Suppression of LPS-induced IFNγ and nitric oxide in splenic lymphocytes by select estrogen-regulated miRNA: A novel mechanism of immune modulation

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Running title: miRNA in estrogen-mediated immune regulation

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ABSTRACT

microRNA (miRNA), recently identified, non-coding, small RNA, are emerging as key regulators in homeostasis of the immune system. Therefore, aberrant expression of miRNA may be linked to immune dysfunction, such as in chronic inflammation and autoimmunity. In this study, we investigated the potential role of miRNA in estrogen-mediated regulation of innate immune responses, as indicated by upregulation of LPS-induced IFNγ, inducible nitric oxide synthase (iNOS), and nitric oxide in splenic lymphocytes from estrogen-treated mice. We found that miR-146a, a negative regulator of Toll-like receptor (TLR) signaling, was decreased in freshly-isolated splenic lymphocytes from estrogen-treated mice compared to placebo controls. Increasing the activity of miR-146a significantly inhibited LPS-induced IFNγ and iNOS expression in mouse splenic lymphocytes. Further, miRNA microarray and Real-time RT-PCR analysis revealed that estrogen selectively upregulates/downregulates the expression of miRNA in mouse splenic lymphocytes. miR-223, which is highly upregulated by estrogen, regulates LPS-induced IFNγ, but not iNOS or nitric oxide in splenic lymphocytes. Inhibition of miR-223 activity decreased LPS-induced IFNγ in splenic lymphocytes from estrogen-treated mice. Our data are the first to demonstrate the selective regulation of miRNA expression in immune cells by estrogen and are indicative of an important role of miRNA in estrogen-mediated immune regulation.

Keywords: microRNAs, estrogen, splenic lymphocytes, TLR4 signaling, mouse
INTRODUCTION

The innate immune system is the first line of defense protecting the host from invasion by diverse microbial pathogens. To date, 13 members of the Toll-like receptor (TLR) family have been identified in mammalian cells, and each TLR recognizes and binds to specific microbial products called pathogen-associated molecular patterns (PAMPs) \(^1,2\). For example, TLR4 recognizes and binds to lipopolysaccharide (LPS), a gram-negative bacterial component, to trigger the myeloid differentiation primary-response protein 88 (MyD88)-dependent signaling pathway and/or the MyD88-independent signaling pathway, resulting in the production of inflammatory molecules such as type I interferon (IFN) and nitric oxide \(^1,3\). While TLR mediated inflammatory responses are important for controlling infections, overwhelming activation of TLR signaling is deleterious and can cause severe inflammatory disease. Thus, the activation of TLRs should be tightly regulated \textit{in vivo}. Various mechanisms employed by different classes of negative regulators have been identified to regulate TLR triggered inflammatory immune responses \(^4,5\). Very recent publications indicate that microRNA (miRNA) fine-tune innate immune responses; thus, an entirely new paradigm of regulation of innate immunity is proposed \(^6,7\).

miRNA are small (18–25 nucleotide long), non-coding RNAs that suppress gene expression at the post-transcriptional level by binding to the 3’UTR of target genes, resulting in either translation inhibition or mRNA degradation \(^8\). Despite their recent identification, the impact of miRNA on gene regulation is profound. miRNA have been shown to be involved in the regulation of a variety of biological processes including development, signal transduction, apoptosis, cell proliferation, and tumorigenesis \(^9-13\).
The role of miRNA in normal immune function, as well as in inflammatory processes, is now emerging. The direct role of miRNA in regulation of innate immune responses was first suggested by a study that indicated that miR-146 is a negative feedback regulator of TLR signaling. Additionally, a recent report indicates that miR-155 and miR-125b, which are induced and inhibited by LPS stimulation, respectively, have opposite effects on TNFα induction and may regulate endotoxin shock responses. Let-7i was shown to target the TLR4 receptor. Microbial infection decreased the expression of let-7i, which is associated with upregulation of TLR4 in infected cholangiocytes.

The direct role of miRNA in innate immunity is further suggested by the function of miRNA in combating viral infections. It was shown that IFNβ inhibits hepatitis C virus replication in the human hepatoma cell line Huh7 by inducing miRNA that target the RNA genome of viruses.

Estrogen, a sex hormone, has been well recognized as an important immune modulator and plays a central role in gender differences in disease susceptibility. Moreover, estrogen has been implicated in autoimmune diseases. Estrogen has also been shown to physiologically regulate the induction of inflammatory molecules by lymphoid cells. We, and others, have shown that in vivo estrogen treatment promotes induction of IFNγ, inducible nitric oxide synthase (iNOS), nitric oxide, monocyte chemoattractant protein-1 (MCP-1), and MCP-5. Estrogen may strengthen innate immune responses by promoting the differentiation of IFNγ-producing killer dendritic cells, or by upregulation of iNOS expression and nitric oxide production. Further, estrogen has been shown to play a key role in controlling bacterial and viral infections in female mouse brains.
In this novel report, we demonstrate that \textit{in vivo} estrogen treatment significantly enhances innate immune responses of splenic lymphocytes to LPS by markedly augmenting induction of iNOS, nitric oxide, and IFN\(\gamma\). Importantly, we show that LPS-induced IFN\(\gamma\) and iNOS are regulated by select miRNA. Microarray and Real-time RT-PCR analysis revealed that estrogen selectively upregulates/downregulates miRNA expression in splenic lymphocytes. By experimentally manipulating the activity of select estrogen-regulated miRNA, we demonstrated that miR-146a and miR-223 regulate LPS-induced IFN\(\gamma\) in splenic lymphocytes. These data are the first to illustrate the mechanistic role of miRNA in estrogen-mediated immune regulation and may have far-reaching implications in therapeutic treatment of estrogen-mediated immune disorders.
MATERIALS AND METHODS

Mice and isolation of splenic lymphocytes

Wild type C57BL/6 male mice (Charles River Laboratories) were orchiectomized and surgically implanted with 17β-estradiol (Sigma-Aldrich) or empty (placebo control) silastic implants as extensively described in our previous studies\(^{25,31-33}\). All animals were housed in the animal facility at the Center for Molecular Medicine and Infectious Diseases (CMMID). The Animal Care and Use Committee at Virginia Polytechnic Institute and State University approved all animal procedures. Mice were euthanized seven to eight wks after implantation, and splenic lymphocytes were isolated and cultured using procedures previously described in detail\(^{23,31}\). In brief, spleens from individual mice were dissociated in phenol red free RPMI-1640 incomplete medium (CellGro). Splenic lymphocytes were isolated with ACK-Tris-NH\(_4\)Cl lysis buffer per our previous studies, and washed with complete RPMI-1640 that was supplemented with steroid-free 10% charcoal-stripped fetal bovine serum (Atlanta Biologicals), two mM L-glutamine (Mediatech), 100 IU/ml penicillin (Mediatech), 100 μg/ml streptomycin (Mediatech), and 1% non-essential amino acids (Mediatech). Splenic lymphocytes were resuspended in complete medium and adjusted to 5 × 10\(^6\)/ml before plating the cells.

Real-time RT-PCR

Total RNA, containing miRNA, was isolated from freshly-isolated splenic lymphocytes using mirVana miRNA isolation kits (Ambion). The Taqman miRNA assay system (Applied Biosystems) was used to quantitatively detect the expression of miRNA following the manufacturer’s instructions. The relative expression level of miRNA was
calculated using the $2^{-\Delta\Delta Ct}$ (Livak) method after normalization to the endogenous small RNA control, snoRNA 202. To analyze the expression of IFNγ and iNOS mRNA, Real-time RT-PCR was performed using iScript one-step RT-PCR kits with SYBR green (Bio-Rad) per our previously reported study. Total RNA was treated with RNAase-free DNAase (Promega) and then used as a template. The mRNA expression level of target genes was normalized to β-actin using the $2^{-\Delta\Delta Ct}$ (Livak) method. PCR primer mixes (10×) for IFNγ, iNOS, and β-actin were purchased from Qiagen.

**miRNA microarray assay and data analysis**

Total RNA was isolated from freshly-isolated splenic lymphocytes from placebo- and estrogen-treated mice (n=2 each group) as described above. Small RNA enrichment and miRNA microarray assays were performed by LC Sciences (http://www.lcsciences.com/). Mouse miRNA array chips (Chip ID miRMouse 10.0 version), which included 568 unique, mature, mouse miRNA, based on the Sanger miRBase Release 10.0, were used in the assay. Each miRNA probe was repeated in sixtuplicate on the chip. The data extracted from the image of each hybridized chip were adjusted by subtracting the background signal (the median of 5% to 25% of the lower signal intensities). Normalization was further carried out using the Variance Stabilization Normalization (VSN) method on background-subtracted data to remove system-related variations. The means of the normalized signal intensities from the six signal spots for each probe were Log2 transformed for further analysis. To visualize the differential expression intensity levels of miRNA in different samples, a heat map was generated using Java Tree View. The Log2 transformed intensity values were centered by
subtracting the mean Log2 values across all the samples for individual miRNA, and then used for cluster analysis to generate the heat map. The significance of miRNA differentially expressed between the two groups of samples (placebo- vs estrogen-treated mice) was determined using the LIMMA package. The raw p values were further corrected by the Benjamini and Hochberg false-discovery rate test. The fold change was calculated by dividing the mean intensity of the miRNA in estrogen treated samples by that in placebo treated samples. If this number was less than one, the negative reciprocal was used. The microarray data set has been submitted to Gene Expression Omnibus (GEO) under the accession number GSE11197.

**Transfection of miRIDIAN miRNA mimics and inhibitors**

miRIDIAN miRNA mimics (double-stranded chemically modified RNA oligonucleotides) and miRIDIAN miRNA inhibitors (single-stranded chemically enhanced oligonucleotides) from Dharmacon were used to supplement and suppress specific miRNA activity in mouse splenic lymphocytes, respectively. Both a nucleofector device and mouse macrophage nucleofector kit (Amaxa) were used to transfect 3.5 μg of miRNA mimics or inhibitors to 1.5 × 10^7 freshly-isolated mouse splenic lymphocytes. Negative miRIDIAN mimics or inhibitors were transfected as matched controls.

**Detection of iNOS, nitric oxide, and IFNγ**

Western Blots were used to analyze iNOS and IFNγ protein expression in whole cell extracts as described before. The blot images were captured and the signal
intensities were analyzed using a Kodak Image Station 440. Griess assays were used to
detect nitric oxide levels in culture supernatants as described $^{23}$. The levels of IFN$\gamma$ in
culture supernatants were determined with ELISAs as described previously $^{24,31}$.

**Statistical analysis**

All values in the graphs are given as means ± SEM. To assess statistical
significance, $t$-tests were performed using GraphPad InStat version 3.0a for Macintosh
(GraphPad Software). For evaluation of the effect of a specific miRNA mimic or
inhibitor on LPS-induced nitric oxide and IFN$\gamma$, the level of nitrite and IFN$\gamma$ in negative
control transfected cells were regarded as 100% and the level of nitrite and IFN$\gamma$ in paired
miRNA mimic or inhibitor transfected cells are shown as a percentage of the level of the
corresponding negative control transfected cells. Paired $t$ tests were performed between
negative control and specific inhibitor or mimic transfected cells.
RESULTS

LPS-induced IFN\(\gamma\) is augmented in splenic lymphocytes by estrogen

Our previous report has shown that estrogen promotes secretion of the proinflammatory cytokine IFN\(\gamma\) in splenic lymphocytes after stimulation with Con-A for only 3 hrs. In the present study, we activated splenic lymphocytes with LPS and then determined the IFN\(\gamma\) level in culture supernatants. At 3 hrs of stimulation with LPS, the IFN\(\gamma\) levels in supernatants of splenic lymphocytes were low (undetected in some samples), and thus, there was no significant difference between placebo- and estrogen-treated mice (data not shown). After 6 hrs of stimulation with LPS, however, we observed significantly higher IFN\(\gamma\) levels in supernatants of splenic lymphocytes from estrogen-treated mice compared to placebo-treated (control) mice (Fig 1A). LPS stimulation induced IFN\(\gamma\) mRNA expression in splenic lymphocytes from both placebo- and estrogen-treated mice.

Intriguingly, in contrast to the increased IFN\(\gamma\) protein levels in the supernatants of LPS stimulated splenic lymphocytes from estrogen-treated mice, the level of IFN\(\gamma\) mRNA was significantly lower than that from placebo-treated mice (Fig 1B). This suggested that the expression of LPS-induced IFN\(\gamma\) in splenocytes from estrogen-treated mice is regulated at both transcriptional and post-transcriptional levels.

In vivo estrogen treatment decreases the expression of miR-146a in splenocytes

Given a recent report indicating the negative regulatory role of miR-146 in TLR signaling in human THP-1 monocytes, we next investigated whether miR-146 plays a similar role in mouse splenic lymphocytes and whether estrogen modulates the response to LPS by altering the expression of miR-146. Quantitative Real-time RT-PCR analysis
indicated that the expression of miR-146a is significantly decreased in freshly-isolated splenic lymphocytes from estrogen-treated mice compared to controls (Fig 2A). In contrast to downregulation of miR-146a levels, estrogen has no apparent effect on miR-146b since the levels of miR-146b were comparable in freshly-isolated splenic lymphocytes from placebo- and estrogen-treated mice (Fig.2A).

**miR-146a downregulates LPS-induced IFNγ in mouse splenocytes**

Next, we further investigated whether miR-146a regulates LPS induced IFNγ in mouse splenic lymphocytes by manipulating its activity using miR-146a mimics, which can supplement the activity of miR-146a in vivo. As indicated, in the cells transfected with miR-146a, the expression of IRAK-1, a confirmed miR-146 target, was decreased compared to that in negative mimic transfected cells (Fig. 2B). With enhanced miR-146a activity, there was a significant decrease in LPS-induced IFNγ cytokine levels in culture supernatants from both placebo- and estrogen-treated mice when compared to negative mimic control transfected cells (Fig. 2C). Moreover, Western blotting clearly showed that IFNγ protein expression in cells transfected with miR-146a mimics was decreased compared to negative mimic transfected cells (Fig. 2D). Together, the data indicated that miR-146a negatively regulates LPS-induced IFNγ in mouse splenic lymphocytes.

**LPS-induced iNOS and nitric oxide in mouse splenic lymphocytes are regulated by miR-146a**

In our previous studies we reported that estrogen markedly enhanced IFNγ-dependent induction of iNOS and nitric oxide in splenic lymphocytes that were activated with Con-
A for 24 hrs\textsuperscript{23}. In this study, we reported that LPS activated splenic lymphocytes from estrogen-treated mice had also enhanced nitric oxide (Fig. 3A) and iNOS protein expression (Fig. 3B). Consistent with increased iNOS protein expression, iNOS mRNA is markedly higher in splenic lymphocytes from estrogen-treated mice compared to placebo controls (Fig. 3C). This suggested that estrogen promotes LPS-induced iNOS at the transcriptional level. Further, we also determined whether increasing the function of miR-146a downregulates LPS-induced iNOS and nitric oxide in splenic lymphocytes from estrogen-treated mice. There was a significant decrease in nitric oxide in culture supernatants from miR-146a mimic transfected splenic lymphocytes compared to negative mimic transfected cells (Fig. 3D, $p = 0.0011$). Further, Western blot analysis also showed decreased iNOS protein expression in cells transfected with miR-146a mimics (Fig. 3E). However, the downregulatory effect of the miR-146a mimics on nitric oxide was not as strong as that observed for LPS-induced IFN$\gamma$. Since the levels of LPS-induced nitric oxide in splenic lymphocytes from placebo-treated mice were too low, downregulatory effects of miR-146a mimics on nitric oxide in cells from placebo-treated mice could not be fully determined. However, we did observe that the miR-146a mimics inhibited LPS-induced iNOS protein expression in some samples from placebo mice in which iNOS could be detected by Western blotting (data not shown). Our data indicate that estrogen promotes LPS-induced iNOS and nitric oxide, which are partially regulated by the activity of miR-146a.

**miRNA expression profiles in mouse splenic lymphocytes are modulated by estrogen**
The above data suggested that estrogen regulates miR-146a, which in turn regulates splenic lymphocyte responses to LPS. It is therefore likely that estrogen may also regulate other miRNA in splenic lymphocytes, as aspect not yet determined. We performed miRNA microarray assays to compare the miRNA expression profiles in freshly-isolated splenic lymphocytes from estrogen-treated mice to those from placebo-treated mice (n=2 per group). The heat map, which was generated using hierarchical cluster analysis, revealed that the expression pattern of miRNA in freshly-isolated splenic lymphocytes from estrogen-treated mice is distinct from that observed in placebo (control) treated mice (Fig. 4A). In Figure 4B, miRNA that were significantly different between placebo- and estrogen-treated mice are listed, based on statistical analysis of the microarray data (p<0.05). Estrogen upregulated several miRNA (e.g. miR-451, miR-223) as depicted by the shade of red in the heat map and by the statistical analysis (Fig 4A and B). Estrogen also significantly downregulated miRNA (e.g. miR-145 and miR-125a), which are shown as shades of green in the heat map (p <0.05) (Fig. 4A and B).

We next confirmed changes in selected differentially expressed miRNA using Real-time RT-PCR with increased sample sizes (n=4-6 per treatment group). Real-time RT-PCR analysis confirmed that estrogen significantly increased the expression of miR-451, miR-486, miR-223, and miR-148a compared to samples from placebo-treated mice (Fig. 5A). Conversely, Real-time RT-PCR results indicated that estrogen also significantly decreased the expression of miR-125a-5p (miR-125a), miR-125b-5p (miR-125b), miR-143, miR-145, let-7e, and miR-126-3p (miR-126) (Fig. 5B). Additionally, Real-time PCR revealed that miR-18a and miR-708, which were increased, but not
statistically significant based on the microarray data, were also significantly upregulated by estrogen.

**Inhibition of miR-223 activity leads to a reduction of LPS-induced IFNγ**

Since estrogen highly upregulates the expression of miR-451, miR-486, and miR-223 (over eight fold based on the Real-time RT-PCR results) in splenic lymphocytes, we further investigated whether these three miRNA play roles in estrogen-mediated enhancement of LPS-induced IFNγ and iNOS expression. We inhibited the function of miR-223, miR-486, and miR-223 using appropriate inhibitors, and then determined the expression of LPS induced IFNγ, iNOS, and nitric oxide. Inhibiting the activity of miR-223, but not miR-451 and miR-486, significantly decreased LPS-induced IFNγ secretion in supernatants of splenic lymphocytes from estrogen-treated mice compared to negative transfected cells (Fig. 6A). However, none of three miRNA had significant effects on nitric oxide production in culture supernatants since there was no significant change in the nitric oxide levels between negative inhibitor and specific inhibitor transfected cells (Fig. 6B). Further, Western blot analysis indicated that IFNγ protein expression is decreased in miR-223 inhibitor, but not in miR-451 and miR-486 inhibitor, transfected cells, compared to negative controls (Fig.6C).
DISCUSSION

In this study, we demonstrated that increasing the activity of miR-146a *in vivo* by transfecting cells with miR-146a mimics significantly decreased LPS-induced IFNγ in both splenic lymphocytes from placebo- and estrogen-treated mice. Our data also provide direct evidence showing that miR-146a targets IRAK-1 and negatively regulates LPS-TLR4 mediated inflammatory responses in splenic lymphocytes. Further, we found that *in vivo* estrogen treatment decreased the expression of miR-146a in freshly-isolated splenic lymphocytes compared to placebo controls. It is therefore conceivable that the lower level of miR-146a in freshly-isolated cells allows earlier induction of IFNγ in splenic lymphocytes from estrogen-treated mice (6 hrs).

Enhancing the activity of miR-146a also inhibited the expression of LPS-induced iNOS and nitric oxide, which are markedly augmented in splenic lymphocytes from estrogen-treated mice. It is notable that the inhibitory effect of miR-146a on LPS-induced IFNγ (about 50%, Fig. 2C) is stronger than that observed for LPS-induced nitric oxide (about 20%, Fig.3C). One possibility is that LPS-induced IFNγ and iNOS are subjected to different regulatory machinery downstream of LPS-TLR signaling. LPS-induced type I interferon and IFNγ can prime the cells and further synergize with LPS to stimulate maximal production of nitric oxide in macrophages\textsuperscript{39,40}. Additionally, we noticed that estrogen enhances the expression of iNOS at both the mRNA and protein levels. However, LPS-induced IFNγ mRNA levels are significantly lower in splenic lymphocytes from estrogen-treated mice after LPS stimulation compared to placebo controls (Fig. 1B). These data suggest that there is a different underlying mechanism in estrogen-mediated promotion of LPS-induced IFNγ compared to LPS-induced iNOS/
nitric oxide. It is possible that estrogen regulates LPS-induced IFNγ at the post-transcriptional level by downregulation of IFNγ targeting miRNA. Identification of IFNγ-targeting miRNA may further delineate whether estrogen regulates IFNγ at the post-transcriptional level by regulating IFNγ-targeting miRNA.

Although limited, recent reports have suggested hormonal regulation of miRNA in non-lymphoid models, such as rat mammary glands and zebrafish. However, so far there are no reports regarding hormonal regulation of miRNA in immune cells. Based on the microarray analysis, 25 miRNAs were significantly regulated by estrogen in splenic lymphocytes (p <0.05, Fig 4B). Selected miRNA were further confirmed using Real-time RT-PCR analysis (Fig 5). Additionally, estrogen significantly decreased miR-146a, and increased miR-18a and miR-708 as determined by Real-time RT-PCR (Fig. 2A and 5A), but not by microarray analysis. The discrepancy in statistical differences between Real-time RT-PCR and microarray analysis (regarding the detection of miR-146a, miR-18a, and miR-708) is most likely due to the difference in the sensitivity of these two assays, biological differences, and the fact that unlike Real-time RT-PCR, a limited number of samples (n=2) were used in the microarray analysis. Moreover, although the miRNA microarray is an efficient and sensitive screening tool for simultaneous analysis of the expression of multiple miRNA, it can also result in false positives/negatives. Therefore, confirmation of microarray data using Real-time RT-PCR or Northern blotting is a necessity. Here, we confirmed the changes of selected miRNA by Real-time RT-PCR with an increased sample size (n=4-6).

Intriguingly, almost all of the miRNAs confirmed in our studies to be downregulated by estrogen (miR-125a, miR-125b, miR-143, miR-145, let-7, miR-126)
are decreased or lost in breast cancer\textsuperscript{45,46}. The decreased expression of these breast cancer related miRNA by estrogen treatment may provide an explanation for increasing breast cancer risk following lengthy exposure to estrogen, such as with post-menopausal hormone replacement therapy\textsuperscript{47,48}.

miR-223 has been shown to regulate granulocyte inflammatory responses\textsuperscript{49}. Here, we found that inhibiting the activity of miR-223 decreased LPS-induced IFN\textgreek{g} in splenic lymphocytes from estrogen-treated mice. Another two miRNA highly upregulated by estrogen, miR-451 and miR-486, had no significant effect on LPS-induced IFN\textgreek{g} and nitric oxide (Fig.6). It is possible that miR-451 and miR-486 are not involved in LPS signaling. We believe that there are multiple miRNA, not single miRNA, which cooperate with each other in the regulation of innate immune responses. Given that estrogen up/downregulates a panel of miRNA, simultaneous inhibition and/or enhancement of the activity of several estrogen-regulated miRNA may result in more significant changes in LPS induced inflammatory molecules compared to negative control transfected cells.

In this study, we used red blood cell (RBC)-depleted splenic lymphocytes as model cells to investigate the role of miRNA in estrogen-mediated regulation of inflammatory responses since in a natural \textit{in situ} setting, cell-cell interactions are critical for regulating the outcome of immune responses. This aspect was highlighted in our previous studies, which showed that Con-A induced IFN\textgreek{g} and nitric oxide can be dramatically decreased in splenic lymphocytes from both placebo- and estrogen-treated mice by blocking binding of the CD28 receptor on T cells with B7 molecules on antigen presenting cells\textsuperscript{23}. Here, we utilized a different approach by using purified subsets of
cells to show that the induction of IFNγ after 6 hours of LPS stimulation and the
induction of nitric oxide levels after 48 hours of LPS stimulation are very low in purified
macrophages, T cells, or macrophage depleted splenic lymphocytes from estrogen-treated
mice. However, there is dramatic induction of IFNγ and nitric oxide when purified
macrophages and T cells are mixed together before LPS stimulation (Supplemental
Fig.S1). These data reinforced the growing belief that splenic lymphocytes simulate the
natural conditions where APCs, T cells, and B cells interact and influence each other to
regulate immune responses. Subsequent studies can potentially focus on addressing a
separate question of whether estrogen differentially regulates miRNA in defined
subpopulations of splenic lymphocytes.

The present report is novel because it is the first study to demonstrate that
estrogen selectively regulates miRNA expression in immune cells. Further, we
demonstrate that select estrogen regulated miRNA, miR-146a and miR-223, regulated
LPS-induced IFNγ, and to lesser extent nitric oxide, in splenic lymphocytes. These data
suggest a novel mechanism for estrogen regulation of the immune system. Further
investigation of biological and immunological functions of estrogen-regulated miRNA is
important to allow comprehensive delineation of the role of estrogen-regulated miRNA in
both normal and dysregulated immune systems. Given that estrogens are thought to be
involved in not only normal immune regulation, but also in many inflammatory disease
conditions, these studies may have profound implications and may offer an entirely new
molecular therapeutics approach for treatment of estrogen-related immune disorders.
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AUTHORSHIP

RD and SAA designed and performed the experiments, analyzed data, and prepared the manuscript. RAP and DK performed experiments and contributed to paper writing. YZ and OC contributed to microarray experiment design and data analysis. RAP and YZ contributed equally to the paper.

All authors declare no competing financial interests.
REFERENCES


FIGURE LEGENDS

Figure 1. LPS-induced IFNγ in mouse splenic lymphocytes is promoted by estrogen at the post-transcriptional level. (A) ELISA analysis of IFNγ levels in supernatants from $5 \times 10^6$ freshly-isolated (t0) splenocytes and those stimulated with LPS (500 ng/ml, the same concentration was used in all the experiments in this study) for 6 hrs. The graphs show the means ± SEM (n = 4 each). (B) Total RNA was prepared from freshly-isolated splenocytes and from those stimulated with LPS for 6 hrs. IFNγ mRNA expression levels were determined by Real-time RT-PCR. The graphs show the relative mRNA expression levels with the means ± SEM (n ≥ 3). * indicates $p < 0.05$.

Figure 2. LPS-induced IFNγ in mouse splenic lymphocytes is regulated by the estrogen-regulated miRNA, miR-146a. (A) Total RNA was isolated from freshly-isolated splenic lymphocytes using mirVana miRNA isolation kits. Relative expression levels of miR-146a and miR-146b between splenic lymphocytes from placebo- and estrogen-treated mice were analyzed using the Taqman miRNA assay system. The graph shows the means ± SEM (n ≥ 6 each). (B-D) Freshly-isolated splenic lymphocytes ($1.5 \times 10^7$) from placebo- and estrogen-treated mice were transfected with either a negative mimic (control) or miR-146a mimic. Twenty-four hours after transfection, cells were left unstimulated (B) or stimulated with LPS for 24 hrs (C and D), and then the supernatants and cell pellets were collected for analysis. (B) Western blot analysis of the expression of IRAK-1 in unstimulated cells at 48 hrs after transfection. (C) The level of IFNγ in supernatants from LPS stimulated miR-146a mimic transfected cells is shown as the percentage expression of negative control mimic transfected cells. Graph shows mean ±
SEM (n ≥ 6 each). (D) Western blot analysis of the expression of IFNγ in cell extracts from negative mimic and miR-146a mimic transfected cells stimulated with LPS. Representative Western blot images are shown from at least three independent experiments. Densitometry analysis of the IFNγ signal in blot images was performed using Kodak molecular imaging software (version 4.5) and normalized to the loading control β-actin. The graph shows relative density with means ± SEM (n=3 each). * and *** indicate p < 0.05 and p < 0.001, respectively.

Figure 3. LPS-induced iNOS and nitric oxide in mouse splenic lymphocytes is enhanced by estrogen and regulated miR-146a. 2.5 × 10^6 splenic lymphocytes (5 × 10^6/ml) from placebo- and estrogen-treated mice (n=4 each) were stimulated with LPS or left unstimulated for 24 hrs (medium only) (A and B). Supernatants and cell pellets were collected for further analysis. (A) The production of nitric oxide in culture supernatants was determined with Griess assays. The graph shows means ± SEM (n=4 each). (B) Western blot analysis of the expression of iNOS in whole cell extracts. (C) The expression of iNOS mRNA in freshly-isolated and splenocytes stimulated with LPS for 6 hrs was analyzed as indicated for Fig.1B. The graphs show the relative mRNA expression level with the means ± SEM (n≥ 3 each). (D) Freshly-isolated splenic lymphocytes were transfected and stimulated with LPS as described for Fig. 2B-D. The level of nitric oxide in supernatants from LPS stimulated transfected cells was determined with Griess assays. The graph shows means ± SEM (n ≥ 6 each). (E) Western blot analysis of the expression of iNOS in cell extracts from negative mimic and miR-146a mimic transfected cells stimulated with LPS. Representative Western blot images are shown from at least three
independent experiments. Densitometry analysis of iNOS signal detected by Western blotting was performed using Kodak molecular imaging software (version 4.5) and normalized to the loading control β-actin. The graph shows relative density with means ± SEM (n=4). * and ** indicate p < 0.05 and p < 0.01, respectively.

Figure 4. Microarray data analysis. (A) The heat map was generated using hierarchical cluster analysis to show distinct miRNA expression patterns in splenic lymphocytes between placebo- and estrogen-treated mice. The intensity values were Log2 transformed, centered by the mean of individual genes across all four samples, and then subjected to cluster analysis for generating the heat map. The color bar was extracted to show the color contrast level of the heat map. Red and green indicate high expression level and low expression level, respectively. (B) miRNA that demonstrated statistically different expression levels between freshly-isolated splenic lymphocytes from placebo- and estrogen-treated mice (p<0.05) are listed. Fold changes in miRNA expression were calculated as the ratio of mean intensity values between estrogen- and placebo-treated mice.

Figure 5. Real-time RT-PCR analysis of miRNA expression in splenic lymphocytes. The expression level of selected estrogen-regulated miRNA in freshly-isolated splenic lymphocytes between placebo- and estrogen-treated mice were further quantified using Taqman miRNA assays. (A-B) The graphs show miRNA that were confirmed to be significantly upregulated or downregulated by estrogen treatment, respectively. Means ±
SEM (n ≥ 4 each) are shown in the graphs. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

**Figure 6.** miR-223, but not miR-451 and miR-486, regulate LPS-induced IFN$\gamma$ in splenic lymphocytes from estrogen-treated mice. 1.5 × 10$^7$ freshly-isolated splenic lymphocytes from estrogen-treated mice were transfected with either a negative inhibitor (control), miR-223, miR-451, or miR-486 inhibitors. Twenty-four hours after transfection, cells were stimulated with LPS for 24 hrs, and the supernatants and cell pellets were collected for analysis. (A-B) The level of IFN$\gamma$ and nitric oxide in culture supernatants of LPS stimulated cells were determined by ELISAs (A) and Griess assays (B), respectively. The level of IFN$\gamma$ and nitric oxide in supernatants from specific inhibitor transfected cells were presented as the percentage level of negative control inhibitor transfected cells. The graphs show the means ± SEM (n=5 each). (C) Western blot analysis of the expression of IFN$\gamma$ protein in miRNA inhibitor transfected cells. Representative Western images are shown from at least three independent experiments. Densitometry analysis of IFN$\gamma$ signal was performed as described for Fig 2. The graph shows relative density with means ± SEM (n=4 each). * indicates $p < 0.05$. 
Figure 2

A

Relative expression

miR-146a  miR-146b

Placebo  Estrogen

B

Placebo  Estrogen

Negative mimic  miR-146a mimic

IRAK-1  β-actin

C

Percent expression

Placebo  Estrogen

IFNγ

Negative mimic  miR-146a mimic

***

D

Placebo  Estrogen

Negative mimic  miR-146a mimic

IFNγ  β-actin

Densitometry
**Figure 4**

### Estrogen upregulated

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<th>p value</th>
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<td>0.029</td>
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### Estrogen downregulated

<table>
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<tr>
<th>miRNA</th>
<th>Fold change</th>
<th>p value</th>
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<tbody>
<tr>
<td>miR-493</td>
<td>-47.14</td>
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<tr>
<td>miR-126-3p</td>
<td>-6.14</td>
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<tr>
<td>miR-125a-5p</td>
<td>-5.28</td>
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<tr>
<td>miR-125b-5p</td>
<td>-5.06</td>
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<td>miR-466a-3p</td>
<td>-5.80</td>
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<td>miR-466b-3-3p</td>
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<tr>
<td>miR-297a</td>
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<tr>
<td>miR-297a</td>
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<tr>
<td>let-7e</td>
<td>-3.97</td>
<td>0.031</td>
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<td>miR-207</td>
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<tr>
<td>miR-142-5p</td>
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<tr>
<td>miR-143</td>
<td>-4.16</td>
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<tr>
<td>miR-206</td>
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<td>miR-200b</td>
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<td>miR-214*</td>
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<td>miR-28*</td>
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Figure 5

A

Relative miRNA expression level

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<td>miR-486</td>
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<td>miR-223</td>
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<tr>
<td>miR-148a</td>
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<tr>
<td>miR-18a</td>
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<td>miR-708</td>
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B

Relative miRNA expression level

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</tr>
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<tbody>
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<td>miR-125a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-125b</td>
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<tr>
<td>let-7e</td>
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<tr>
<td>miR-126</td>
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<tr>
<td>miR-145</td>
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<tr>
<td>miR-143</td>
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</table>
Suppression of LPS-induced IFN$\gamma$ and nitric oxide in splenic lymphocytes by select estrogen-regulated miRNA: A novel mechanism of immune modulation

Rujuan Dai, Rebecca A. Phillips, Yan Zhang, Deena Khan, Oswald Crasta and S. Ansar Ahmed