JLB

Article

TLR4, TLR7/8 agonist-induced miR-146a promotes macrophage tolerance to MyD88-dependent TLR agonists

M. Abu Nahid,[1] Lia M. Benso, John D. Shin,[2] Huseyin Mehmet,[3] Alexandra Hicks,[4] and Ravisankar A. Ramadas[5]

Immunology, Oncology, and Immunomodulatory Receptors Discovery Research, Merck Research Laboratories, Boston, Massachusetts, USA

RECEIVED MAY 8, 2015; REVISED DECEMBER 15, 2015; ACCEPTED FEBRUARY 5, 2016. DOI: 10.1189/jlb.2A0515-197R

ABSTRACT

TLRs facilitate the recognition of pathogens by immune cells and the initiation of the immune response, leading to the production of proinflammatory cytokines and chemokines. Production of proinflammatory mediators by innate immune cells, such as macrophages, is tightly regulated to facilitate pathogen clearance while limiting an adverse impact on host tissue. Exposure of innate immune cells to TLR ligands induces a state of temporary refractoriness to a subsequent exposure of a TLR ligand, a phenomenon referred to as "tolerance." This study sought to evaluate the mechanistic regulation of TLR4 and TLR7/8 ligand-induced tolerance to other TLRs by microRNA-146a. With the use of THP-1 macrophages, as well as human classic and alternative macrophages, we demonstrate that priming with a TLR4 agonist (LPS) or a TLR7/8 agonist (R848) induces homologous and heterologous tolerance to various TLR ligands in macrophages, leading to the impaired production of cytokines and chemokines. We also demonstrate that overexpression of microRNA-146a is sufficient to mimic LPS or R848-induced hyporesponsiveness. Conversely, inhibition of microRNA-146a activity leads to LPS- or R848-induced TLR hyper-responsiveness in TLR signaling tolerance. Furthermore, we demonstrate that microRNA-146a dampens cytokine production following a primary stimulus with MyD88-dependent but not MyD88-independent TLR pathways. Collectively, these data provide comprehensive evidence of the central role of microRNA-146a in TLR signaling tolerance to plasma membrane, as well as endosomal TLR ligands in human macrophages. *J. Leukoc. Biol.* 100: 339–349; 2016.

Abbreviations: C_t = cycle threshold, Flag = flagellin, IRAK1 = IL-1R-associated kinase 1, IRF = IFN response factor, M1 = classic macrophage, M2 = alternative macrophage, MCP = monocyte chemoattractant protein, MDC = macrophage-derived chemokine, miR/miRNA = microRNA, MSD = Meso Scale Diagnostics, ODN = oligodeoxynucleotide, Pam2 = palmitoyl-2-cysteine-serine-lysine-4, PBST = PBS-Tween, PGN = peptidoglycan, poly I:C = polyinosinic:polycytidylic acid, PRR = pattern recognition receptor, qRT-PCR = quantitative real-time RT-PCR, TRAF6 = TNFR-associated factor 6

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Inflammation is an intricate pathophysiological state orchestrated by various immune cells in response to microbial infection and/or tissue damage. Innate immune cells, such as monocytes and macrophages, recognize the invading pathogens by conserved PRRs and respond appropriately to resolve the infection [1]. TLRs, a well-characterized cluster of prototype PRRs, play a central role in the recognition and response to infection by macrophages, as well as cells in the adaptive immune system [2]. Most TLRs are located on the plasma membrane, with the exception of TLR3, TLR7, TLR8, and TLR9, which are localized in the endosomal compartment [3, 4]. Whereas engagement of TLRs by cognate ligands leads to the production of proinflammatory mediators that are essential for clearing the pathogen, unchecked production of proinflammatory mediators might lead to systemic inflammatory damage and the development of pathologic states, such as sepsis, autoimmune diseases, metabolic diseases, and cancer [5–8].

Previous studies have described endogenous mechanisms that limit such overproduction of proinflammatory mediators, such as endotoxin tolerance and pyroptosis, as a caspase-mediated programmed cell death pathway [9, 10]. For example, leukocytes from septic patients exhibit a refractory state to a subsequent LPS challenge and no longer produce undesirable levels of inflammatory mediators [11, 12]. This phenomenon, referred to as endotoxin tolerance (or refractoriness), is a host mechanism that limits overactivation of the immune system upon continuous exposure to pathogenic insults. This could manifest as a dampened response to a subsequent stimulus with the same ligand (homologous tolerance) or to a different ligand (heterologous tolerance) [13–17]. Whereas this phenomenon has been

1. Current affiliation: Tripler Army Medical Center, Honolulu, Hawaii, USA.
2. Current affiliation: Biogen, Cambridge, Massachusetts, USA.
3. Current affiliation: Proteostasis Therapeutics, Cambridge, Massachusetts, USA.
4. Current affiliation: Onkaido, Cambridge, Massachusetts, USA.
5. Correspondence: Immunology, Oncology, and IMR Discovery Research, Merck Research Laboratories, 33 Ave. Louis Pasteur, Boston, MA 02115, USA. E-mail: ravisankar.ramadas@merck.com

0741-5400/16/0100-339 © Society for Leukocyte Biology

described extensively in the context of TLR4 signaling, similar tolerance phenomenon in response to endosomal TLR ligands, such as TLR7/8, remains underexplored.

miRNAs have recently emerged as major regulators of gene expression [18–20], primarily by targeting the 3′-untranslated region of mRNAs to facilitate mRNA degradation or translational inhibition. The potential role of miRNA in the regulation of autoimmunity and cancer is an active area of investigation [21]. The role of miR-146a in the regulation of IRAK1 and TRAF6 expression downstream of TLR4 activation, as well as its role in facilitating TLR4 homologous signaling tolerance, has been demonstrated previously in monocytic cell lines [16, 22–25]. However, the role of miR-146a in other TLR signaling pathways and other TLR signaling tolerance mechanisms and its role in primary human macrophages has not been investigated extensively. Therefore, in this study, we evaluated the expression profile of miR-146a, as well as its role in regulating the homologous and heterologous TLR signaling tolerance in both human monocyte/macrophage cell lines, as well as primary human macrophages.

METHODS

Cell culture and innate immune ligand stimulation

Human THP-1 cells, an undifferentiated promonocytic cell line (American Type Culture Collection, Manassas, VA, USA), were maintained by twice-weekly passage in RPMI-1640 medium (Life Technologies, Thermo Fisher Scientific, Grand Island, NY, USA) containing 25 mM HEPES and L-glutamine, 10% FBS, and 100 U/ml penicillin-streptomycin at 37°C with 5% CO_2. Log-phase cells were used in all experiments and cultured at the density of 5×10^5 cells/ml. To determine the kinetics of ligand-induced cytokine and/or chemokine production in vitro, fresh THP-1 monocytes were suspended in complete RPMI-1640 culture medium and seeded at 5×10^5 cells/ml in 24-well plates. Cells were stimulated with the indicated concentrations of the following TLR agonists: 1000 ng/ml LPS (TLR4 ligand) from *Escherichia coli*, serotype O111:B4; palmitoyl-3-cysteine-serine-lysine-4 (TLR2/TLR1 ligand); PGN (TLR2 ligand); Pam2 (TLR2 ligand); imidazoquinoline compounds, such as R848 (TLR7/8 agonist), or imiquimod (TLR7 agonist); CpG-ODN (TLR9 ligand); poly I:C (TLR3 ligand); and 300 ng/ml recombinant Flag (TLR5 ligand). All of these TLR ligands were ultrapure, obtained from InvivoGen (San Diego, CA, USA), and reconstituted in endotoxin-free water, which was used at previously reported concentrations [16, 26].

THP-1 monocytic cells were differentiated into macrophages (THP-1 macrophages), following the methods described previously [27, 28], and can be considered as a suitable, noninvasive tool for macrophage-associated studies. In brief, cellular differentiation was induced by subjecting THP-1 monocytic cells (10^6 cells/ml) to 10–50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO, USA) in RPMI-1640 medium for 48 h in a 12-well plate. The plates were then washed 3 times with complete growth medium to remove nonadherent cells. The adherent macrophages were incubated further for 8–10 h in fresh culture medium for better recovery and differentiation. Additionally, human PBMC-derived monocytes were polarized to M1 and M2 macrophages, following previous methods with minor modifications [29]. In brief, leukopheresis packs were obtained from Research Blood Components (Brighton, MA, USA). PBMCs were isolated using Ficoll-Paque density centrifugation and frozen before monocyte isolation. Human $CD14^+/CD16^+$ monocytes were negatively selected PBMCs using magnetic beads (Stemcell Technologies, Vancouver, BC, Canada). Purified cells were cultured for 6 d with RPMI-1640 media containing 100 ng/ml GM-CSF or 50 ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA). Suspension cells were then discarded, and adherent cells were gently scraped off of the tissue-culture flask for use in functional assays. The cells were harvested, and culture supernatants were collected over 24 h and stored at −80°C until assays were performed for mediator release, such as TNF-α, IL-1β, MIP-1β, MIP-1α, MCP-1, and MDC. Cell pellets were washed in PBS and stored at −80°C after flash-freezing for total RNA isolation in subsequent analysis.

In vitro induction of homologous and heterologous TLR signaling tolerance

LPS- or R848-induced tolerance and cross-tolerance using monocytes and/or macrophages were adapted from methods described previously with minor modifications [16, 23, 26, 30–32]. In the TLR signaling tolerance assays, PMA-treated, adherent THP-1 macrophages were treated with a low dose of LPS (50 ng/ml) for 18 h to prime the cells. In some tolerance or cross-tolerance studies, cells were primed for 18 h with R848 (100 ng/ml). After washes with tissue-culture grade PBS, the cells were restimulated with TLR ligands (LPS, PGN, Pam2, Flag, R848, imiquimod, ODN, and poly I:C) at the mentioned concentrations or cultured without stimulation (untolerized negative control). Likewise, M1 and M2 macrophages were incubated for 4 h in complete medium to become adherent before priming with LPS or R848 (100 ng/ml) for 18 h, followed by washing, and challenged with different inflammatory ligands. Six to 24 h following secondary challenge, supernatants were harvested and stored at −80°C until assays were performed for secreted inflammatory mediators, such as TNF-α, MIP-1β, MIP-1α, MCP-1, and MDC. Cell pellets were used for RNA isolation and qRT-PCR analysis. To ensure that the priming doses of TLR ligands did not reduce cell viability over the duration of the experiment, we used CellTiter-Glo reagent (Promega, Madison, WI, USA) to evaluate cell viability in THP-1 monocytes and THP-1-differentiated macrophages, 2, 4, and 24 h following stimulation with 100 ng/ml LPS.

miRNA microarray and quantification of miRNA and mRNA expression levels

Total RNA from microbial ligand-treated and untreated cells was prepared using the *mir*Vana miRNA isolation kit (Life Technologies, Thermo Fisher Scientific). RNA yield and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop , Thermo Scientific, Wilmington, DE, USA) or with an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). High-quality RNA was used for the miRNA expression analyses using a TaqMan Low Density Array v3.0 (Applied Biosystems, Thermo Fisher Scientific) to detect and quantify ~754 human miRNAs, according to the manufacturer's protocol. Data were analyzed by Expression Suite Software (v1.03) and TIBCO Spotfire (v 5.5.1). Validation or quantification of mature miRNA expression in different conditions was performed using the TaqMan qRT-PCR, as described before [23]. Equal amount of each RNA (6.7 ng for miRNA and 33 ng for mRNA) were used for qRT-PCR analysis. For mRNA analysis, a high-capacity cDNA RT kit (Applied Biosystems, Thermo Fisher Scientific) and TaqMan mRNA assay primers for mRNA expression were used. The C_t values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, was determined. miRNA expression values were calculated by RQ Manager software (version 1.2) using RNU44 as an endogenous control (Applied Biosystems, Thermo Fisher Scientific), following the $2^{-\Delta\Delta Ct}$ method [33]. mRNA expression values were quantified in the same way after normalization to mammalian 18S rRNA.

Transient transfection of miR-146a modulators

Synthetic miR-146a mimic or inhibitor (100 nM) was transfected in PMA-treated THP-1 macrophages using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. miR-146a mimic and mock-transfected (miRNA mimic-negative control) macrophages were incubated for 24 h, followed by washing twice with complete growth medium. The washed cells were treated with TLR ligands at the indicated concentration for 6 h. For miR-146a inhibitor experiments, transfected macrophages and mock (miRNA inhibitor-negative control) were incubated for 24 h, followed by washing with complete growth medium. Cells were then challenged with TLR ligands for 6 h. Supernatants from cell cultures were harvested and assayed for cytokine and/or chemokine secretion, and cell pellets were used for RNA isolation and qRT-PCR analysis.

Cytokine and chemokine detection

Cytokine and/or chemokine concentrations in cell culture supernatants were measured by highly sensitive MSD electrochemiluminescence assay (MSD, Rockville, MD, USA), according to the manufacturer's specifications. In brief, single- or multispot (7- or 9-plex), 96-well MSD plates, precoated with the antibodies against inflammatory cytokines or chemokine, were incubated with 1% blocker A solution for 1 h at room temperature on a plate shaker set at 700 rpm. After the plates were washed with PBST, 50 μl calibrators (standard) and harvested supernatants were added for 2–3 h and incubated at room temperature on a plate shaker set at 700 rpm. Following wash with PBST, 25 μl SULFO-TAG-labeled anti-human inflammatory cytokine or chemokine detection antibody solution was added for 2 h and incubated at room temperature on a plate shaker set at 700 rpm. After the plates were washed with PBST, 2× MSD Read Buffer T was added to the plates (150 μl/well) and read immediately on MSD Sector Imager 2400 at (620 nm).

Flow cytometry

THP-1 monocytes or PMA-treated THP-1 macrophages were stimulated with LPS, poly I:C, R848, or ODN for 30 min. Untreated and treated cells were subjected to surface TLR4 and endosomal TLR3, TLR7, and TLR9 expression analysis by FACScan. In brief, following ligand stimulation, naive and differentiated cells were transferred onto ice and washed with ice-cold FACS stain buffer (BD Biosciences, San Jose, CA, USA) before fluorescent staining with directly labeled antibodies. Cells were blocked with Human TruStain FcX (FcR blocking solution; BioLegend, San Diego, CA, USA) for 10 min at room temperature. For endosomal TLR expression analysis, the cells were fixed and permeabilized with the BD Cytofix/Cytoperm kit (BD Biosciences). Cells were then stained with a 1/10 dilution of Alexa Fluor 488-conjugated anti-human CD284 (TLR4; mouse monoclonal; eBioscience, San Diego, CA, USA), PE-conjugated anti-human CD283 (TLR3; mouse monoclonal; eBioscience), PE-conjugated anti-human TLR7 antibody (mouse IgG1; Thermo Fisher Scientific), Alexa Fluor 488-conjugated anti-human TLR9 (sheep polyclonal IgG; R&D Systems), or respective isotype control antibody for 30 min at 4°C. Following washing with FACS buffer, flow cytometry was performed by using LSRFortessa and FACSDiva software (BD Biosciences). Data were analyzed by FlowJo software, version 6.0 (Tree Star, Ashland, OR, USA).

Western blot analysis

LPS-primed and unprimed THP-1 macrophages were collected 2 h after secondary challenge with various ligands and then lysed with lysis buffer containing cOmplete protease inhibitors cocktail (Roche, Indianapolis, IN, USA), as described [23]. Likewise, R848, poly I:C-treated macrophages, and miR-146a mimic or miR-146a inhibitor-transfected cell lysate were prepared. Soluble lysates were quantitated for protein concentration by the Bradford assay (Coomassie Plus protein assay reagent), and Western blot for an equal amount of protein was performed using LI-COR Odyssey Western blotting kits (LI-COR Biotechnology, Lincoln, NE, USA). In brief, proteins were separated by 10% SDS-PAGE precast gel (Bio-Rad Laboratories, Hercules, CA, USA) and electrotransferred to a polyvinylidene difluoride membrane using the iBlot gel transfer device (Life Technologies, Thermo Fisher Scientific). Membranes were blocked for 1 h at room temperature with LI-COR blocking buffer and were probed with primary rabbit polyclonal antibody (1:300; Santa Cruz Biotechnology, Dallas, TX, USA) against human IRAK1, TRAF6, IRAK4, MyD88 adaptor proteins, or mouse monoclonal anti-tubulin (1:5000; Sigma-Aldrich), overnight at 4°C. The membranes were then washed with PBS-T and incubated for 1 h with secondary Alexa Fluor 700 conjugated goat anti-rabbit or anti-mouse IgG (1:5000; Life Technologies, Thermo Fisher Scientific). After washing with PBST, reactive protein bands were visualized by Odyssey infrared imaging system.

Statistical analysis

Data are presented in the figures as means ± SE. For multiple group comparisons, a 1- or 2-way ANOVA test was performed, followed by post hoc Dunnett's or Bonferroni test to correct for multiple comparisons. Unpaired 2-tailed Student's *t*-test was used to compare 2 independent groups. For all statistical analysis, Prism for Windows, version 6.0 (GraphPad Software, San Diego, CA, USA), was used, and $P < 0.05$ was considered statistically significant.

Online Supplemental material

These materials include Supplemental Figs. 1–3 and their legends.

RESULTS

Kinetics of LPS-induced expression of TNF-α and miRNAs in innate immune cells

We evaluated the expression kinetics of TNF-α and miR-146a expression in THP-1 cells, PMA-differentiated THP-1 macrophages (THP-1 macrophages), as well as polarized human M1 and M2 macrophages in response to a single stimulation with LPS (**Fig. 1**). A significant increase in TNF-α levels was observed, 2–4 h following LPS stimulation, followed by a gradual decline over a period of 24 h in THP-1 cells (Fig. 1A) and THP-1 macrophages (Fig. 1B). In human M1 and M2 macrophages, TNF-α levels were increased at 8 h, followed by a decline at 24 h (Fig. 1C). There was a time-dependent increase in miR-146a expression in these samples (Fig. 1D–F), which negatively correlated with TNF-α levels. This trend was similar in THP-1 cells, THP-1 macrophages, as well as human M1/M2 macrophages in the evaluated time points. To determine if other innate immune-relevant miRNAs also display a similar expression pattern, we evaluated miR-132 and miR-155 expression in THP-1 macrophages, as well as human M1 and M2 macrophages following LPS stimulation (Supplemental Fig. 1). We observed that neither miR-132 (Supplemental Fig. 1A) nor miR-155 (Supplemental Fig. 1B) was increased significantly in THP-1 macrophages upon LPS stimulation.

Cytokine and chemokine responses by TLR ligand-stimulated monocytes and macrophages

Whereas LPS-induced TNF-α production in macrophages is a commonly used readout for TLR4 responsiveness and endotoxin tolerance studies, such reliable readouts for other TLR agonists have not been characterized extensively. Therefore, a panel of TLR agonists was used for stimulating THP-1 cells and THP-1 macrophages to evaluate other cytokines and chemokines. First, we confirmed successful THP-1 differentiation (increased CD11b expression), as well as the expression of a subset of TLRs, such as TLR4, TLR7, TLR9, and TLR3, in THP-1 monocytes and THP-1 macrophages by flow cytometry (Supplemental Fig. 2). Among these, TLR4, -7, and -9 were strongly expressed in THP-1 cells and THP-1 macrophages, whereas TLR3 was only modestly expressed (Supplemental Fig. 2). The production of TNF-α following TLR ligand stimulation was comparable between THP-1 cells (**Fig. 2A**) and THP-1 macrophages (Fig. 2F), where LPS, PGN, and Pam2 induced the most robust responses. However, whereas R848, imiquimod, ODN, and poly I:C induced only modest MIP-1α, MIP-1β, MCP-1, and MDC production in THP-1 cells Fig. 2B–E), the production of these cytokines was significantly higher in the THP-1 macrophages (Fig. 2G–J). Flag-induced production of TNF-α, MCP-1, and MDC was significantly

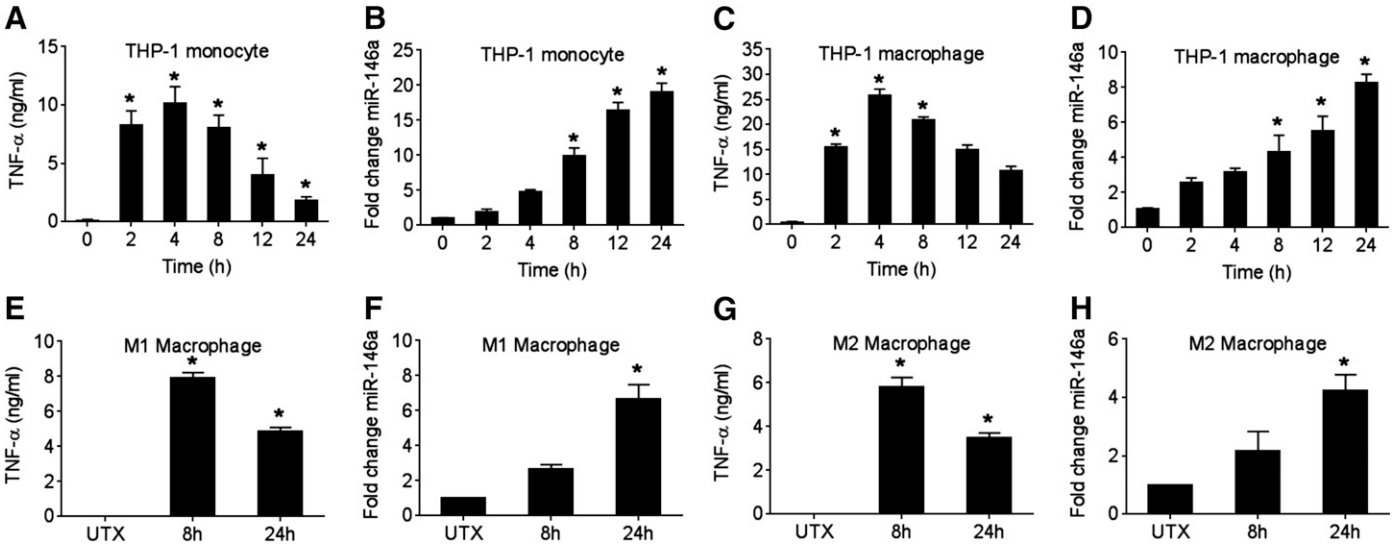

Figure 1. LPS induction of TNF-α and miRNA expression kinetics in monocytes and macrophages. THP-1 monocytes, PMA-treated (50 ng/ml) THP-1 monocyte-derived macrophages (THP-1 macrophage), and polarized human macrophages were treated with 100 ng/ml LPS and incubated for 0, 2, 4, 8, 12, or 24 h. Culture supernatants were collected from LPS-treated THP-1 monocytes (A), THP-1 macrophages (C), as well as M1 (E) or M2 (G) macrophages at the indicated time points, and TNF-α protein secretion was analyzed using the MSD ELISA kit. qRT-PCR analysis of miR-146a expression kinetics in the same set of LPS-treated THP-1 monocytes (B), THP-1 macrophages (D), M1 (F), and M2 (H) macrophages. miRNA expression was normalized to control RNU44. Data are expressed as means ± SD from 3 technical replicates from 1 of 2 independent experiments. $^*P < 0.05$ compared with untreated macrophages. UTX, Untreated.

higher than the untreated controls in THP-1 macrophages (Fig. 2H–J), but not in THP-1 cells (Fig. 2C–E).

LPS-induced miR-146a may account for tolerance to a panel of cell surface and endosomal ligands in macrophages

Consistent with previous findings on homologous LPS tolerance, a primary stimulation (priming) with LPS significantly reduced the production of TNF-α in response to a subsequent LPS stimulus (**Fig. 3A**). In addition, LPS priming significantly reduced the production of TNF-α in response to a subsequent stimulus with a range of other TLR ligands, such as PGN, Pam2, Flag, R848, and imiquimod, demonstrating TLR-induced heterologous tolerance to other TLR ligands (Fig. 3A). Similar trends in homologous and heterologous tolerance to LPS were also observed in the production of MIP-1β (Fig. 3B), whereas the level of miR-146a induction in all of the LPS-primed treatment groups was comparable (Fig. 3C), suggesting that a second challenge with TLR ligands does not modify the miR-146a expression levels. However, the expression of other miRNAs, such as miR-132 or miR-155, was unchanged under the same conditions (data not shown). It has been demonstrated previously that LPS-primed M1 and M2 macrophages produce less TNF-α following a secondary LPS challenge [34]. Here, we demonstrate that the LPS-primed M1 macrophages are hyporesponsive to a subsequent stimulation with LPS, PGN, Pam2, Flag, as well as R848, as evidenced by reduced TNF-α or MIP-1β protein production (Fig. 3D and E), and are inversely correlated with miR-146a expression (Fig. 3F). Likewise, with the confirmation of LPS-induced homologous tolerance in M2 macrophages, we also demonstrate a similar heterologous LPS tolerance to PGN, Pam2, Flag and R848, as evaluated by TNF-α (Fig. 3G) and MIP-1β (Fig. 3H) production, as well as the inverse correlation with miR-146a expression in M2 macrophages (Fig. 3I). Evaluation cell viability following LPS priming demonstrated that there is a small but significant loss of viability in THP-1 macrophages (Supplemental Fig. 3C) but not monocytes (Supplemental Fig. 3A), whereas the production of TNF-α was compared with the responses observed in the other studies (Fig. 3B and D). As homologous and heterologous TLR signaling tolerance to LPS was observed in the THP-1 monocytes and macrophages, it is unlikely that loss of viability contributes to reduced cytokine and chemokine production upon secondary TLR challenge following an initial TLR ligand priming. IRAK1 and TRAF6 are the 2 important molecular targets of miR-146a, based on the TargetScan prediction for miRNA targets [35]. It has been demonstrated previously that LPS priming of THP-1 monocytes induces the degradation of IRAK1 and TRAF6 [16]. We also observed a reduction of IRAK1 and TRAF6 protein levels under heterologous LPS tolerance conditions compared with nontolerized control cells (Fig. 3J), suggesting that the miR-146a-induced reduction of these adaptor molecules might contribute to LPS-induced heterologous tolerance to other TLR ligands.

miR-146a expression is increased in macrophages following TLR7/8 stimulation

With the use of a human miRNA array, we demonstrate that miR-146a was up-regulated significantly in THP-1 macrophages upon R848 stimulation compared with unstimulated controls (**Fig. 4A**). Whereas miR-146a was up-regulated upon stimulation with R848 and LPS compared with untreated controls, other innate immune system-relevant miRNAs, such as miR-132 and miR-155, were not up-regulated following R848 stimulation (Fig. 4A).

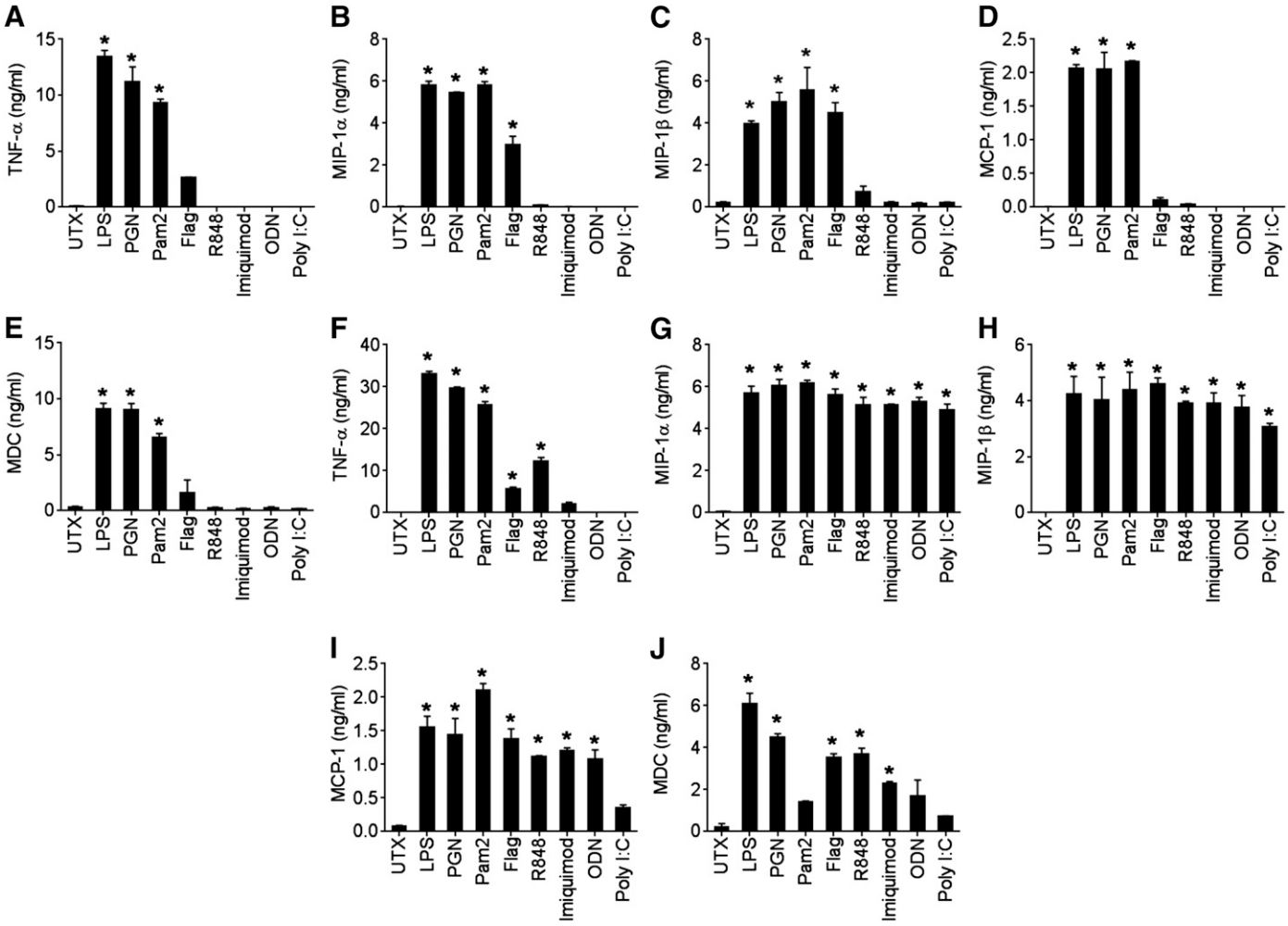

Figure 2. Proinflammatory mediator production by THP-1 monocytes and THP-1 macrophages. Undifferentiated THP-1 monocytes (A–E) or PMA-differentiated THP-1 macrophages (F–J) were stimulated with a range of surface and endogenous TLR agonists for 6 h, and the proinflammatory mediators, such as TNF-α (A, F), MIP-1α (B, G), MIP-1β (C, H), MCP-1 (D, I), and MDC (E, J), were measured by the MSD ELISA kit, as described in Methods. Data are expressed as means ± SD from 3 technical replicates from 1 of 2 independent experiments. $*P < 0.05$ compared with unstimulated macrophages.

Upon R848 stimulation, a time-dependent increase in miR-146a expression was not observed in THP-1 monocytes (Fig. 4B) but observed in THP-1 macrophages (Fig. 4C). The expression of miR-132 was unchanged in the THP-1 macrophages upon stimulation with R848 (Fig. 4D). Stimulation of THP-1 monocytes or macrophages with poly I:C or ODN did not induce a similar time-dependent increase in miR-146a expression (Fig. 4E and F). Evaluation of miR-146a at a select time point (24 h) following LPS stimulation in the same experiments confirmed previous findings that LPS induced miR-146a in THP-1 monocytes and macrophages (Fig. 4E and F). In primary human macrophages, TLR ligand-induced miR-146a expression was much more robust in the M1 macrophages, whereas LPS-, PGN-, Pam2-, Flag-, and R848-induced miR-146a expression was significantly higher than the untreated controls (Fig. 4G). Whereas the induction of miR-146a expression was still observed in the M2 macrophages, only Flag- and R848-induced miR-146a levels were significantly higher than the untreated controls (Fig. 4H).

R848-induced homologous or heterologous tolerance to TLR ligands in macrophages is associated with increased miR-146a expression

Next, with the use of monocytes and macrophages, we evaluated if homologous and heterologous TLR signaling tolerance is also observed following priming by endosomal TLR ligands, such as R848. In unprimed samples, an increase in TNF-α production was observed in response to all TLR ligands evaluated, except for ODN and poly I:C (**Fig. 5A**). Priming with R848 resulted in a significant decrease in TNF-α production in response to a second stimulation with R848, as well as LPS, PGN, Pam2, and Flag (Fig. 5A). The expression of miR-146a was significantly higher in the R848-primed samples compared with unprimed controls (Fig. 5B). There level of TNF-α production was increased in unprimed M1 macrophages upon stimulation with LPS, PGN, Flag, and R848, along with a significant reduction in the TNF-α level in the R848-primed M1 macrophages (Fig. 5C). As observed with THP-1 macrophages, the expression of miR-146a was significantly higher

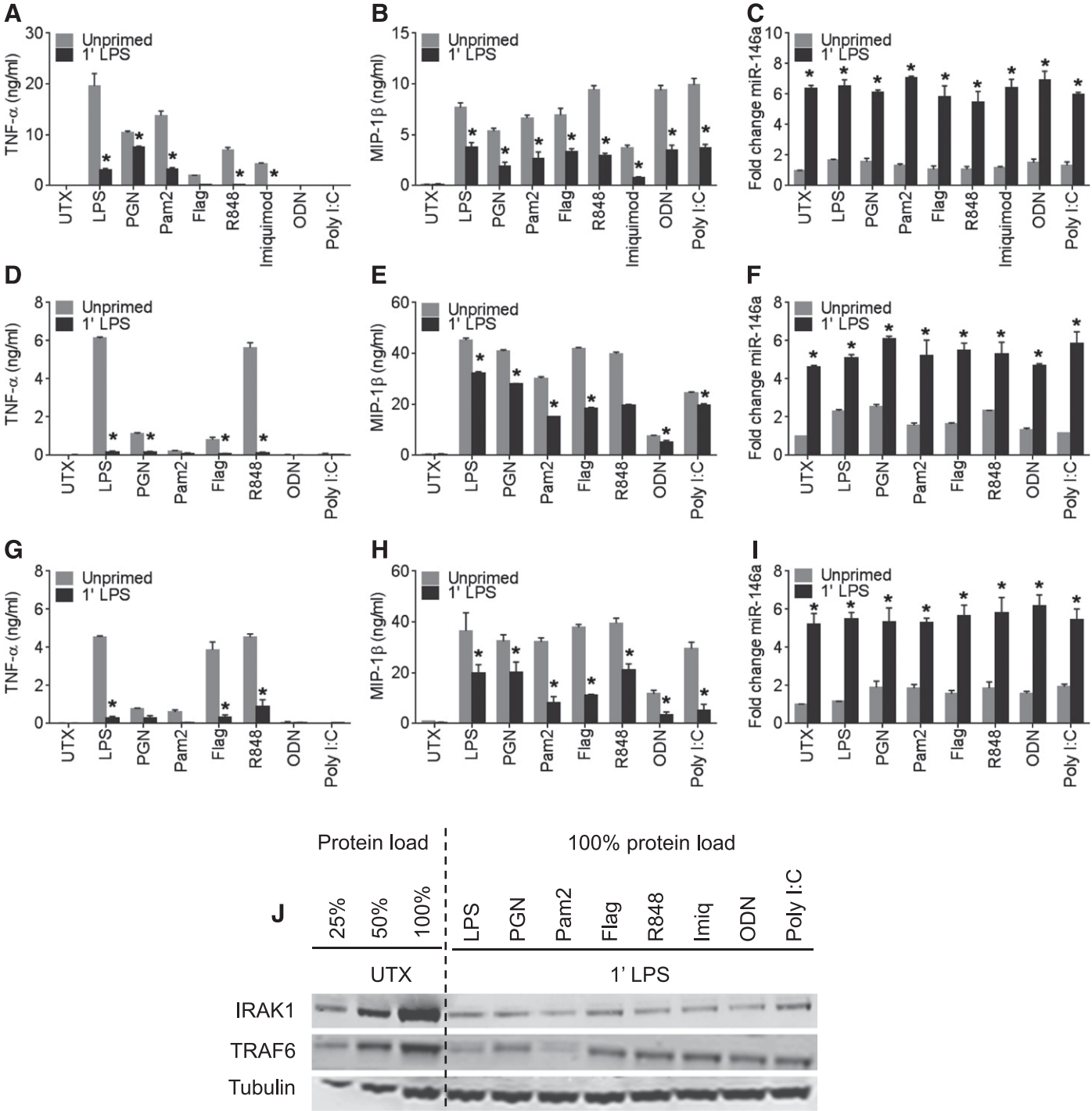

Figure 3. Homologous and heterologous TLR signaling tolerance in macrophage cell lines and primary human macrophages following LPS priming. THP-1 (A–C), M1 (D–F), and M2 (G–I) macrophages were primed with 50 –100 ng/ml LPS (1′ LPS) for 18 h, and unprimed controls incubated for the same time period were washed twice with complete growth medium and then challenged with various ligands, as indicated in the footnotes for 6 h. Culture supernatant levels of TNF-α (A, D, G) and MIP-1β (B, E, H) were analyzed using the MSD ELISA kit. miR-146a expression in the same set of samples was analyzed by qRT-PCR in THP-1 (C), M1 (F), as well as M2 (I) macrophages. Immunoblot analyses (J) for IRAK1, TRAF6, and tubulin (loading control) in the untreated control or LPS-primed THP-1 macrophages challenged with a panel of TLR ligands for 2 h. Serial dilutions of untreated cell lysates (100, 50, and 25%) were included in the Western blot to facilitate semiquantitative measurement for IRAK1 and TRAF6 expression. Data are expressed as means ± SD from 3 technical replicates from 1 of 2 independent experiments. *$P < 0.05$ compared with unprimed macrophages.

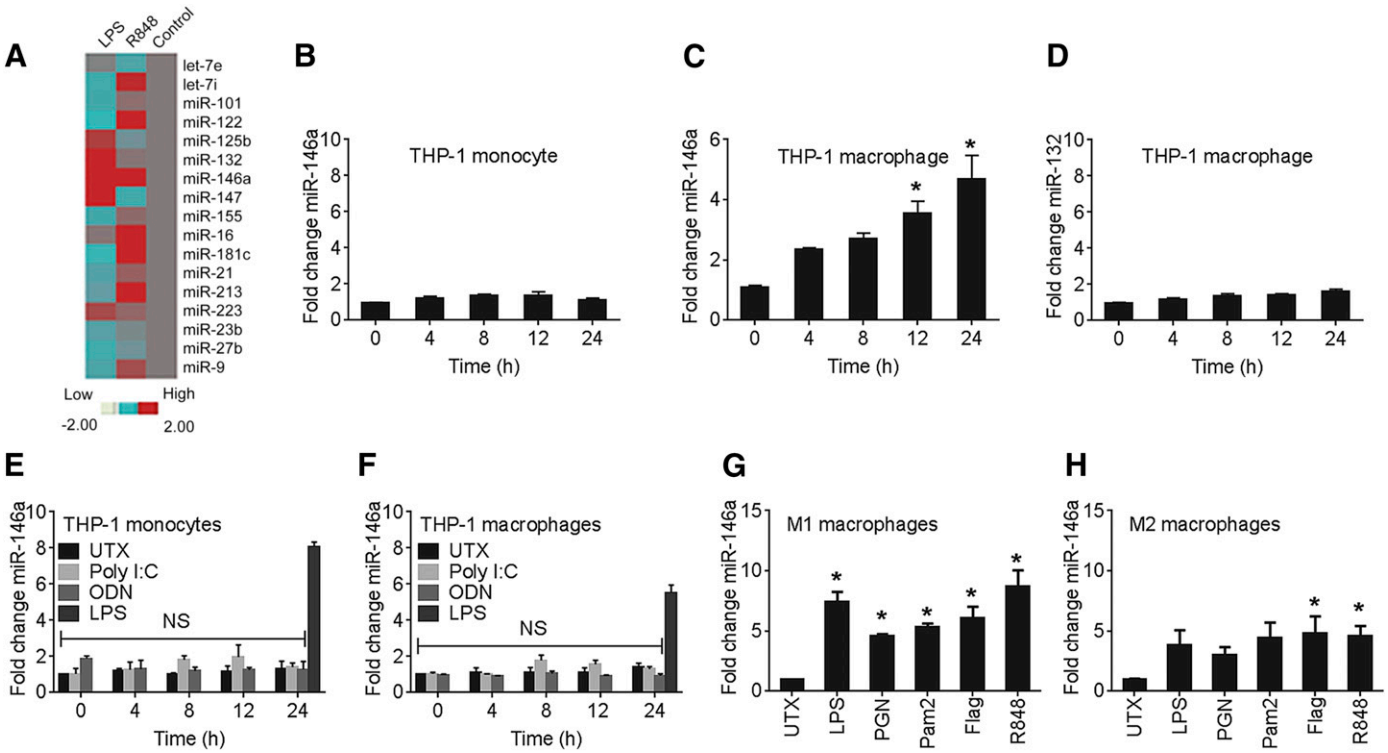

Figure 4. miR-146a expression in R848-stimulated macrophages. THP-1 macrophages were treated with LPS or R848 (1000 ng/ml) for 12 h and analyzed for miRNA expression by the TaqMan array human miRNA gene card (A). Expression of miR-146a in R848-stimulated THP-1 monocytes (B) or THP-1 macrophages (C) following R848 stimulation. Expression of miR-132 in THP-1 macrophage (D) following R848 stimulation. Expression of miR-146a in THP-1 macrophages following stimulation with 2000 ng/ml poly I:C or ODN (E, F), where LPS-stimulated miR-146a production at 24 h served as the positive control. Expression of miR-146a in M1 (G) and M2 (H) macrophages, 24 h following stimulation with the indicated TLR ligands. Data are expressed as means ± SD from 3 technical replicates from 1 of 2 independent experiments. $*P < 0.05$ compared with untreated macrophages.

in the R848-primed samples compared with unprimed samples (Fig. 5D). There was an increase in TNF-α production in unprimed M2 macrophages upon stimulation with LPS, PGN, Pam2, Flag, and R848, along with a significant reduction in the TNF-α level in the R848-primed macrophages (Fig. 5E). The expression of miR-146a was significantly higher in the R848-primed M2 macrophages compared with unprimed macrophages (Fig. 5F). As miR-146a inhibits the translation of IRAK1 and TRAF6, we evaluated the protein levels of these molecules in THP-1 macrophages. Consistent with previous findings, LPS stimulation reduced IRAK1 and TRAF6 protein levels (Fig. 5G). However, we observed that R848 stimulation reduced only IRAK1 but not TRAF6 levels, whereas poly I:C stimulation did not result in decreased levels of either IRAK1 or TRAF6 (Fig. 5G). As expected, the levels of MyD88 remained unchanged under all of the stimulation conditions (Fig. 5G).

Homologous or heterologous tolerance to LPS or R848 is proportional to the priming dose of TLR ligand and miR-146a levels

We then evaluated if there is a dose-dependent regulation of miR-146a expression and miR-146a-driven functional regulation in response to endosomal TLR ligands. When stimulated with increasing concentrations of LPS, we observed a priming dose-dependent reduction in TNF-α production in THP-1 macrophages following a second stimulation with LPS or R848 (**Fig. 6A**). There was also a LPS dose-dependent increase in miR-146a expression in THP-1 macrophages (Fig. 6B), as well as reduction in IRAK1 and TRAF6 protein levels (Fig. 6C). Likewise, we also observed that when stimulated with increasing concentrations of R848, there was a priming dose-dependent reduction in TNF-α production in THP-1 macrophages, following a second stimulation with LPS or R848 (Fig. 6D). As observed with LPS priming, there was an R848 dose-dependent increase in miR-146a expression in THP-1 macrophages (Fig. 6E), as well as a reduction in IRAK1 levels, but not TRAF6 levels (Fig. 5F). These data suggest that homologous and heterologous TLR signaling tolerance is priming dose dependent and is calibrated at the miRNA level for cell surface TLRs, as well as endosomal TLRs.

miR-146a alone can mimic TLR ligand priming to induce tolerance to endosomal TLR ligands

To examine the miR-146a intrinsic role in homologous and heterologous TLR signaling tolerance, we used miR-146a mimic or inhibitor in modulating cytokine and chemokine production responses against a panel of TLR ligands. Transfection of THP-1 macrophages with an miR-146a mimic resulted in overexpression of mature miR-146a compared with mock transfection controls (**Fig. 7A**). Overexpression of miR-146a mimic resulted in reduced IRAK1 and TRAF6 protein levels, but not IRAK4 protein levels,

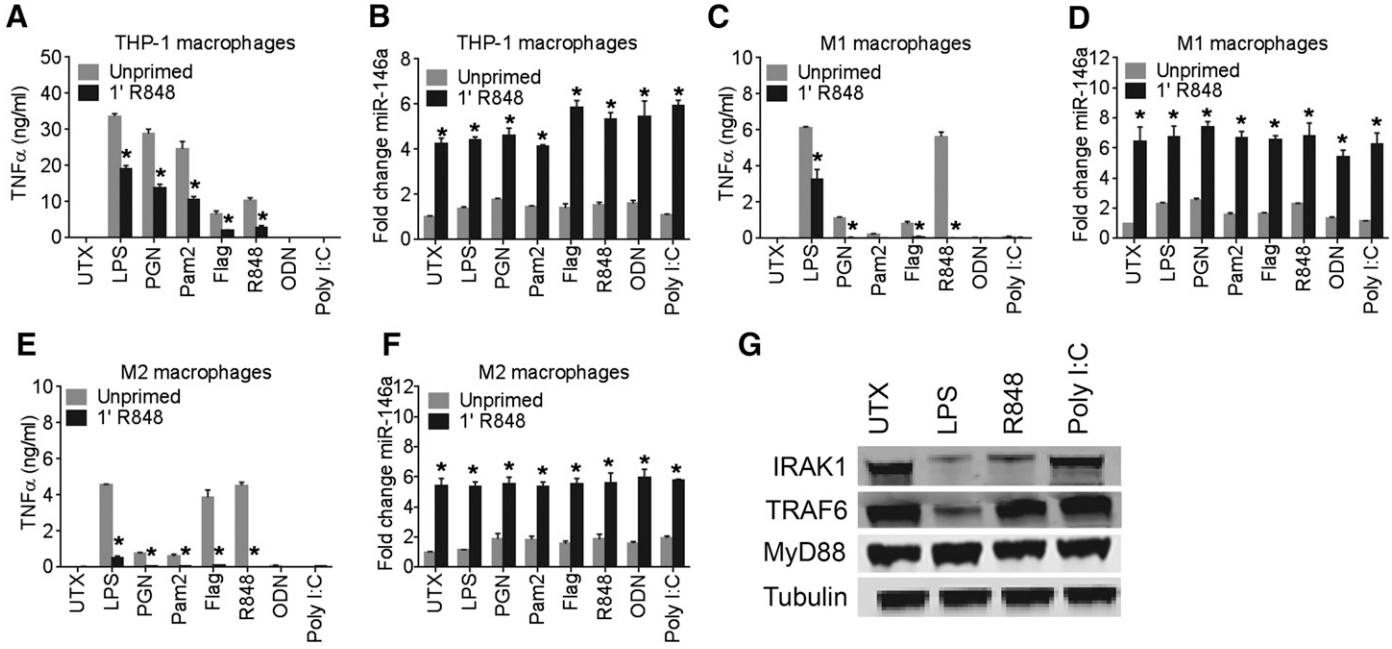

Figure 5. Homologous and heterologous TLR signaling tolerance in macrophage cell lines and primary human macrophages following R848 priming. THP-1 macrophages were primed with 100 ng/ml R848 (1′ R848) for 18 h and then challenged with a panel of TLR agonists for 6 h. Cell culture supernatants were analyzed for the production of TNF-α from THP-1 (A), M1 (C), and M2 (E) macrophages using the MSD ELISA kit. Expression of miR-146a from the same samples was evaluated by qRT-PCR in THP-1 (B), M1 (D), or M2 (F) macrophages. Data are expressed as means ± SD from 3 technical replicates from 1 of 2 independent experiments. $*P < 0.05$ compared with untreated macrophages. Immunoblot analysis of IRAK1, TRAF6, and MyD88 protein levels, 18 h following treatment with LPS, R848, and poly I:C in THP-1 macrophages (G), where tubulin serves as a loading control. Different loading amounts (25, 50, and 100%) proteins.

confirming the specificity of miR-146a overexpression (Fig. 7B). Conversely, transfection of an miR-146a inhibitor reduced miR-146a expression compared with mock transfection controls in LPS-stimulated macrophages (Fig. 7C). Transfection with miR-146a inhibitor increased the level of IRAK1 and TRAF6 protein levels without modifying IRAK4 protein levels in LPS-treated samples, confirming the specificity of the miR-146a inhibitor (Fig. 7D). The production of TNF-α upon stimulation with LPS, PGN, Pam2, Flag, and R848 was reduced significantly upon miR-146a mimic transfection and significantly increased upon miR-146a inhibitor transfection (Fig. 7E). Stimulation with ODN and poly I:C did not induce the production of TNF-α (Fig. 7E). Likewise, the production of MIP-1β upon stimulation with LPS, PGN, Pam2, Flag, R848, and ODN was decreased significantly by miR-146a mimic and induced significantly by miR-146a inhibitor (Fig. 7F). Interestingly, whereas poly I:C induced the production of MIP-1β, neither miR-146a mimic nor miR-146a inhibitor modulated MIP-1β production in THP-1 macrophages (Fig. 7F).

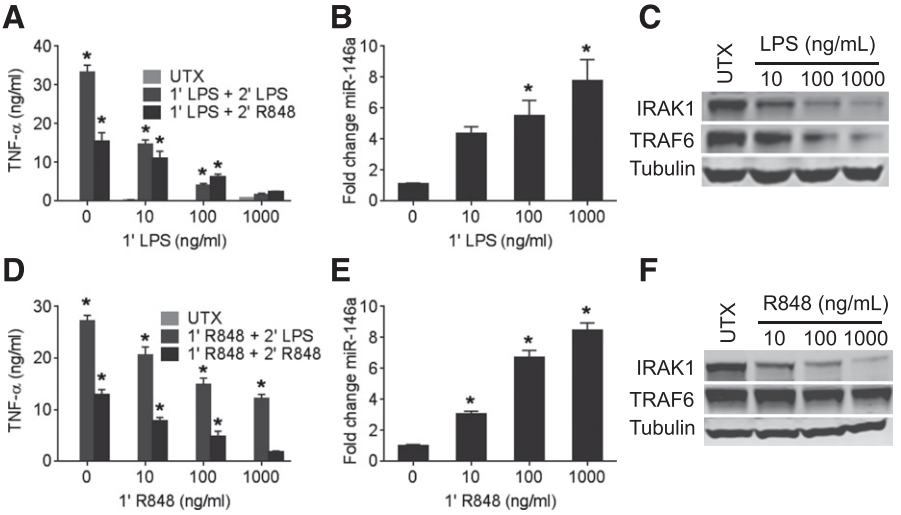

Figure 6. The priming of TLR dose determines the magnitude of homologous and heterologous tolerance and miR-146a expression. Production of TNF-α in THP-1 macrophages primed with LPS (A) or R848 (B) for 18 h and then rechallenged with LPS or R848 evaluated by the MSD ELISA kit. Expression of miR-146a in LPS-primed (B) or R848-primed (E) macrophages evaluated by qRT-PCR. Immunoblot analyses for IRAK1, TRAF6, and tubulin in the cell lysates from untreated, as well as LPS (C)- or R848 (F)-treated (0–1000 ng/ml) THP-1 macrophages, 18 h following stimulation. Data are expressed as means ± SD from 3 technical replicates from 1 of 2 independent experiments. $*P < 0.05$ compared with untreated macrophages.

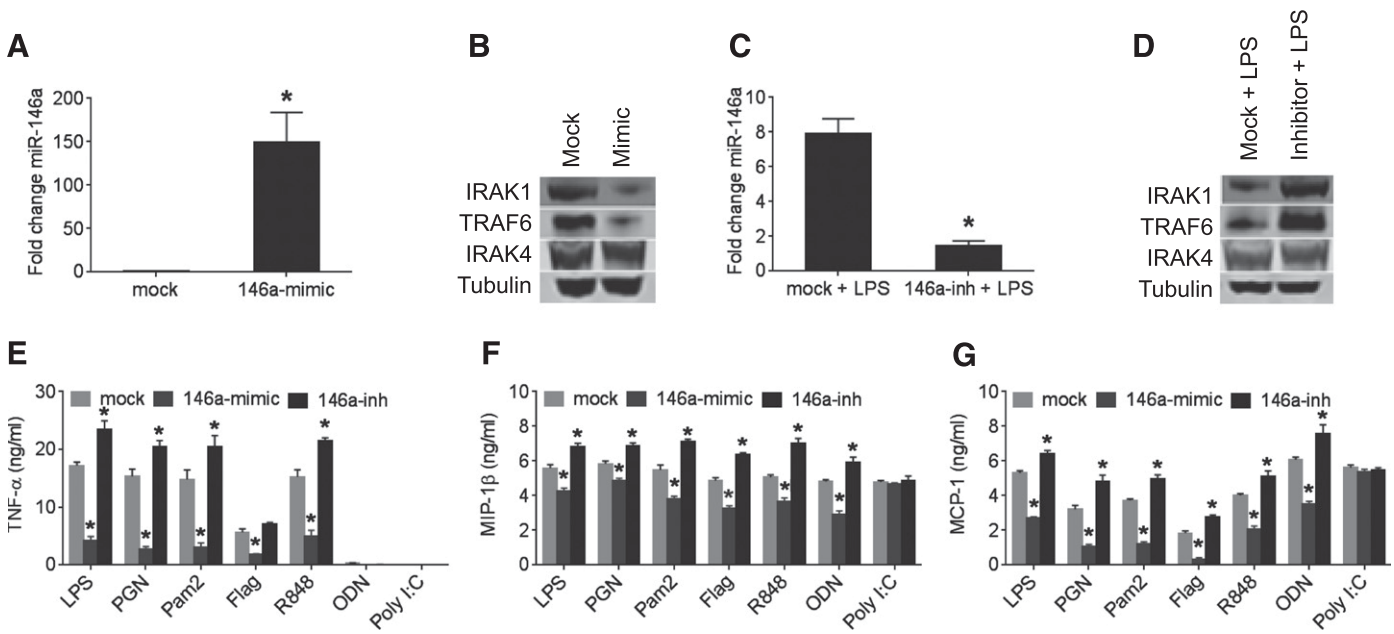

Figure 7. miR-146a intrinsic ability to promote homologous and heterologous tolerance to MyD88-dependent TLR signaling. THP-1 macrophages were transfected with 100 nM miR-146a mimic (146a-mimic) or inhibitor (146a-inh) for 24 h, along with mock-transfected controls (miRNA mimic or inhibitor-negative control) before challenge with TLR ligands for 6 h. Expression of miR-146a, following transfection with miR-146a mimic (A) or following transfection miR-146a inhibitor and an additional LPS challenge for 16 h (C), was evaluated by qRT-PCR. Protein levels of IRAK1, TRAF6, IRAK4, and tubulin following transfection with miR-146a mimic (B) or with miR-146a inhibitor, followed by an LPS challenge for 16 h (D), were evaluated by immunoblot. The production of TNF-α (E), MIP-1β (F), and MCP-1 (G) in cell culture supernatants in miR-146a mimic or inhibitor-transfected THP-1 macrophages, 6 h following TLR agonist stimulation, was evaluated by the MSD ELISA kit. Data are expressed as means ± SD from 3 technical replicates from 1 of 2 independent experiments. $*P < 0.05$ compared with mock-transfected controls.

Likewise, miR-146a modulation did not have an impact on MCP-1 production following stimulation with all of the TLR-evaluated TLR ligands except poly I:C (Fig. 7G).

DISCUSSION

Emerging studies suggest that there is mutual regulation between TLR signaling and key regulatory miRNAs, such as miR-146a, miR-132, and miR-155 [36]. Specifically, the induction of miR-146a, in response to LPS and its role in inflammatory cytokine production, has been characterized in THP-1 monocytes [23]. Whereas a majority of studies has investigated miR-146a regulation of TLR responses in monocytic cell lines, in this study, we characterized miR-146a-mediated TLR signaling responses and underpinning mechanisms in PMA-differentiated THP-1 macrophages, as well as human M1 and M2 macrophages. Here, we demonstrate that miR-146a is expressed in response to TLR agonists in THP-1 monocytes—THP-1 cells differentiated to macrophages as well as human M1 and M2 macrophages. With the confirmation of the well-characterized role of miR-146a in LPS-induced TNF-α responses, we have identified several novel cytokine and chemokine readouts, such as MIP-1β and MCP-1, which can be used to interrogate TLR ligand-induced tolerance and cross-tolerance mechanisms in macrophages. In addition, we have demonstrated that tolerance to TLR7/8 agonists, as well as TLR7/8 agonist-induced cross-tolerance to other TLR agonists, is mediated by miR-146a. In addition, we have demonstrated that miR-146a does not play a significant role in the regulation of macrophage chemokine responses to TLR3 agonists. Collectively, these data may help further elucidate the role of miR-146a in TLR signaling responses, providing a broader context for its role as a functional mediator or a biomarker in innate immune responses.

miR-146a has been characterized extensively as a critical mediator of both innate [36] and adaptive [37] immune responses, tumor-suppressive functions, as well as in other biologic processes, such as hematopoiesis, cellular differentiation, and migration [38]. The role of miRNAs in LPS-induced endotoxin tolerance mechanisms has been investigated extensively in THP-1 monocytes. Here, we demonstrate that the miR-146a expression profile following LPS and R848 stimulation is similar in THP-1 macrophages, as well as human M1 and M2 macrophages. We detected a time-dependent increase in miR-146a expression up to 24 h following TLR stimulation, suggesting that the concentrations of TLR ligands used do not result in reduced viability. Although we did not evaluate the impact of miR-146a on M1 or M2 macrophage polarization, the induction of miR-146a was more robust in the polarized M1 macrophages compared with M2 macrophages following LPS and R848 stimulation, which is, in part, similar to previously reported findings from mouse macrophages for a subset of TLR ligands [26, 39]. Among the LPS-induced miRNAs, miR-146a has been shown to be NF-κB dependent and IRF-3/7 independent [35, 40]. Whereas the role of IRF family members has been well characterized in the context of M1 and M2 macrophage

polarization [41], we have not evaluated the transcriptional mechanisms that regulate miR-146a expression in M1 and M2 macrophages. In the current study, several other chemokines, such as MIP-1α, MIP-1β, MCP-1, and MDC, were produced more robustly in PMA-differentiated THP-1 macrophages compared with THP-1 monocytes in response to endosomal TLR ligands. Therefore, this expanded repertoire of readouts could potentially be used to evaluate endosomal TLR signaling tolerance in differentiated macrophages in future studies.

We also demonstrate that there are overlapping and distinct patterns of miR-146a induction by TLR ligands, as well as regulation of TLR responses by miR-146a. In our studies, we did not find CpG- or poly I:C-induced expression of miR-146a in THP-1 monocytes or macrophages, which suggests that there is specificity among endosomal TLRs in their differential regulation of miR-146a expression in human macrophages. Previous reports have demonstrated that CpG priming in the RAW264.7 cell line led to the disruption of IRAKs and development of cross-tolerance to LPS or CpG [42] and that there was an increase in miR-146a expression in CpG-stimulated plasmacytoid dendritic cells [43]. Therefore, it is possible that CpG-induced miR-146a expression could be differentially regulated among the cells in the myeloid lineage and across species. It has been demonstrated previously that LPS-primed mice were hyporesponsive to CpG DNA [39, 44]. We neither observed poly I:C-induced miR-146a in human macrophages nor poly I:C-induced tolerance in human macrophages (data not shown), concordant with previous studies with similar results in mouse peritoneal macrophage [45].

During innate immune activation, excessive TLR signaling is controlled by miR-146a-mediated degradation or translation inhibition of the adaptor kinases IRAK1 and TRAF6, leading to endotoxin tolerance [23, 30, 46–48]. In this study, we observed that homologous or heterologous tolerance to LPS led to the reduction of IRAK1 and TRAF6 protein in THP-1 macrophages. Stimulation with R848 led to reduction in IRAK1 but not TRAF6 protein levels, similar to the observations from mouse primary macrophages [49]. Thus, miR-146a-associated reduction in TRAF6 protein levels might vary depending on the type of cells or ligands used for stimulation. In a previous study, the silencing of the adaptor kinases IRAK1 or TRAF6 rendered THP-1 monocytes hyporesponsive, leading to a partial but not complete suppression of cytokine production in response to TLR2, TLR4, and TLR5 ligands [16]. Our current study expands on these observations and demonstrates that LPS priming or miR-146a overexpression alone in THP-1 macrophages leads to only a partial reduction the production of cytokines or chemokines following TLR stimulation, including stimulation by the endosomal TLR7/8, TLR9, and TLR3 ligands, such as R848, ODN, and poly I:C. In addition, we demonstrate that miR-146a does not have an intrinsic role in the regulation of MyD88-independent pathways in THP-1 macrophages, suggesting pathway selectivity for miR-146a-mediated TLR signaling tolerance mechanisms as well. The lack of complete suppression of cytokine and chemokine response during homologous and heterologous TLR tolerance following miR-146a overexpression suggests that other LPS- or R848-induced miRNAs, regulatory molecules, or compensatory mechanisms might influence TLR signaling tolerance.

A tight regulation of immune responses is essential for a balance between protective and detrimental immunity, necessitating the regulation of excessive immune activation. Whereas the dampening of excessive immune responses by miRNA-mediated signaling tolerance mechanisms has been described before [11, 36], it remains to be determined if the potentially beneficial outcomes driven by these mechanisms in autoimmune and chronic inflammatory settings might be counterproductive in tumor immunotherapy [50], where enhancement of the host immune response is necessary for tumor clearance. In summary, this study provides new evidence for homologous and heterologous TLR signaling tolerance in macrophages and more importantly, in M1- and M2-activated macrophages. Although miR-146a appears to regulate TLR tolerance against most plasma membrane and endosomal TLR ligands, there appears to be some pathway selectivity in the miR-146a-regulated TLR responses, as miR-146a does not appear to impair MyD88-independent TLR signaling through TLR3. The relevance and impact of the collective observations and the pathway selectivity in miR-146a-mediated TLR signaling tolerance in infectious, inflammatory, or autoimmune disease settings remain to be investigated.

AUTHORSHIP

M.A.N., L.M.B., J.D.S., H.M., A.H., and R.A.R. conceived of and designed the experiments and interpreted the data. M.A.N. and L.M.B. performed experimentation. M.A.N. and R.A.R. wrote the paper.

ACKNOWLEDGMENTS

This research was fully supported by Merck & Co. The authors thank Philip Garrett-Engele for his technical expertise and assistance.

DISCLOSURES

There are no conflicts of interest to disclose.

REFERENCES

1. Akira, S., Uematsu, S., Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124,** 783–801.
2. Akira, S., Takeda, K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.* **4,** 499–511.
3. Nishiya, T., DeFranco, A. L. (2004) Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. *J. Biol. Chem.* **279,** 19008–19017.
4. O'Neill, L. A., Golenbock, D., Bowie, A. G. (2013) The history of Toll-like receptors—redefining innate immunity. *Nat. Rev. Immunol.* **13,** 453–460.
5. Beutler, B., Cerami, A. (1987) Cachectin: more than a tumor necrosis factor. *N. Engl. J. Med.* **316,** 379–385.
6. Danner, R. L., Elin, R. J., Hosseini, J. M., Wesley, R. A., Reilly, J. M., Parillo, J. E. (1991) Endotoxemia in human septic shock. *Chest* **99,** 169–175.
7. Liew, F. Y., Xu, D., Brint, E. K., O'Neill, L. A. (2005) Negative regulation of Toll-like receptor-mediated immune responses. *Nat. Rev. Immunol.* **5,** 446–458.
8. Shishodia, S., Aggarwal, B. B. (2002) Nuclear factor-kappaB activation: a question of life or death. *J. Biochem. Mol. Biol.* **35,** 28–40.
9. Bergsbaken, T., Fink, S. L., Cookson, B. T. (2009) Pyroptosis: host cell death and inflammation. *Nat. Rev. Microbiol.* **7,** 99–109.

10. Biswas, S. K., Lopez-Collazo, E. (2009) Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol.* **30,** 475–487.
11. Cavaillon, J. M., Adib-Conquy, M. (2006) Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Crit. Care* **10,** 233.
12. McCall, C. E., Grosso-Wilmoth, L. M., LaRue, K., Guzman, R. N., Cousart, S. L. (1993) Tolerance to endotoxin-induced expression of the interleukin-1 beta gene in blood neutrophils of humans with the sepsis syndrome. *J. Clin. Invest.* **91,** 853–861.
13. Beutler, B. (2004) SHIP, TGF-beta, and endotoxin tolerance. *Immunity* **21,** 134–135.
14. Biswas, S. K., Bist, P., Dhillon, M. K., Kajiji, T., Del Fresno, C., Yamamoto, M., Lopez-Collazo, E., Akira, S., Tergaonkar, V. (2007) Role for MyD88-independent, TRIF pathway in lipid A/TLR4-induced endotoxin tolerance. *J. Immunol.* **179,** 4083–4092.
15. De Vos, A. F., Pater, J. M., van den Pangaart, P. S., de Kruif, M. D., van 't Veer, C., van der Poll, T. (2009) In vivo lipopolysaccharide exposure of human blood leukocytes induces cross-tolerance to multiple TLR ligands. *J. Immunol.* **183,** 533–542.
16. Nahid, M. A., Satoh, M., Chan, E. K. (2011) Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. *J. Immunol.* **186,** 1723–1734.
17. O'Neill, L. A., Bowie, A. G. (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* **7,** 353–364.
18. Ambros, V. (2004) The functions of animal microRNAs. *Nature* **431,** 350–355.
19. Chen, C. Z., Schaffert, S., Fragoso, R., Loh, C. (2013) Regulation of immune responses and tolerance: the microRNA perspective. *Immunol. Rev.* **253,** 112–128.
20. Lodish, H. F., Zhou, B., Liu, G., Chen, C. Z. (2008) Micromanagement of the immune system by microRNAs. *Nat. Rev. Immunol.* **8,** 120–130.
21. O'Connell, R. M., Rao, D. S., Chaudhuri, A. A., Baltimore, D. (2010) Physiological and pathological roles for microRNAs in the immune system. *Nat. Rev. Immunol.* **10,** 111–122.
22. Boldin, M. P., Taganov, K. D., Rao, D. S., Yang, L., Zhao, J. L., Kalwani, M., Garcia-Flores, Y., Luong, M., Devrekanli, A., Xu, J., Sun, G., Tay, J., Linsley, P. S., Baltimore, D. (2011) miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J. Exp. Med.* **208,** 1189–1201.
23. Nahid, M. A., Pauley, K. M., Satoh, M., Chan, E. K. (2009) miR-146a is critical for endotoxin-induced tolerance: implication in innate immunity. *J. Biol. Chem.* **284,** 34590–34599.
24. Nahid, M. A., Yao, B., Dominguez-Gutierrez, P. R., Kesavalu, L., Satoh, M., Chan, E. K. (2013) Regulation of TLR2-mediated tolerance and cross-tolerance through IRAK4 modulation by miR-132 and miR-212. *J. Immunol.* **190,** 1250–1263.
25. Zhao, J. L., Rao, D. S., Boldin, M. P., Taganov, K. D., O'Connell, R. M., Baltimore, D. (2011) NF-kappaB dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. *Proc. Natl. Acad. Sci. USA* **108,** 9184–9189.
26. Tsukada, K., Kitazawa, T., Fukushima, A., Okugawa, S., Yanagimoto, S., Tatsuno, K., Koike, K., Nagase, H., Hirai, K., Ota, Y. (2007) Macrophage tolerance induced by stimulation with Toll-like receptor 7/8 ligands. *Immunol. Lett.* **111,** 51–56.
27. Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K., Dockrell, D. H. (2010) The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One* **5,** e8668.
28. Park, E. K., Jung, H. S., Yang, H. I., Yoo, M. C., Kim, C., Kim, K. S. (2007) Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm. Res.* **56,** 45–50.
29. Verreck, F. A., de Boer, T., Langenberg, D. M., Hoeve, M. A., Kramer, M., Vaisberg, E., Kastelein, R., Kolk, A., de Waal-Malefyt, R., Ottenhoff, T. H. (2004) Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc. Natl. Acad. Sci. USA* **101,** 4560–4565.
30. Jacinto, R., Hartung, T., McCall, C., Li, L. (2002) Lipopolysaccharide- and lipoteichoic acid-induced tolerance and cross-tolerance: distinct alterations in IL-1 receptor-associated kinase. *J. Immunol.* **168,** 6136–6141.
31. Maeda, S., Akanuma, M., Mitsuno, Y., Hirata, Y., Ogura, K., Yoshida, H., Shiratori, Y., Omata, M. (2001) Distinct mechanism of *Helicobacter pylori*-mediated NF-kappa B activation between gastric cancer cells and monocytic cells. *J. Biol. Chem.* **276,** 44856–44864.
32. Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K., Akira, S. (2000) Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface Toll-like receptor 4 expression. *J. Immunol.* **164,** 3476–3479.
33. Livak, K. J., Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods* **25,** 402–408.
34. Porta, C., Rimoldi, M., Raes, G., Brys, L., Ghezzi, P., Di Liberto, D., Dieli, F., Ghisletti, S., Natoli, G., De Baetselier, P., Mantovani, A., Sica, A. (2009) Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. *Proc. Natl. Acad. Sci. USA* **106,** 14978–14983.
35. Taganov, K. D., Boldin, M. P., Chang, K. J., Baltimore, D. (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. USA* **103,** 12481–12486.
36. Nahid, M. A., Satoh, M., Chan, E. K. (2011) MicroRNA in TLR signaling and endotoxin tolerance. *Cell. Mol. Immunol.* **8,** 388–403.
37. Curtale, G., Citarella, F., Carissimi, C., Goldoni, M., Carucci, N., Fulci, V., Franceschini, D., Meloni, F., Barnaba, V., Macino, G. (2010) An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes. *Blood* **115,** 265–273.
38. Rusca, N., Monticelli, S. (2011) MiR-146a in immunity and disease. *Mol. Biol. Int.* **2011,** 437301.
39. Dalpke, A. H., Lehner, M. D., Hartung, T., Heeg, K. (2005) Differential effects of CpG-DNA in Toll-like receptor-2/-4/-9 tolerance and cross-tolerance. *Immunology* **116,** 203–212.
40. Hou, J., Wang, P., Lin, L., Liu, X., Ma, F., An, H., Wang, Z., Cao, X. (2009) MicroRNA-146a feedback inhibits RIG-I-dependent type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. *J. Immunol.* **183,** 2150–2158.
41. Günthner, R., Anders, H. J. (2013) Interferon-regulatory factors determine macrophage phenotype polarization. *Mediators Inflamm.* **2013,** 731023.
42. Yeo, S. J., Yoon, J. G., Hong, S. C., Yi, A. K. (2003) CpG DNA induces self and cross-hyporesponsiveness of RAW264.7 cells in response to CpG DNA and lipopolysaccharide: alterations in IL-1 receptor-associated kinase expression. *J. Immunol.* **170,** 1052–1061.
43. Karrich, J. J., Jachimowski, L. C., Libouban, M., Iyer, A., Brandwijk, K., Taanman-Kueter, E. W., Nagasawa, M., de Jong, E. C., Uittenbogaart, C. H., Blom, B. (2013) MicroRNA-146a regulates survival and maturation of human plasmacytoid dendritic cells. *Blood* **122,** 3001–3009.
44. Lehner, M. D., Morath, S., Michelsen, K. S., Schumann, R. R., Hartung, T. (2001) Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different Toll-like receptors independent of paracrine mediators. *J. Immunol.* **166,** 5161–5167.
45. Sato, S., Takeuchi, O., Fujita, T., Tomizawa, H., Takeda, K., Akira, S. (2002) A variety of microbial components induce tolerance to lipopolysaccharide by differentially affecting MyD88-dependent and -independent pathways. *Int. Immunol.* **14,** 783–791.
46. Li, C. H., Wang, J. H., Redmond, H. P. (2006) Bacterial lipoprotein-induced self-tolerance and cross-tolerance to LPS are associated with reduced IRAK-1 expression and MyD88-IRAK complex formation. *J. Leukoc. Biol.* **79,** 867–875.
47. Medvedev, A. E., Kopydlowski, K. M., Vogel, S. N. (2000) Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and Toll-like receptor 2 and 4 gene expression. *J. Immunol.* **164,** 5564–5574.
48. Siedlar, M., Frankenberger, M., Benkhart, E., Espevik, T., Quirling, M., Brand, K., Zembala, M., Ziegler-Heitbrock, L. (2004) Tolerance induced by the lipopeptide Pam3Cys is due to ablation of IL-1R-associated kinase-1. *J. Immunol.* **173,** 2736–2745.
49. Kawagoe, T., Takeuchi, O., Takabatake, Y., Kato, H., Isaka, Y., Tsujimura, T., Akira, S. (2009) TANK is a negative regulator of Toll-like receptor signaling and is critical for the prevention of autoimmune nephritis. *Nat. Immunol.* **10,** 965–972.
50. Pardoll, D. M. (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer* **12,** 252–264.

KEY WORDS:
microRNA · tolerance