 μg/ml PGN for 6–24 h. At least three PBMCs from different healthy subjects were analyzed. (E) qRT-PCR analysis of miR-132 expression kinetics in mouse RAW264.7 cells stimulated for 2–24 h with 1 μg/ml PGN, Pam, or LPS. (F) TNF-α secretion by mouse primary macrophages treated for 6–24 h with 0–10 μg/ml PGN. Mouse macrophages were pooled from multiple animals, and assays were analyzed in triplicate. (G) qRT-PCR analysis of miR-132 expression. Human and mouse miRNA expression was normalized with control RNU44 and SnoRNA202, respectively. Data are from three independent experiments (A–E, mean ± SD), or experiments were performed three times and a set of representative data is shown (F, G, mean ± SD). *p < 0.05, **p < 0.01 (two-tailed unpaired t test) compared with untreated control.
Rapid induction of miR-132/-212 in PGN/Pam-stimulated THP-1 monocytes is mediated through CREB-dependent machinery. (A) Western blot analysis demonstrating phosphorylation of CREB (Ser133, p-CREB) within 15 min after PGN (top panel) or Pam (bottom panel) stimulation of THP-1 cells. Relative intensity is calculated as ratios of p-CREB to total CREB in cell lysates. (B) qRT-PCR analysis of CREB mRNA expression in THP-1 cells transfected without (mock) siRNA or with 100 nM siCREB or siLMNA. (C) Western blot analysis showing 70% reduction of CREB expression in THP-1 cells transfected with 100 nM siCREB using tubulin as a loading control. (D) miR-132 expression in siCREB-knockdown THP-1 cells stimulated for 5 h with PGN, Pam, and LPS. (E) Two-hour pretreatment of synthetic kinase inhibitors PD98059 and U0126 blocked phosphorylation of CREB in THP-1 monocytes stimulated with 1 μg/ml PGN for 30 min. Western blot analysis showed 5.22-fold increase in relative intensity of p-CREB in PGN-treated versus untreated control (UTX) with addition of DMSO alone and then stimulated for 30 min with 1 μg/ml PGN. (F and G) qRT-PCR analysis of primary and mature miR-132, miR-212, and miR-146a expression in THP-1 monocytes pretreated for 2 h with inhibitors PD98059 and U0126 and then stimulated for 8 h with PGN or Pam. Data points and error bars represent three independent experiments (B, D, F, G, mean ± SD). *p < 0.05, **p < 0.01 (two-tailed unpaired t test) compared with mock-transfected THP-1 monocytes (D) or corresponding cells not treated with inhibitors (F, G). Western blot data are representative of two independent experiments (A, C, E).

miR-132/-212 may account for PGN- and Pam-induced tolerance

Previously, PGN-induced homologous and heterologous tolerance has been shown in vitro and in vivo (8, 9). In this paper, to evaluate this tolerance phenomenon in greater depth, the most commonly used THP-1 cell model was used again. THP-1 monocytes were primed with PGN (500 ng/ml) or Pam (100 ng/ml) for 18 h, followed by challenge exposure with various TLR agonists (1000 ng/ml). After 3 h, the TNF-α protein levels were assessed by ELISA (Fig. 3A). As expected, TNF-α production was reduced by 80–90% in tolerized cells compared with untreated controls, after challenge with a panel of inflammatory ligands, including LPS Se, PGN, Pam, and LPS Pg. Analysis of TNF-α mRNA by qRT-PCR showed profoundly lower expression in tolerized cell (Fig. 3B), consistent with the data at protein level. Besides TNF-α, production of IL-1β, IL-6, and IL-8 was also assessed in PGN- or Pam-tolerized conditions (Supplemental Fig. 2A-C) from the same supernatant used in Fig. 3A. PGN-tolerized THP-1 monocytes showed significant IL-1β reductions by 20% against LPS Se challenge, 60% against PGN, and 50% against Pam but not against LPS Pg (Supplemental Fig. 2A). A similar pattern of IL-1β reduction was apparent in Pam-tolerized cells with clear effect observed in challenge with PGN or Pam but not LPS Se or LPS Pg (Supplemental Fig. 2A). IL-6 was clearly diminished across all conditions by ~50–70% in PGN- or Pam-tolerized THP-1 cells against the same or different ligands (Supplemental Fig. 2B). For IL-8, PGN- or Pam-tolerized THP-1 monocytes showed 20–30% reduction after PGN, Pam, or LPS Pg challenge, whereas no effects were observed against LPS Se challenge (Supplemental Fig. 2C). Thus, PGN- or Pam-tolerized cells showed uniform hyporesponsiveness for TNF-α and IL-6 but variable hyporesponsiveness for IL-1β and IL-8 when challenged with different inflammatory ligands. Because the levels of miRNA examined are not different (Fig. 3C), the variable hyporesponsiveness for IL-1β and IL-8 has...
to be explained by other means, such as other unidentified miRNA or additional regulatory factors other than IRAK4.

Having examined the tolerance in PGN- or Pam-primed THP-1 monocytes, we then investigated whether there was any association of miR-132/-212 expression with the observed tolerance. miRNA expression in the tolerized THP-1 cells were examined compared with the control (Fig. 3C). As expected, miR-132 and miR-212 in the PGN-tolerized sample showed ∼40- and ∼20-fold higher expression, respectively, over 18 h of initial incubation plus 3 h of challenge compared with untolerized controls; in contrast, miR-146a showed ∼10-fold increase, whereas miR-155 had little or no changes in expression over the same time period (Fig. 3C).

Analysis of TNF-α and miR-132 expression in the PGN-tolerized human PBMCs showed similar results to that obtained in THP-1 monocytes (Fig. 3D, 3E). Similar to PGN-primed PBMCs, which can show cross-tolerance to multiple TLR ligands (3), Pam-primed PBMCs showed a reduction of TNF-α and IL-6 against PGN, Pam, and LPS challenges, and this was negatively correlated with miR-132 expression (Supplemental Fig. 2D-F). PGN-primed mouse primary macrophages showed a similar reduction of TNF-α and increase in miR-132 level after challenge with PGN, Pam, or LPS (Fig. 3F, 3G). Murine macrophage RAW264.7 cells showed similar PGN-induced tolerance and cross-tolerance with LPS (Supplemental Fig. 2G), similar to previously described (9); in this case, there was ∼5-fold increase in miR-132 compared with control unprimed cells (Supplemental Fig. 2H). In addition to PGN or Pam, the capacity of flagellin to induce tolerance was also examined. Flagellin-primed THP-1 monocytes after challenge with flagellin, PGN, or Pam showed reduced TNF-α production and increase in miR-132/-212 expression (Fig. 4A–C). Furthermore, PGN- or Pam-primed THP-1 monocytes showed cross-tolerance to flagellin challenge (Fig. 4E), and this was consistent with the high level of miR-132 expression (Fig. 4F).

Next, we investigated changes in adaptor kinase expression, which has been implicated in hyporesponsiveness. IRAK4 is an important candidate based on its indispensable role in the MyD88-dependent TLRs pathway, and we identified it to be a putative target of miR-132/-212 based on TargetScan prediction for miRNA
targets (37). To find such a potential correlation between IRAK4 and miR-132/-212, IRAK4 expression after priming with either PGN or Pam and challenged with different ligands was analyzed by Western blot, in which a moderate reduction of ∼32–76% was observed in all cases when normalized to tubulin level and compared with unprimed control (Fig. 3H). A similar decrease of IRAK4 protein was evident in PGN-treated RAW264.7 cells (up to 64% reduced at 8 h; Supplemental Fig. 2I), in flagellin-treated THP-1 cells (up to 41% reduced at 6 h; Fig. 4D), and in THP-1 monocytes treated with PGN or Pam for 18 h (up to 90% reduced; Fig. 4G). To investigate the probable cause for the variations in IRAK4 protein reduction, IRAK4 expression under increasing priming concentrations of PGN or Pam was examined. The data showed that increasing priming concentrations from 0.01 to 1 µg/ml yielded a greater reduction in IRAK4 protein, as evidenced by Western blot analysis (Fig. 5G, 5H). Notable tolerance was demonstrated by significant reduction of TNF-α at priming with 0.5 or 1 µg/ml PGN (Fig. 5A) or Pam (Fig. 5B). With the increase of priming dose, miR-132/-212 expression was also increased as expected, and priming with 1 µg/ml PGN or Pam caused a >90% reduction (p < 0.01) of
TNF-α, which correlated with the higher miR-132/-212 levels that were not observed at lower PGN/Pam priming concentrations (Fig. 5A–F). In our recent report, elevated miR-146a expression depended on continuous exposure to LPS or PGN (20). Similarly, in this paper, after 12 h of PGN withdrawal, cells started to regain PGN responsiveness and were almost completely recovered from tolerance after 22 h (Fig. 6A). At this point, miR-132/-212 expression was significantly lower (p < 0.01) compared with 18 h of continuous PGN priming plus 5 h of PGN challenge (Fig. 6B), indicating the importance of their presence at high levels to keep cytokine levels under control.

Taken together, these data suggest the likely role of miR-132/-212 in PGN- or Pam-induced tolerance, based on the inverse correlation with proinflammatory cytokine production and the repressed levels of IRAK4. Another promising concept that can be introduced is that bacterial components seem to cause priming of miR-132/-212, which renders innate immune cells hyporesponsive to subsequent challenge. The half-lives (t1/2) of miR-132/-212 estimated from this experiment were ~9–10 and ~10–12 h, respectively, based on the simple difference in fold change between the data points (Fig. 6B). The t1/2 of these miRNAs may in part determine the programmed control in how long the PGN-induced tolerance takes effect. It is acknowledged that the actual t1/2 is likely shorter than these estimated t1/2 because of continuous miRNA synthesis. In any case, to ensure that physiological changes in the level of IRAK4 was indeed acknowledged that the actual t1/2 is likely shorter than these estimated t1/2 because of continuous miRNA synthesis. In any case, to ensure that physiological changes in the level of IRAK4 is the molecular target of miR-132/-212 expression.

Upregulation of miR-132/-212 alone can mimic PGN/Pam priming to induce tolerance

Earlier in this paper, the dramatic induction of miR-132/-212 by PGN or Pam was shown (Fig. 1B). To monitor the direct consequence of miR-132/-212 expression in TLR2 ligand–induced tolerance, THP-1 cells were transfected with 40 nM miR-132- or miR-212-mimic alone or in combination with miR-146a-mimic for 24 h. After an 8-h challenge of transfected THP-1 cells (>95% viable) with PGN, TNF-α production decreased 58% by miR-132-mimic, 47% by miR-212-mimic, 50% by miR132- plus miR-212-mimic, 63% by miR-146a-mimic transfection (positive control), 70% by miR-132- plus miR-146a-mimic transfection, and 67% by miR-212- plus miR-146a-mimic transfection compared with mock transfected control (Fig. 7A). Transfection with NS control or unrelated miR-375-mimic did affect the level of TNF-α production by THP-1 monocytes transfected in parallel as in (A) substituted only with miRNA inhibitor(s) (total 40 nM). Effective inhibition of miR-132/-212 expression in THP-1 cells transfected with 40 nM miRNA respective inhibitors (132-inh, 212-inh) for 24 h and then stimulated for 16 h with 100 ng/ml PGN. (D) Recovery of TNF-α production by THP-1 monocytes transfected for 24 h with miRNA inhibitor (total 40 nM) and primed/tolerized with PGN (100 ng/ml) for 8 h and then challenged with 500 ng/ml PGN for 3 h. There was no apparent change in proliferation and cell survival in cells treated with miRNAs-mimics and inhibitors compared to the controls. Data represent three independent experiments (A–C) or an experiment with triplicate sample analyzed (D, mean ± SD). *p < 0.05, **p < 0.01 (two-tailed unpaired t test) compared with mock transfected controls.
miR-146a-mimic in combination with either miR-132-mimic (70% reduction) or miR-212-mimic (67% reduction) showed only slightly increased reduction of TNF-α production compared with individual miRNA-mimic alone suggesting this may be a limitation of the experimental system or that the contribution of these miRNAs to PGN-tolerance is only up to 70%. To corroborate such a role of miR-132 or miR-212, a parallel experiment in THP-1 cells was performed by substituting the miRNA-mimics with miRNA inhibitors (Figure 7B). TNF-α production increased by 36–54% in miRNA inhibitors transfected cells compared with the mock transfected control (Fig. 7B). TNF-α production was not significantly affected when cells were transfected with unrelated miR-375 inhibitor. Similar results were obtained in a parallel experiment in THP-1 cells transfected with miRNA mimics and inhibitors but challenged with Pam instead of PGN (data not shown). The function of the miR-132/-212 inhibitors was documented in transfection with up to 90% reduction of these miRNAs (p < 0.01) compared with mock transfected THP-1 monocytes (Fig. 7C). To address the contribution of miR-132 and miR-212 in PGN-tolerance, their respective miRNA inhibitors were transfected to THP-1 cells for 24 h prior to the tolerance assay. Normalizing the TNF-α production of the untransfected unprimed and PGN-primed cells (1'PGN) to 100% (untolerized) and 0% (tolerized), respectively, the effect of each miRNA inhibitor alone on the increase in TNF-α production ranged from 28 to 37% (Fig. 7D). Of note, miR-132 or miR-212 inhibitor plus miR-146a inhibitor showed more pronounced increase of TNF-α (61–64%) than each inhibitor alone, suggesting a cooperative effect. Transfection of unrelated miR-375 inhibitor did show significant effect compared with control without miRNA inhibitor. These data support the dominant role of PGN- or Pam-induced miR-132 and/or miR-212 to mediate tolerance.

**IRAK4 is the molecular target of miR-132/-212**

In the above experiments, the levels of IRAK4 showed a negative correlation with miR-132/-212. According to the TargetScan algorithm, IRAK4 shares the same single 3'-UTR binding site for both miR-132/-212 (Fig. 8A). The interaction between IRAK4 and miR-132/-212 was analyzed by luciferase assay using the pMir-3'IRAK4 vector containing the 3'-UTR of IRAK4 cloned downstream of a firefly luciferase reporter. HEK293 cells were cotransfected with pMir-3'IRAK4 vector (reporter IRAK4-wt), RL luciferase control vector, and miR-132-mimic, miR-212-mimic, or miR-146a mimic. Luciferase expression was significantly reduced with miR-132 mimic (~50%) and miR-212-mimic (~25%), whereas no significant modulation was observed with miR-146a-mimic or for the reporter with 4 nt of the seed sequence mutated (IRAK4-mut; Fig. 8B). To further verify the direct regulation of IRAK4

![Figure 8](http://www.jimmunol.org/)
by miR-132/-212, the expression of IRAK4 mRNA and protein was assayed in THP-1 cells, which showed 35–40% reduction after miR-132- and miR-212-mimic transfection compared with mock or miR-146a-mimic transfection (Fig. 8C, 8D). The specificity for IRAK4 repression was demonstrated because miR-132-mimic and miR-212-mimic had no apparent effect on IRAK1, TRAF6, and LMNA mRNA (Fig. 8C). Western blot analysis showed no change in NF-κB p65 and ERK 1/2 levels in miR-132-mimic–transfected cells compared with NS control–transfected cells (Fig. 8D, right panel). Conversely, IRAK4 mRNA was moderately increased after blocking of mature miR-132/-212 using respective miRNA inhibitors in THP-1 monocytes (Fig. 8E). Fig. 8F further shows the functional regulation of IRAK4 3′-UTR in THP-1 cells during PGN stimulation because the IRAK-wt reporter showed 40% reduction in luciferase activity compared with IRAK-mut control and only after PGN stimulation. Having confirmed IRAK4 as a molecular target of miR-132 and/or miR-212 using various approaches, the next question was whether silencing of IRAK4 would affect cytokine secretion. siRNA targeting IRAK4 (siIRAK4) showed >50% reduction at the mRNA level (Fig. 8G) and 69% decrease in protein by Western blot analysis (Fig. 8H). With the silencing of IRAK4, TNF-α, IL-6, and IL-8 (data not shown) were markedly reduced (40–60%) after PGN, Pam, or LPS challenge (Fig. 8I); these reductions in cytokines are similar to those in previous reports with IRAK4 knockdown (23, 39). The overall findings is consistent with IRAK4 being targeted by miR-132 and/or miR-212, a promising mechanism to prevent excessive cytokine production.

Bacteria-induced miR-132/-212 contribute to resistance to bacterial infection

In the preceding experiments, PGN- or Pam-induced tolerance or cross-tolerance was observed against purified TLR2, TLR4, and TLR5 ligands. However, in nature, hosts are exposed to whole bacteria that usually display more than one type of TLR ligands. Thus, it is important to determine whether miR-132/-212 are induced during periodontal bacterial infection (49). In this study, HK P. gingivalis and T. forsythia significantly induced miR-132 (p < 0.01) and miR-212 to a similar extent (data not shown), whereas HK T. denticola had a much milder effect (Fig. 9A). When infected with live T. forsythia, THP-1 cells showed a similar miR-132 expression, whereas P. gingivalis and T. denticola showed relatively low levels of induction (Fig. 9B, left panel). As expected, in the same infected monocytes, miR-212 showed a similar expression pattern as miR-132 (Fig. 9B, right panel). In contrast, miR-146a expression was significantly lower than miR-132 or miR-212 (data not shown), similar to our previous results (40). HK T. forsythia–primed THP-1 cells showed significant reduction (p < 0.01) of TNF-α after challenge with HK bacteria and various ligands (Fig. 9C), and a similar reduction was observed in live T. forsythia–infected THP-1 monocytes (data not shown). HK T. forsythia–tolerized THP-1 cells showed a significant increase (p < 0.001) of miR-132 (Fig. 9D) compared with untreated controls. In line with higher miR-132/-212 expression, HK and live P. gingivalis– and T. forsythia–treated THP-1 monocytes showed substantial reduction (50–60%) of IRAK4 protein expression versus control (Fig. 9E). These data were consistent with the above findings in PGN- and Pam-tolerized THP-1 monocytes. Of note, none of these bacteria induced miR-132/-212 to a similar extent (especially T. denticola), suggesting their expression might be bacteria and ligand specific. In summary, higher expression of miR-132/-212 plays an important role in providing tolerance or cross-tolerance in the in vitro THP-1 cell model against various TLRs ligands as well as bacterial infection.

Discussion

Emerging results indicate that TLR activation affects the expression of a few key miRNAs (26, 41, 42). Recently, an increased expression of miR-146a in response to LPS in THP-1 monocytes was described (21), whereas a relatively smaller amount of miR-132 induction was noted in the same condition, although no detailed expression analysis of miR-132/-212 by other TLR ligands has been documented. Of note, induction of miR-212 by innate immune ligand has not been described previously. In this study,

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miR-132/-212 was selected for further study because of its unusual high fold change in expression. Note that the PGN-induced miR-132/-212 (this study) and LPS-induced miR-146a responses (20, 21) are not typical of the “fine-tuner” character described for miRNAs in TLR signaling (26). Other studies have characterized miRNAs as “rheostats” that make fine-scale adjustments to protein output (43, 44). The induced expression of miR-132/-212 suggests their role in a more elaborated programmed feedback mechanism.

The rapid and high levels of miR-132/-212 induction by PGN, Pam, or flagellin stimulation are analogous to miR-146a induction by LPS. In this article, miR-132/-212 expression kinetics following several microbial ligands were examined in THP-1 monocytes, human PBMCs, and mouse primary macrophages. Unlike LPS, TLR2/TLR5 ligands triggered a sharp increase in miR-132/-212 expression at earlier time points compared with miR-146a in THP-1 monocytes (Fig. 10A, PGN priming). miR-132 remained at significantly high levels over 48 h. On the basis of these observations, we can conclude that compared with miR-146a, miR-132/-212 have earlier expression kinetics, which may be very important during acute infection. It is noted that although miR-132/-212 showed higher fold changes than miR-146a (~80-versus 15-fold, respectively, at 12 h), the copy number of miR-132 (~100 copies/cell) was lower than miR-146a (~500 copies/cell). The fact is that untreated THP-1 cells have a lower basal level of miR-132 than miR-146a. Upon PGN or Pam stimulation, TNF-α production was negatively correlated with miR-132/-212 expression similar to previous observations on kinetics of miR-146a and TNF-α after LPS stimulation (21). Similar to LPS-induced tolerance mediated by miR-146a (21), the higher fold change of miR-132/-212 induced by TLR2/TLR5 ligands might represent the early response to infection, followed by the miR-146a response as reported for TLR4 stimulation (20, 21).

miR-132/-212 induction by PGN, Pam, or flagellin stimulation is controlled by CREB activation. miR-132/-212 is located on chromosome 17p13 and transcriptionally activated by CREB in neurons. Its rapid upregulation has been observed in KSHV-infected cells through phosphorylation of CREB (31). In this article, LPS induced a lower level of miR-132/-212 compared with PGN/Pam stimulation. Although both LPS and PGN have been reported to interact with CD14 (45, 46), differences in binding affinity to different receptors (TLR4 versus TLR2) and other unknown factors may all contribute to differences in the specificity of the dominant miRNA induction (miR-146a versus miR-132/-212). PGN-induced phosphorylation of CREB was not due to endotoxin contamination (36); this demonstration of PGN specificity is consistent with the fact that only ultrapure ligands were used in this study. Moreover, PGN and LPS induce differential activation of MAPKs, with LPS strongly inducing all three families of kinases (ERK, JNK, and p38), whereas PGN only induces ERK and JNK without affecting p38 (36). Inhibition of activation of these kinases by U0126 and PD98059, reduced both in primary and mature miR-132/-212, whereas miR-146a was not diminished, indicating their specificity without affecting other general miRNA-processing function. CREB-mediated expression of miR-132/-212 induced by PGN or Pam is a novel finding, and this opens a new horizon to evaluate their kinetics in depth in innate immunity regarding the mechanism of cross-tolerance. CREB-regulated rapid miR-132 induction may serve as an antiapoptotic response in macrophages. Thus, CREB activity is important in innate immunity against certain bacteria, such as Salmonella spp., Shigella spp., and Yersiniae spp., which inhibit survival signals and induce apoptosis of macrophages as a mechanism to evade the host immune response (47).

miR-132/-212 play important roles in TLR2 ligand-induced tolerance and cross-tolerance. Although our data and other reports suggest that PGN is a potent trigger for cytokine production, tolerance induced by this Gram-positive bacteria cell wall component has not been studied as extensively as LPS tolerance. The ability of PGN to induce heterologous tolerance shown in this study is congruent with previous findings (8, 9). Accordingly, monocytes primed with PGN or Pam showed hyporesponsiveness to TLR2 ligands (PGN, Pam, and LPS Pg) or TLR5 ligand flagellin. Moreover,
flagellin-primed cells showed tolerance to itself, PGN, or Pam with notably higher miR-132/-212 expression. Thus, these new findings support that tolerance or cross-tolerance is linked to miR-132/-212 overexpression. The dominant effect of miR-132/-212 alone, or in combination with miR-146a, in tolerance was verified by transfection experiments with the corresponding miRNA-mimics; all miRNA mimics showed significantly less TNF-α response to either PGN or Pam. In contrast, knockdown of miR-132/-212 expression using miRNA inhibitors, also known as antagonirs (48), alone or in combination with knockdown of miR-146a in THP-1 cells tampered the tolerance effect with an increased inflammatory response to TLR2 ligand. Taken together, miR-132/-212 plays an important regulatory role in cytokine production, PGN-induced tolerance, and cross-tolerance. The high levels of miR-132/-212 also may contribute to other regulation downstream of IRAK4. miR-132 may also targets p300 as reported in an earlier study in neuronal cells (31). Because p300 is a known regulator of the innate immune response, and specifically binds the TNF promoter (49), miR-132 may also exert its regulation via p300. However, in THP-1 cells used in the current study, expression of p300 is very low, and no significant change is detected in LPS-, PGN-, or Pam-stimulated cells as monitored by both qRT-PCR and Western blot (Supplemental Fig. 3).

Regulation of IRAK4 by miR-132/-212 is the major mechanism for PGN-induced tolerance. miR-132/-212 share an identical seed sequence and thus would be expected to regulate a similar subset of target genes. However, miR-132/-212 can be used for similar (50) or distinct functions in different cell types (30, 33). In this article, IRAK4 mRNA was validated as a molecular target for miR-132/-212 with apparently different degree of efficiency. In experiments to evaluate the effect of IRAK4 knockdown on PGN-stimulated tolerance, cytokine response was not eliminated but significantly reduced, suggesting that other IRAK family members may compensate in part during infection. This mechanism seems biologically relevant because IRAK4-deficient mice were viable against *S. typhimurium* infection (39). Fig. 10A and 10B outlines the model that demonstrates how PGN-induced miR-132/-212 play an important role in the response to microbes or its components at early stages of infection and limits the overstimulation of proinflammatory cytokines by suppressing IRAK4. Low-dose PGN-primed THP-1 cells (10 ng/ml; Fig. 10A) produce TNF-α rapidly and continue to do so for 4-6 h. As soon as regulatory miR-132 starts to increase, then TNF-α production decreases. At 18 h postpriming, a profound difference between miR-132 expression and TNF-α secretion is established because of the negative effect on IRAK4 by upregulated miR-132, which leads to tolerance (Fig. 10A). Unlike the untolerized control (Fig. 10B), tolerized cells do not respond to even a high dose of PGN challenge (Fig. 10A).

Our findings fully support the dominant role of miR-132/-212 in the PGN- and related ligand-induced tolerance. Besides purified ligands, whole HK or live *P. gingivalis* and *T. forsythia* stimulation showed significant expression of miR-132/-212 in monocytes. Subsequently, *T. forsythia*-primed monocytes showed significant reduction of TNF-α after challenge with various ligands because of the reduction of IRAK4 by higher miR-132/-212 expression. It is interesting to speculate that miR-132/-212 induced by bacteria or its components may play a role in immune-inflammatory diseases, such as periodontitis, by affecting IRAK4 or other targets like matrix metalloprotease-9 as shown in a report on the critical role of miR-132/-212 for epithelial–stromal interactions via targeting matrix metalloprotease-9 (50).

The important biological significance in TLR ligand–induced tolerance is that it is a part of innate immune response to TLR ligands (danger signals) and macrophage/monocyte response by producing proinflammatory cytokines such as TNF-α. The affected cells have done their job by sounding the cytokine “alarm,” and TLR ligand–induced tolerance is a mechanism to dampen cytokine production in a programmed manner. As the alarm is rung, further stimulation with the same TLR ligand (tolerance) or different TLR ligands (cross-tolerance), even at a high dosage, does not generate a strong cytokine response and thus prevent overproduction of proinflammatory cytokines that are capable to induce tissue damages. In TLR signaling, the current understanding from structural studies is that binding of TLR ligand to receptor activates the formation of mydosome, which involves the helical assembly of the MyD88-IRAK4-IRAK2/IRAK1 complex (22). Thus, IRAK4 is recruited to MyD88 earlier than IRAK2/IRAK1, both targets of miR-146a (18, 21, 25). As discussed above, the miR-132/-212 response to TLR2 ligand appears earlier acting on IRAK4, whereas miR-146a affects IRAK2/1 and TRAF6 somewhat later.

The most interesting part is that both miR-146a and miR-132/-212 target key adaptors of the MyD88-dependent TLR signaling pathway—like a one-two punch—to ensure that the blockage of this pathway will lead to tolerance state to inhibit further stimulation and limiting cytokine production. The speculation of a one-two punch is illustrated in Fig. 10C where bacterial infection releases LPS, PGN, and flagellin, which first induces TLR signaling via the MyD88-dependent pathway leading to the activation of NF-κB and production of TNF-α and other cytokines. In the TLR1 cell model, there is an ~2-h delay in the appearance of miR-132/-212 (this study) and miR-146a (20, 21). These two miRNA sets serve as a one-two punch to the MyD88-dependent pathway by specific translational repression of IRAK4 and IRAK2/1, which are the critical components for the formation of the mydosome complex. This consideration is appropriate, as in any given bacterial infection, the host cell is likely presented by different TLR ligands, and the cooperative response by these miRNAs on the MyD88-dependent pathway is potentially advantageous to ensure cross-tolerance is achieved to prevent overproduction of proinflammatory cytokines.

MyD88-dependent TLR pathways use IRAK4 and patients deficient in IRAK4 failed to respond to IL-1, IL-18, and six TLRs (TLR1–5 and TLR9) as expected (1). Accordingly, the upregulated miR-132/-212 in PGN-mediated tolerance is likely to affect other pattern recognition receptor activity in innate immunity. As all TLRs, with the exception of TLR3, use IRAK4 and the MyD88-dependent pathway, they all are likely to be regulated by miR-132/-212 in a comparable manner. Detection and activation of immune cells in response to PGN also can occur through alternate pattern recognition receptors including NOD1 and NOD2 (51, 52), CD14, and a family of PGN recognition proteins (53), and it remains possible that these receptor signaling pathways are involved in the mechanism of PGN-induced tolerance. As a consequence, PGN-induced tolerance associated with the upregulated miR-132/-212 may have a broader role in regulating TNF-α by TLR pathways.

Innate immune response to the invading microorganism in animals may be influenced by miR-132/-212. PGN-tolerant mice were significantly resistant to both gram-positive (*S. aureus*) and gram-negative (*Pseudomonas aeruginosa*) bacteria (8). Overexpression of miR-132/-212 associated with bacteria or PGN tolerance is likely to have important consequences in host innate immunity responding to myriad bacterial infections. More extensive studies on the expression kinetics are needed to fully explore the role of these miRNAs, especially in terms of its half-life in PGN-tolerant animals. In vivo investigations, such as the phenotypic analysis of mice with targeted deletion of miR-132/-212,
may be necessary to fully elucidate the role of these miRNAs in innate immunity. Note that mir-132-212 have been reported as dysregulated in cancer (33, 54, 55), and overexpression of mir-132 has been shown in such inflammatory diseases as rheumatoid arthritis and osteoarthritis (56). Therefore, mir-132-212 expression may be associated with inflammation and tumorigenesis and, given its role in innate immunity, might be an important link between inflammation and cancer.

In summary, a series of evidence provides mechanistic insights into the function of mir-132-212 in Toll2/TLR5 ligand-induced tolerance, which operates as a negative regulatory feedback mechanism to prevent uncontrolled inflammatory reaction potentially comparable to that observed in sepsis. The major milestone of this study has been highlighted using various cell types, including human and murine monocytes/macrophage cell lines, murine primary macrophages, and human PBMCs. In these cells, miR-132 and/or miR-212 were upregulated in response to purified ligands as well as bacteria. It should be noted that key components of MyD88 pathway such as IRAK2/1 and TRAF6 are regulated by mir-146a, and our article shows that IRAK4 is targeted by miR-132-212 via mRNA degradation and/or translational repression. These miRNAs have now been worked in to what appear to be a complementary fashion in response to the various ligand stimulations or bacterial infections. Thus, further investigations of the modulation of the levels of mir-132-212 alone and/or in combination with miR-146a may be very important because these are interesting targets for therapeutic intervention for boosting or limiting TLR activation.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure S1. Supplemental data on the characterization of miRNA expression and TNF-α production during TLR-ligand stimulation of THP1 monocytes. (A) Time-course analysis of copy number of miR-132 and miR-146a in PGN-stimulated THP-1 monocytes. Copy number of miR-132 and miR-146a in THP-1 cells stimulated for 0-24 h (horizontal axis) with PGN (2500 ng/ml). (B) Induction of miR-132 and miR-212 by LPS treatment in THP-1 monocytes. qRT-PCR analysis of dose- and time-dependent expression of miR-132 and miR-212 in THP-1 monocytes stimulated for 2-24 h with 0-1000 ng/ml LPS. (C) TNF-α production by THP-1 cells stimulated with Pam. THP-1 monocytes were incubated for 2-48 h with 0-1000 ng/ml Pam and TNF-α in culture supernatant was measured by ELISA. (D-E) qRT-PCR analysis of miR-146a, miR-132, miR-212, and miR-155 expression kinetics in Pam-treated THP-1 cell. (F) TNF-α production by THP-1 monocytes stimulated for 0-24 h with 300 ng/ml flagellin. (G) qRT-PCR analysis of miR-146a, miR-132, and miR-212 expression in the same THP-1 cells stimulated with 300 ng/ml flagellin. All miRNA expressions were normalized to RNU44. Data are from three independent experiments (mean ± s.d.). *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with time 0 controls (A,F-G) or untreated controls (B-D).
Supplementary Figure S2. Supplemental data on cytokine production in THP-1 cells, human PBMCs, and mouse RAW264.7 cells primed with PGN or Pam and challenged by other TLR-ligands. (A-C) Diminished proinflammatory cytokine secretions by PGN- and Pam-primed THP-1 monocytes. ELISA of IL-1β (A), IL-6 (B), and IL-8 (C) production by THP-1 monocytes primed with or without PGN or Pam (500 ng/ml) for 18 h and then challenged with various ligands (horizontal axis) for 3 h (IL-1β) or 24 h (IL-6 and IL-8). (D-F) High levels of miR-132 may account for Pam-induced tolerance in human PBMCs. TNF-α (D) and IL-6 (E) production by human PBMCs primed with or without Pam (1000 ng/ml) for 18 h and then challenged with various ligands (horizontal axis) for 3 h (TNF-α) or 24 h (IL-6). (F) qRT-PCR analysis of miR-132 in the PBMC treated as in D. (G-I) High levels of miR-132 may promote PGN-induced tolerance in mouse RAW264.7 cells. (G) TNF-α production by RAW264.7 cells primed with or without PGN (1000 ng/ml) for 18 h and then challenged with various ligands (horizontal axis) for 3 h. (H) qRT-PCR analysis of miR-132 in the purified total RNA obtained from cells described in G. (I) Immunoblot analysis of IRAK4 in RAW264.7 cells stimulated for 4 and 8 h with PGN (2000 ng/ml). Tubulin serves as a loading control. Data and error bars (mean ± s.d.) are from either from two (A-C and G-H) or three (D-F) independent experiments. *P < 0.05; **P < 0.01 (two-tailed unpaired t-test) compared with unprimed control.
Supplementary Figure S3. p300 expression in THP-1 cells stimulated by LPS, PGN, Pam, and flagellin. (A) Little or no expression of p300 mRNA detected in THP-1 cells untreated (UTX) or treated with 1 μg/ml of LPS, PGN, or Pam for different time points as indicated up to 24 h. Positive control RNA from HeLa cells was included for the qRT-PCR analysis. (B) Immunoblot analysis of p300 in THP-1 cells stimulated for 2 or 8 h with 1 μg/ml of LPS, PGN, or flagellin. Cell lysate from HeLa cells was included as a positive control and tubulin analyzed as a loading control.