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Abstract

Reactive oxygen species (ROS) produced in macrophages is critical for microbial killing, but they also take part in inflammation and antigen presentation functions. MicroRNAs (miRNAs) are endogenous regulators of gene expression, and they can control immune responses. To dissect the complex nature of ROS-mediated effects in macrophages, we sought to characterize miRNAs that are responsive to oxidative stress-induced with hydrogen peroxide (H_2O_2) in the mouse macrophage cell line, RAW 264.7. We have identified a set of unique miRNAs that are differentially expressed in response to H_2O_2. These include miR-27a*, miR-27b*, miR-29b*, miR-24-2*, and miR21*, all of which were downregulated except for miR-21*. By using luciferase reporter vector containing nuclear factor-kB (NF-kB) response elements, we demonstrate that overexpression of miR-27b* suppresses lipopolysaccharide-induced activation of NF-kB in RAW 264.7 cells. Our data suggest that macrophage functions can be regulated by oxidative stress-responsive miRNAs by modulating the NF-kB pathway.

Sivasubramani Thulasingam and Chandirasegaran Massilamany contributed equally to this work.
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Keywords: Innate immunity, Oxidative stress, Hydrogen peroxide, microRNAs, RAW 264.7 cells

Introduction

The innate immune response is primarily mediated by phagocytes (neutrophils and macrophages), dendritic cells, natural killer cells, and γδ T cells. Phagocytic cells recognize evolutionarily conserved structures which are common to different classes of microbes termed pathogen-associated molecular patterns (PAMPs), e.g., bacterial lipopolysaccharide (LPS). The receptors that recognize PAMPs are called pattern recognition receptors (PRRs) and among these, the family of toll-like receptors (TLRs) is well-studied [1]. The interactions between PAMPs and PRRs provide signals to the host and trigger inflammatory responses while alerting the adaptive immune system. Upon engulfing, microbes are killed by the generation of lethal reactive oxygen species (ROS) in macrophages through the nicotinamide adenine dinucleotide phosphate oxidase complex and reactive nitrogen intermediaries namely nitric oxide [2]. Produced in excess, ROS can contribute to tissue damage and inflammation as a result of oxidative stress, one of the pathogenic mechanisms of inflammatory and degenerative disorders. ROS, however, can be beneficial in that, they can act as secondary messengers in various physiological processes including the induction of adaptive immune responses.

CD4 T helper (Th) cells play a central role in the generation of adaptive immune response against intracellular microbes. Antigen presenting cells provide two signals for T cell activation: antigen presentation (signal 1) and costimulation (signal 2). ROS can upregulate costimulatory molecules [3] and act as a third signal for T cell activation [4]. Based on the redox state, two types of macrophages have been described: oxidative and reductive macrophages and they can influence Th1 and Th2 cytokine responses, respectively [5, 6]. How ROS regulate these complex functions is a fundamental question.

miRNAs are small non-coding RNAs of 20-22 nt and they regulate many cellular processes. Evidences suggest that miRNAs can control the differentiation of immune cells and immune responses [7, 8]. For example, miR-181a is critical for T cell selection in the thymus [9], the mice transgenically expressing miR-17-92 die prematurely due to lymphoproliferative autoimmune disorders [10]. Likewise, miRNAs such as miR-150, miR-146a/b, and miR-155 play a role in innate immune reactions. Deficiency of miR150 leads to the expansion of B1 B cell subset [11], whereas LPS induced expression of miR-146a/b negatively regulates innate immune response by attenuating TLR signaling and miR-155 is induced during the inflammatory response in macrophages [12, 13]. Recent research demonstrates

that miR-21 protects H_2O_2-induced injury in cardiac myocytes and vascular smooth muscle cells from H_2O_2-induced apoptosis via interaction with programmed cell death 4 suggesting that miRNAs can regulate oxidative stress-induced effects [14, 15]. Importantly, each miRNA can negatively regulate hundreds of target genes and alter their functions [16, 17]. Identifying miRNAs provides opportunities to dissect the mechanisms by which oxidative stress can modulate various immune functions. To this end, we sought to characterize miRNAs using hydrogen peroxide (H_2O_2) as a defined stimulus in mouse macrophage cell line, RAW 264.7. We identified a unique set of miR-NAs namely miR-27a*, miR-27b*, miR-29b*, miR-24-2*, and miR-21* that are differentially expressed in response to H_2O_2 and our data suggest that miR-27b* can modulate nuclear factor-kB (NF-kB) pathway.

Materials and methods

Cell culture, measurement of intracellular ROS, and cell viability

RAW 264.7 cells (clone, TIB-71) obtained from American Type Culture Collection (Manassas, Virginia) were maintained in antibiotic-free 1× DMEM supplemented with 10% fetal bovine serum (FBS) hereafter called growth medium. To optimize the conditions for H_2O_2-induced oxidative stress in RAW 264.7 cells, 5-(and-6′)-chloromethyl-2′,7′dichlorodihydroflorescein diacetate (CM-H_2DCFDA), an oxidation-sensitive dye was used as an ROS indicator [18].

Briefly, 1×10^6 cells/ml were plated in 96-well plates and loaded with 0 to 4 μM of CM-H_2DCFDA in serum-free 1× HBSS medium for 20 min at 37°C and after washing twice with 1× PBS, cells were exposed to different amounts of H_2O_2 (0-1000 μM) in growth medium for 1 h. Following washing, cells were resuspended in 1× PBS containing cell death marker, 7-amino-actinomycin D (7-AAD) (Invitrogen, California), and acquired by FACScan flow cytometer (BD Pharmingen, California). The fluorescence intensity of CMH2DCFDA in live (7-AAD $^-$) cells was analyzed by Flow Jo software (Tree Star, Oregon) which also allowed us to obtain percentages of live (7-AAD $^-$) and dead (7-AAD$^+$) cells.

Measurement of H_2O_2 and glutathione (GSH)

The degree of oxidative stress-induced with H_2O_2 in RAW 264.7 cells was determined by measuring the utilization of H_2O_2 and GSH levels in culture medium with or without cells by spectrophotometry at 480 and 412 nm, respectively [19].

Microarray analysis

miRNA microarray analysis was performed at the University of Nebraska Medical Center (UNMC) core facility (Omaha, Nebraska). To analyze the expression of H_2O_2-responsive miRNAs, we used locked nucleic acid (LNA) arrays, version 10 (Exiqon, Denmark) consisting of 1154 capture probes for mature miRNAs which included spike-in controls [20]. Briefly, RAW 264.7 cells were treated with or without 800 μM H_2O_2 in growth medium for 1 h at 37°C with four replicates for each and total RNA was isolated using mirVana miRNA-isolation kit (Applied Biosystems, Cailfornia). Four replicates of control (untreated) and H_2O_2-treated samples were randomly divided into four sets, each constituted one control and one treated sample. Five hundred nanogram of total RNA from each control and H_2O_2-exposed samples in three of the four sets were labeled with Cy3 (control) or Cy5 (treated) dyes using Exiqon labeling system (Exiqon) and the dyes were swapped in the 4th set. Arrays were scanned after hybridization by using GenePix 4000B microarray scanner and GenePix 6.3 software (Axon Instruments, California).

miRNA detection by TaqMan PCR

To validate the miRNAs that are differentially expressed in microarray analysis, we used TaqMan real-time PCR method. We selected miR-27a* and miR-27b* and analyzed their expression using the TaqMan PCR probes and primers obtained commercially (Applied Biosystems). Total RNA was extracted as above and cDNAs were prepared by using TaqMan RT kit as recommended (Applied Biosystems) and miRNA expression was analyzed using the cDNAs derived from three replicates each for control and H_2O_2 (800 μM)-exposed samples by using iCycler (Bio-Rad Laboratories, CA). In each sample, expression of miR-27a* and miR-27b* was normalized to endogenous control, RNU48 (Applied Biosystems) and the relative expression was calculated.

Measurement of NF-kB activity based on luciferase reporter assay

The activity of NF-kB in RAW 264.7 cells was measured by using luciferase reporter assay (Promega, Wisconsin). This involved two reporter vectors namely, Firefly pGL4.32 (Luc 2p/NF-kB-response elements (RE)/Hygro) plasmid containing five tandem repeats of NF-kB-RE and Renilla pRL-TK luciferase plasmid (internal control) (Promega). RAW 264.7 cells were harvested by using 0.25% trypsin ethylenediaminetetraacetic acid and 2×10^6 cells were mixed with 100 μl of electroporation solution (Lonza, Maryland). Cocktails of pGL4.32 (400 ng) and pRL-TK (20 ng) (20:1) vectors supplemented with or without miRNA mimics, and scrambled miRNAs (negative control mimics) (Dharmacon, Colorado) (40 nM) were nucleofected using the Amaxa nucleofector system (Lonza). One milliliter

of growth medium was added immediately following transfection and the cells were incubated for 24 h. Cells were treated with or without LPS (1 μg/ml) and 6 h later, cell lysates were prepared using passive lysis buffer (Promega). Luciferase activities were measured by using dual luciferase system (Promega) in 20/20n luminometer (Turner Biosystems, California) and expressed as relative luciferase units (RLU). First, ratios were obtained by dividing the RLU for Firefly (NF-kB-RE) by RLU for Renilla (internal control) in each sample treated with or without LPS. Second, fold induction of Firefly luciferase activity representing NF-kB activation was obtained by dividing the values obtained for LPS-treated samples by the values from untreated samples.

NF-kB p65 nuclear translocation assay

To determine nuclear translocation of p65 subunit of NFkB, TransAM NF-kB transcription factor assay was used as described previously [21, 22]. RAW 264.7 cells were transfected with or without miR-27b* mimics or scrambled miRNAs (control mimics) as above, and the cells were plated in 6-well plates for 24 h. The cells were then treated with LPS for 6 h and the nuclear fractions were used for evaluating the translocation of p65 subunit of NF-kB by colorimetric reaction according to the manufacturer's recommendations (Active Motif, California). Briefly, the nuclear fractions representing the control (untransfected) or transfected with control mimics and miR-27b* mimics were added to 96-well plates in which oligonucleotides for NFkB consensus site (5′-GGGACTTTCC-3′) were immobilized. After incubating for 1 h, the plates were washed, and anti-NF-kB p65 was added followed by horse radish peroxidase (HRP)-conjugated secondary antibody and developing and stop solutions supplied by the manufacturer. The absorbance was then measured by spectrophotometry at 450/655 nm.

Cytokine mRNA expression

RAW 264.7 cells were transfected with or without miR-27b* mimics or control mimics and plated in 6-well plates for 24 h as above. The cells were then treated with LPS (1 μg/ml) for 6 h. To extract total RNA, the medium was removed and the cells were lysed using a buffer containing guanidium thiocyanate (RNeasy kit, Qiagen, California), and the samples were treated with RNAse-free DNAse I according to the manufacturer's recommendations (Qiagen). To make certain that the RNA samples were free of residual DNA, a second round of DNAse digestion was performed using amplification grade DNAse I (Invitrogen), and cDNAs were synthesized utilizing Superscript III reverse transcriptase kit as recommended (Invitrogen). The relative fold inductions of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and chemokine (C-C motif) ligand 2 (Ccl2) mRNA expression was done utilizing commercially obtained TaqMan PCR probes and primers (Applied Biosystems) using an iCycler (Bio-Rad Laboratories, California). Three replicates of the cDNAs obtained from each treatment were used for analysis, and the fold induction of the mRNA expression was calculated by normalizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression [23, 24].

Statistics

To determine the differential expression of miRNAs in the microarray analysis, linear models for microarrays package in R/Bioconductor with Benjamini-Hochberg adjusted P values were used [25, 26]. The miRNAs having a False Discovery rate adjusted $P \leq 0.05$ values were taken for further analysis. The miRNAs that are up or downregulated by 1.5-fold or higher were identified. Differences in the levels of H_2O_2 utilization and GSH in cultures treated with or without H_2O_2 and expression of miR-27a*, miR-27b* and cytokine mRNAs by TaqMan PCR analysis were tested by Students *t*. $P \leq 0.05$ values were considered significant.

Results and discussion

We report here that H_2O_2 induces differential expression of a unique set of miRNAs in RAW 264.7 cells and one of the downregulated miRNAs namely miR-27b* suppresses the NF-kB pathway. miRNAs are endogenous regulators of gene expression and they can alter the functions of multiple target proteins. Thus, miRNAs can be used as powerful tools to modulate a functional phenotype that involves the participation of multiple proteins as in the case of ROS-mediated events. To this end, we sought to characterize the oxidative stress-induced miRNAs in RAW 264.7 cells.

We used H_2O_2 as a defined stimulus to induce oxidative stress. RAW 264.7 cells loaded with CM-H_2DCF-DA were exposed to different concentrations of H_2O_2 (0-1000 μM) in growth medium for 1 h and after staining with a cell death marker, 7-AAD, the fluorescent intensity of CM-H_2DCFDA was measured by flow cytometry. CM-H_2DCFDA is a cell-permeant and it is non-fluorescent until it is oxidized by ROS. As shown in Figure 1a, there was a dose-dependent increment in the fluorescence intensity of CM-H_2DCFDA oxidation with the increase in H_2O_2 and dye concentrations. However, as the concentration of CM-H_2DCFDA was increased from 2 to 4 μM, the background staining was also increased even in the cells that were not exposed to H_2O_2 (30.69% at 4 μM) (Figure 1a). Therefore, we based our evaluations at 2 μM of CM-H_2DCFDA and chose 800 μM of H_2O_2 and 1 h exposure as the optimum concentrations to induce oxidative stress based

on two criteria: (a) Majority of cells (85%) showed positive staining for CM-H_2DCFDA at 800 μM of H_2O_2 (Figure 1a) and at this concentration, there was no significant cell death when compared with untreated cells (21.85 vs. 13.97%) (Figure 1b). (b) At the end of 1 h, H_2O_2 was almost completely utilized (Figure 1c) with a corresponding decrease in the levels of GSH ($P = 0.03$) (Figure 1d), an indicator of oxidative stress which otherwise protects cells from oxidative stress by dethiolating the protein-thiol mixed disulfides. It should be noted that the consumption of H_2O_2 (800 μM) occurred when supplemented into the medium alone leaving ~ 200 μM at the end of 1 h as opposed to 40 μM in treated samples (Figure 1c). Utilization of H_2O_2 in control samples may be due to antioxidants present in the FBS in growth medium. Overall, ~200 μM of H_2O_2 was used up by the cells at any given time point (Figure 1c). Similar concentrations of H_2O_2 have been used to induce oxidative stress in RAW 264.7 cells [27].

By using the optimized conditions of H_2O_2, we then sought to characterize oxidative stress-responsive miRNAs in RAW 264.7 cells. Cells were incubated with or without 800 μM of H_2O_2 for 1 h in growth medium with four replicates each. Total RNA was extracted and subjected for microarray analysis using LNA probes and the data were analyzed by bioinformatics tools and the differentially expressed miRNAs of 1.5-fold or higher were identified. Figure 2 shows differential expression of five miRNAs in response to H_2O_2 treatment and all of them carry an annotation star (miRNA*). These include miR-27a*, miR27b*, miR-29b*, miR-24-2*, and miR-21* (Figure 3a). We verified the expression pattern of these miRNAs in dye swap analysis in which the dyes used to label the control and H_2O_2-treated samples were swapped. It was expected that the miRNAs that are downregulated in samples in which controls were labeled with Cy3 and H_2O_2-treated samples with Cy5 (Figure 2a) follow a reverse pattern in dye swapping and it was the case (Figure 2b). Furthermore, we verified in the microarray analysis that none of the miR-

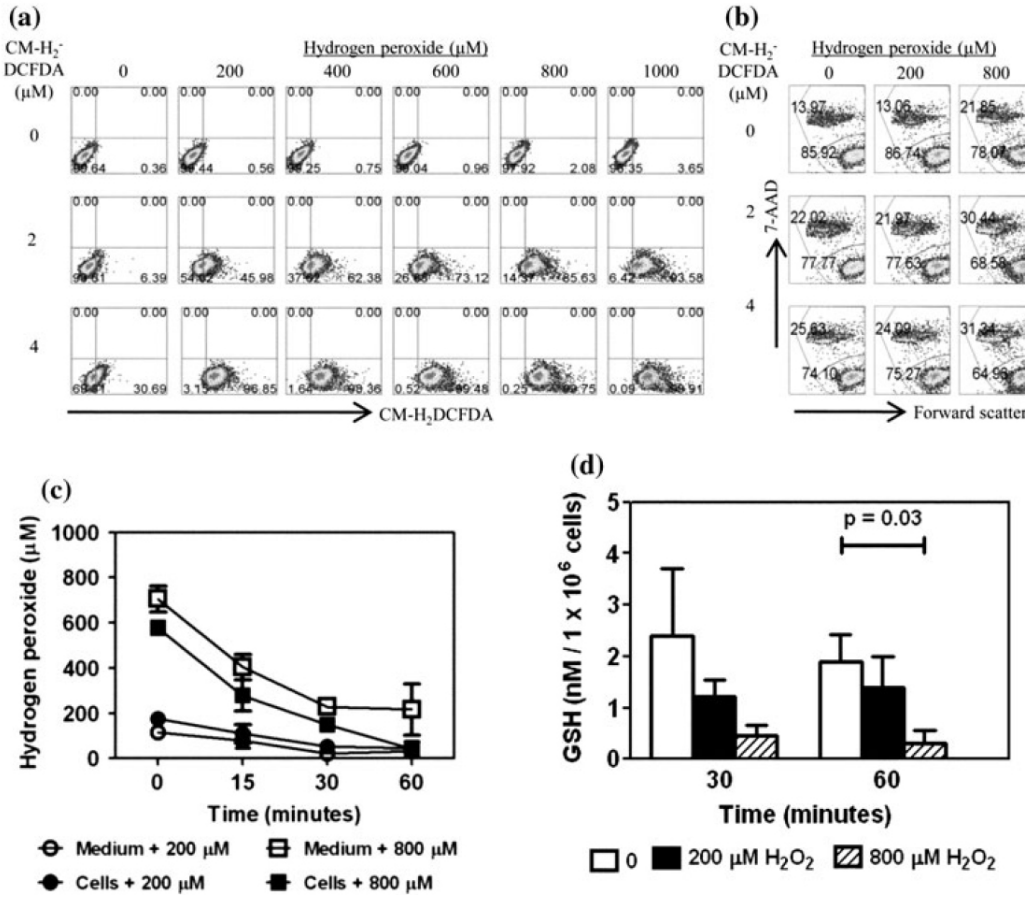

Figure 1 Induction of oxidative stress by H_2O_2 in RAW 264.7 cells. a) Intracellular detection of ROS. RAW 264.7 cells were loaded with CM-H_2DCFDA in serum-free DMEM and after washing, cells were exposed to the indicated concentrations of H_2O_2 in growth medium for 1 h followed by staining with 7-AAD. Cells were acquired by flow cytometer and the fluorescent intensity of CM-H_2DCFDA was analyzed in live (7-AAD $^-$) population. A representative experiment is shown. b) Cell viability. Percentages of live cells (7-AAD $^-$) were determined in the above cultures by plotting dead cells (7-AAD$^+$) against forward scatter. c) H_2O_2 utilization. To measure the degree of oxidative stress-induced with H_2O_2, consumption of H_2O_2 was evaluated spectrophotometrically in growth medium with or without cells ($n = 3$). d) GSH levels. RAW 264.7 cells were exposed to the indicated amounts of H_2O_2 and the GSH levels were measured spectrophotometrically ($n = 5$). Mean ± SEM values are shown.

NAs included in the Exiqon probe sets were altered in RAW 264.7 cells when exposed to 200 µM H_2O_2 for 15 min (data not shown). This may be due to insufficient availability of H_2O_2 to induce oxidative stress (Figure 1c). The differentially expressed miRNAs included both up (miR-21*, 1.6-fold) and downregulated miRNAs [miR27b* and miR-27a* (*3-fold); miR-29b* and miR-24-2* (*2-fold)] (Figure 3a). It appears that expression of oxidative stress-responsive miRNAs differ by cell types. For example, miR-21 was upregulated in cardiomyocytes treated with H_2O_2 and human fibroblasts exposed to the stress-induced with ionizing radiation and H_2O_2 express characteristic miRNAs [14, 15, 28]. Nonetheless, the differentially expressed miRNAs induced with H_2O_2 may have a role in modulating the effects of oxidative stress as relevant to each cell type.

It is believed that miRNAs and miRNAs* are both mature miRNAs arising from the same precursors. miR-NAs* are less abundant than miRNAs and they are likely to be non-functional [29, 30]. These notions have been debated recently and suggested that both species of miRNA can exist at equal levels and can alter the expression of target messages [31, 32]. Under oxidative stress conditions, antioxidant defense system tries to scavenge the prooxidants to maintain redox homeostasis, but insufficient production of antioxidants can lead to oxidative stress in the cells. By using GSH as readout, we demonstrated that the RAW 264.7 cells were in a state of oxidative stress when exposed to 800 µM of H_2O_2 (Figure 1d). This raises a question whether the oxidative stress-responsive miRNAs have a role in altering the antioxidant gene expression that quench ROS to maintain redox homeostasis or control the genes downstream of H_2O_2 stimulation that mediate the effects of oxidative stress.

To determine the role of oxidative stress-responsive miRNAs, we chose miR-27a* and miR-27b* for further analysis. First, we validated the expression pattern of miR27a* and miR-27b* in RAW 264.7 cells treated with or without H_2O_2 by TaqMan PCR analysis. Figure 3b shows that the expression of miR-27a* and miR-27b* was significantly downregulated approximately five-fold in H_2O_2-treated samples as compared with controls. Second, we searched for target genes that might be regulated by miR27a* and miR-27b* in miRBase (http://microrna.sanger.ac.uk). We found that each miRNA can bind to about 700 targets and some of these such as protein kinase c and mitogen activated protein kinases have been implicated in the activation of NF-kB pathway [33, 34].

After engulfing microbes, macrophages produce ROS which can activate activator protein-1 and NF-kB via MyD88-dependent pathway and induce inflammatory gene expression [1, 35]. ROS can also influence adaptive immune response by acting as a third signal for T cell activation by upregulating costimulatory molecules [4, 36]. The fact that some of the target proteins of miR-27a* and miR-27b* are implicated in the activation of NF-kB prompted us to examine the effects of oxidative stress-responsive miRNAs in the modulation of the NF-kB pathway. To provide a proof of concept, we

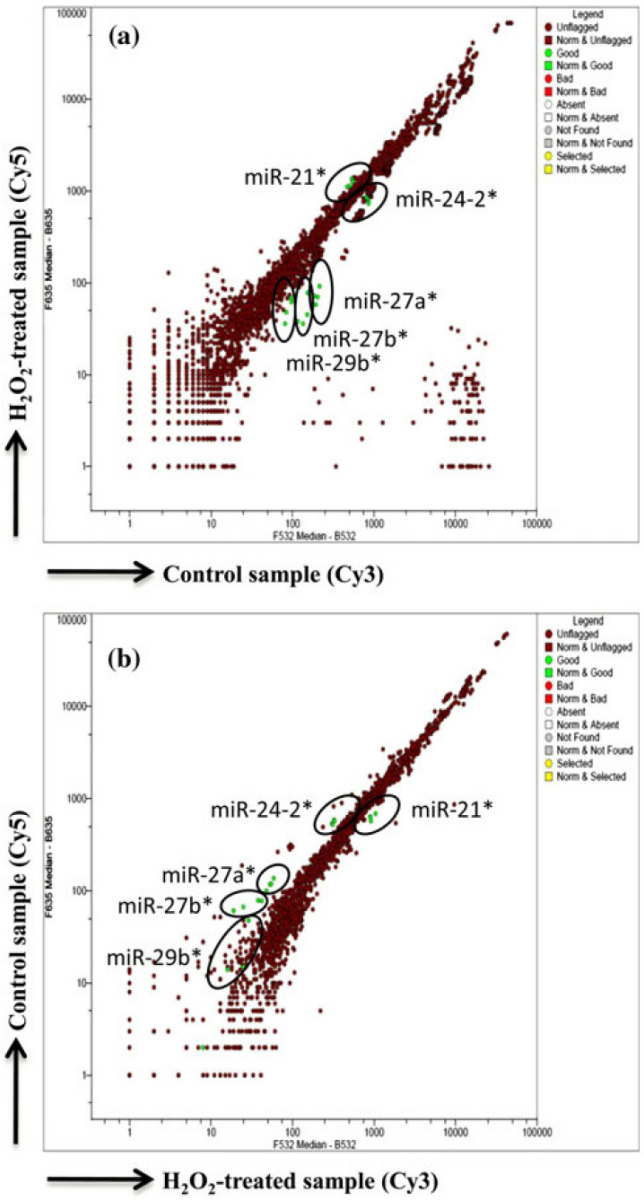

Figure 2 Microarray analysis. Total RNA was isolated from RAW 264.7 cells with four replicates each treated with or without 800 µM H_2O_2 for 1 h. In three of the replicates, H_2O_2-treated samples were labeled with Cy5, and the controls with Cy3 (*top panel*) and the dyes were swapped in the fourth set (*bottom panel*). After hybridization, arrays were scanned by using GenePix software and the differentially expressed miRNAs were identified. Four dots in each circle represent one miRNA and the miRNA expression pattern was reciprocal between the sample sets labeled with Cy5 for H_2O_2, and Cy3 for controls (*top panel*) and the sample sets in which the dyes were swapped (*bottom panel*). Representative experiments are shown.

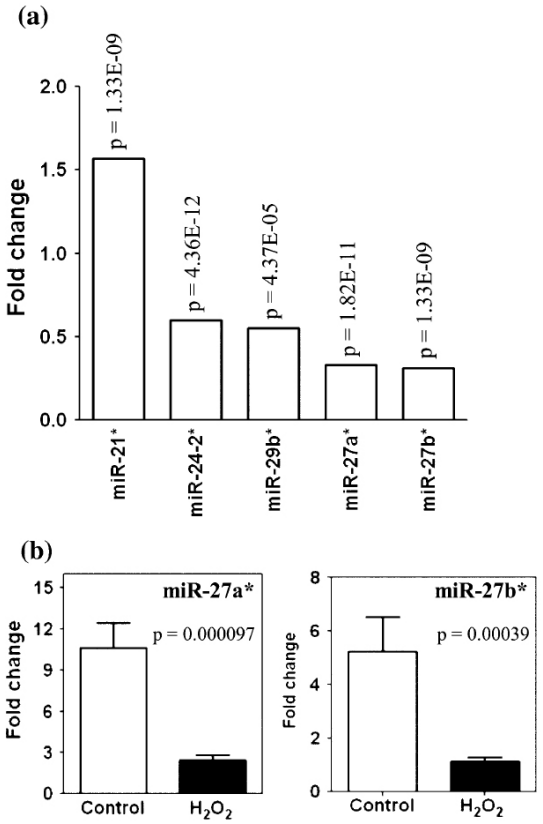

Figure 3 H_2O_2-induced miRNA expression in RAW 264.7 cells. a) Differentially expressed miRNAs. The data obtained from microarray analysis for control (untreated) and H_2O_2-treated samples were analyzed by bioinformatics tools. The miRNAs that are up or downregulated by 1.5-fold or higher were identified. False discovery adjusted *P* values ≤ 0.05 were taken as significant. b) Quantification of miR-27a* and miR-27b* expression in response to H_2O_2 by TaqMan PCR. Total RNA was extracted from RAW 264.7 cells treated with or without 800 μM of H_2O_2 for 1 h and cDNAs were prepared. Expression levels of miR-27a* and miR-27b* were quantified by normalizing to RNU48 (endogenous control) and the fold change was determined. Mean ± SEM values are shown (*n* = 3).

chose miR27b*, one of the most downregulated miRNAs noted in our studies (Figure 3). We tested this hypothesis by using the luciferase reporter system which involves Firefly pGL4.32/5× NF-kB luciferase reporter vector and Renilla pRL-TK luciferase vector as an internal control. RAW 264.7 cells were cotransfected with reporter plasmids and miR-27b* mimics or their control mimics. After 24 h, LPS-induced luciferase activity was measured. As expected, LPS induced the activation of NF-kB in the cells transfected with the reporter vectors (positive control), and negative control mimics had no significant effect on NF-kB activation (Figure 4a). However, in response to LPS stimulation, NF-kB activity was significantly reduced in the cells cotransfected with reporter vectors and miR-27b* mimics when compared with the positive control (no miRNAs) ($P = 0.03$) or with the cells cotransfected with the negative control mimics ($P = 0.02$). We further verified the nuclear translocation of p65 subunit, the transactivating subunit in the canonical NF-kB pathway [37-39]. Figure 4b shows that the translocation of p65 subunit was significantly affected in the cells transfected with miR-27b* when compared with untransfected cells ($P = 0.007$) or cells transfected with control mimics ($P = 0.009$). In the latter, however, some degree of suppression of p65 translocation also occurred indicating that the transfection procedure per se or scrambled (control) miRNAs can have some nonspecific effects on transcription factor activities. Overexpression of miR-27b* mimics might negatively regulate its target proteins, which may be critical for NF-kB activation and the absence of these proteins in cells transfected with miR-27b* mimics might have led to either failure of NFkB activation or active suppression of NF-kB. To verify whether overexpression of miR-27b* can alter NF-kB-dependent gene expression, we measured mRNA expression for IL-1β, IL-6, TNF-α, and Ccl2 by TaqMan PCR analysis [40-43]. While there were no differences in the expression of IL-1β, IL-6, and TNF-α, Ccl2 mRNA expression was lower in the cells transfected with miR27b* mimics as compared with that of control mimics (Figure 4c). As noted above, such suppression also occurred in the cells transfected with the control mimics but to a lesser degree which might be due to the non-specific effects of transfection procedures with scrambled miRNAs. Taken together, the results suggest that the oxidative stress-responsive miRNAs can regulate the effects of ROS via NF-kB pathway but the identification of putative target proteins that modulate this pathway remains to be determined.

In summary, we have identified a set of unique miRNAs* that are differentially expressed in response to H_2O_2 in RAW 264.7 cells. miRNAs* are generally considered to be non-functional and here, we provide a proof of concept that one of the oxidative stress-responsive miRNAs namely miR-27b* has a modulating effect on NF-kB activation. Our data provide new insights into the molecular mechanisms by which macrophage-derived ROS could modulate innate immune functions.

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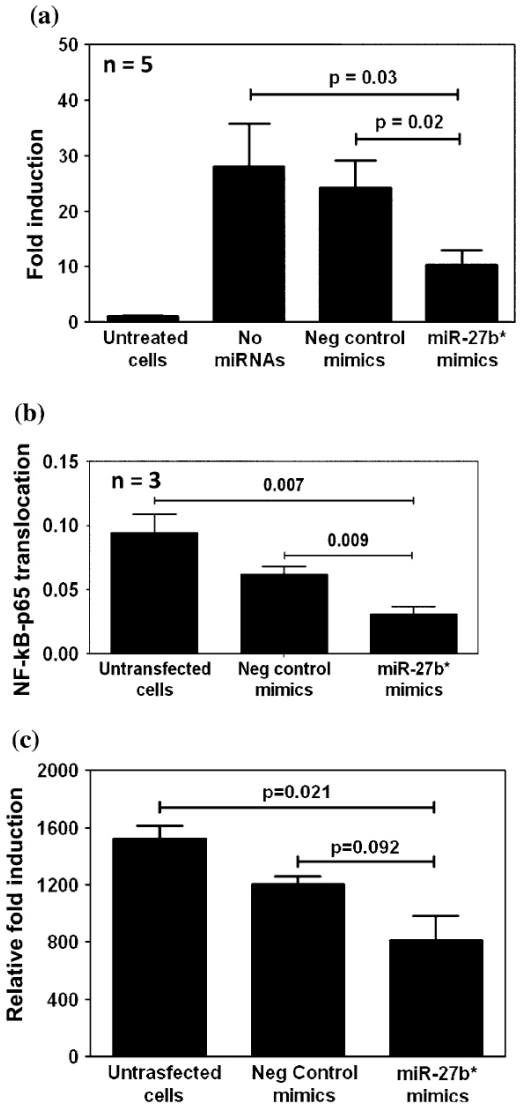

Figure 4 Determination of the effect of miR-27b* mimics on NF-kB pathway. a) Luciferase reporter assay. RAW 264.7 cells were cotransfected by nucleofection with a cocktail containing 20:1 of Firefly pGL4.32/5× NF-kB luciferase reporter vector and Renilla pRL-TK luciferase vector (internal control) and miR-27b* mimics or negative control mimics (40 nM). The cells were cultured for 24 h prior to treatment with LPS and 6 h later, Firefly luciferase activities were measured in cell lysates and normalized to Renilla luciferase activities. The fold induction of NF-kB was calculated as described in the methods. Mean ± SEM values are shown (n = 5). b) NF-kB p65 nuclear translocation assay. RAW 264.7 cells were transfected with or without miR27b* mimics or scrambled miRNAs (negative control mimics) and after 24 h, the cells were treated with LPS for 6 h. Nuclear fractions representing the above samples were added to the plates containing oligonucleotides for NF-kB consensus site followed by primary anti-NF-kB p65 and HRP-conjugated secondary antibodies and absorbance was then measured by spectrophotometry (n = 3). c) Ccl2 mRNA expression. cDNAs were derived from RAW 264.7 cells transfected with or without miR-27b* and control mimics. TaqMan PCR analysis was performed to determine the relative fold induction of Ccl2 mRNA by normalizing the expression levels of Ccl2 to GAPDH mRNA. Mean ± SEM values representing three replicates are shown.

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