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MicroRNA-127 Inhibits Lung Inflammation by Targeting IgG Fcγ Receptor I

Ting Xie,*† Jiurong Liang,* Ningshan Liu,* Qingguo Wang,† Yuhang Li,† Paul W. Noble,* and Dianhua Jiang*

The molecular mechanisms of acute lung injury are incompletely understood. MicroRNAs (miRNAs) are crucial biological regulators that act by suppressing their target genes and are involved in a variety of pathophysiologic processes. miR-127 appears to be downregulated during lung injury. We set out to investigate the role of miR-127 in lung injury and inflammation. Expression of miR-127 significantly reduced cytokine release by macrophages. Looking into the mechanisms of regulation of inflammation by miR-127, we found that IgG FcγRI (CD64) was a target of miR-127, as evidenced by reduced CD64 protein expression in macrophages overexpressing miR-127. Furthermore, miR-127 significantly reduced the luciferase activity with a reporter construct containing the native 3′ untranslated region of CD64. Importantly, we demonstrated that miR-127 attenuated lung inflammation in an IgG immune complex model in vivo. Collectively, these data show that miR-127 targets macrophage CD64 expression and promotes the reduction of lung inflammation. Understanding how miRNAs regulate lung inflammation may represent an attractive way to control inflammation induced by infectious or noninfectious lung injury. The Journal of Immunology, 2012, 188: 2437–2444.
MicroRNAs (miRNAs) are critical regulators of gene expression. Mature miRNAs bind target mRNAs at complementary sites in 3' untranslated regions (UTRs) or coding sequences and thereby trigger downregulation and suppression of target gene expression (19). Emerging evidence also shows that miRNAs play an important role in both adaptive and innate immunity (20). miRNAs are involved in innate immunity through the regulation of TLR signaling and cytokine responses. For example, reports showed that miR-146, miR-155, and miR-132 are strongly upregulated after LPS treatment in human monocyte THP1 (21–23). miR-146 may target IL-1R-associated kinase-1 and TNFR-associated factor 6, two essential components of the TLR signaling pathways, thereby acting as a negative regulator of the inflammatory response (21). miR-147 is induced by TLR stimulation and can regulate the macrophage inflammatory response by decreasing LPS-induced TNF-α and IL-6 production (24). miRNAs also regulate adaptive immune responses. For example, silencing of miR-126 by antagonor impairs Th2 responses in the lung (25). miR-181a appears to act as a negative regulator of TCR signaling by targeting Bcl-2 and CD69, mediating positive selection (26, 27). Furthermore, miR-155 has a role in regulating Th cell differentiation and the germinal center reaction to produce an optimal T cell-dependent Ab response (28).

We have recently analyzed miRNA expression after bleomycin-induced lung injury and repair (29) and identified that miR-127 expression was decreased 1 d after bleomycin treatment. In this report, we examined miR-127 expression in both noninfectious and infectious lung injury. To examine the role of miR-127 in macrophages, we established a miR-127 stable overexpression of MH-S cells. We demonstrated that miR-127 directly downregulates CD64 and attenuated lung inflammation in vivo. Taken together, these data suggest that miR-127 plays a previously unrecognized role in preventing exaggerated inflammatory responses to complement injury by negatively regulating FcγRI.

Materials and Methods

Reagents

Peptidoglycan (PGN) from Bacillus subtilis and LPS from Escherichia coli 0111:B4 were purchased from Sigma-Aldrich. HA fragments (∼20,000 kDa in molecular mass. LPS-free) were from ICN. IC were prepared according to previous reports (30). Briefly, IgG-BSA IC were produced by slowly adding four volumes IgG anti-BSA (5 mg/ml; MP Biomedicals) to one volume BSA (10 mg/ml; Sigma-Aldrich) and incubating at 37˚C for 30 min. The complexes were recovered by centrifugation and resuspended in RPMI 1640 medium. Macrophage cell lines MH-S and RAW246.7, as well as human monocyte cell line U937, were stimulated with IgG-BSA IC (100 µg/ml) or RPMI 1640 medium as control. Cells

The mouse macrophage cell line RAW264.7, alveolar macrophage cell line MH-S and human monocyte cell line U937 were purchased from the American Type Culture Collection. Cells were cultured in RPMI 1640 plus 10% FBS at 37°C and 5% CO2. U937 were differentiated and activated with PMA (100 nM; Sigma-Aldrich) before other treatment.

miRNA oligonucleotides

2'-O-methyl (2'-OME) modified miRNAs were chemically synthesized by IDT. All bases were 2'-OME modified, and a cholesterol moiety was linked to the 3' terminus. miR-127: 5'-mUmGmCmGmAmUmGmCmGmCmUmGmAmGmUmCmUmCmAmGmCmUmU/m3CholTEG/-3', in which "m" denotes 2'-O- modified and termed pMIR-REPORT [mFcLuciferase report vector and 0.08 µg control vector containing Renilla luciferase report vector and 0.08 µg control vector containing Renilla luciferase report vector]. The full-length FcγRI 3' UTR was amplified by PCR from mouse genomic DNA with the following primers: FcγRI-Forward, 5'-GGT GAC TAG TGG ACC GCA GCA GCC-3'; and FcγRI-Reverse, 5'-AAG TGA CAG GTC CTC TGG AAG-3'. The amplified 1404-bp product was inserted into the Spel/Sacl sites of the luciferase expression vector pmIR-REPORT (Applied Biosystems) immediately downstream of the luciferase gene and termed pmIR-REPORT [FcγRI-3' UTR]. A mutant construct was generated with two primers: FcγRI-3m-Forward, 5'-AGC TGG CCG CTA GGA CAA CAA GAG GGA AGC CGA GCA GCC-3' and FcγRI-3m-Reverse, 5'-GGC GGG ACC GTG TTA ATT TGG TTT ATT TAA GAG GTG CAT GCC-3' cloned into pmIR-REPORT, resulting in pmIR-REPORT [mFcγRI-3' UTR]. Transfection. RAW264.7 cells were cotransfected with 0.4 µg firefly luciferase reporter vector and 0.08 µg control vector containing Renilla luciferase reporter vector, and control probes were purchased from Ambion.

Expression constructs and stable transduction

miR-127 lentiviral construct was generated as follows: a region of 101 nucleotides containing premiR-127 was amplified from mouse genomic DNA by using the following primers: 5’ MIR127, 5’-GAT CAC TGT TAA CCA GCC TGC T-3’; and 3’ MIR127, 5’-TAA ACT CGA GGG AGG CAG ATG A-3’. They were then cloned into the Hpal/Xhol sites of a pSico vector (31) named pSico-miR-127. Viral particles were produced by calcium phosphate-mediated transfection into 293T cells as described (32). Lentiviral supernatants were collected 48 h after transfection, passed through a 0.22-µm filter, and used directly to infect MH-S cells. GFP-positive cells were sorted 3 to 4 d postinfection and resulted in MH-S-SicoGFP and MH-S-miR127GFP cells. Recombinant adenoviral stocks expressing Cre recombinase were purchased from the Gene Transfer Vector Core facility of the University of Iowa College of Medicine (Iowa City, IA). Infections of GFP-positive cells were performed by using 100 PFU virus per cell. Four days after adenovirus infections, the GFP-negative cells were sorted from GFP expressing with aden-oCre as MH-S-Sico and MH-S-miR-127 cells. Cre recombination was discriminated by PCR amplification of genomic DNA with the following primers: pSico-Cre-UP: 5’-GGG ACA GCA GAG ATC CAG TT-3’ and pSico-Cre-DN: 5’-ACC GCA ACC CTT AAA CAT GA-3’. The uncombined viral DNA results in a 2149-bp band, and the recombination viral DNA results in a 495-bp band.

Animals, IgG IC-induced lung injury, bleomycin-induced lung injury, and bronchoalveolar lavage analysis

All experiments were carried out using 8–12-wk-old C57BL/6 female mice (The Jackson Laboratory). All studies were conducted in accordance with National Institutes of Health guidelines for the care and use of animals and with approval from the Duke University Animal Care and Use Committee. Rabbit anti-BSA IgG (catalog number 55275; MP Biomedicals; 10 µg in 20 µl PBS) was administered intranasally, and 5 mg/kg BSA (A2412; Sigma-Aldrich) in 200 µl PBS was injected i.v. immediately thereafter. Control mice received BSA i.v. in the absence of an intranasal dose of anti-BSA. To assess permeability, 0.25% Evans blue (catalog number A16774; Alfa Aesar) was administered with BSA i.v. Because neither CEMR67 nor PBS had any effect, either CEMR67 or PBS was used as control in certain experiments. When miR-127 and control probe CEMR67 were used, 100 µg in 40 µl PBS was instilled intranasally 2 h before anti-BSA IgG administration.

Animals were sacrificed 4 h after IgG IC-induced alveolitis according to reports (33). The trachea was catheterized, and lungs were lavaged with 0.8 ml ice-cold PBS three times. The first 0.8 ml bronchoalveolar lavage fluid (BALF) was collected for ELISA, and the total and differential cells in BALF were counted by a hemocytometer. Cell differentials were performed on cytospin preparations stained with the protocol Hema3 stain (Fisher Healthcare). Evans blue concentration in BALF was determined by an ELISA reader under 405 nm for lung permeability analysis. Total protein level was determined by bichinonic acid protein assay.

Under anesthesia, 2.5 µg/kg bleomycin (Blenoxane; Mayne Pharma, Paramus, NJ) dissolved in sterile PBS was administered via trachea as previously described (6, 34, 35). The trachea was catheterized, and lungs were lavaged with 0.8 ml ice-cold PBS three times. The first 0.8 ml bronchoalveolar lavage fluid (BALF) was collected for ELISA, and the total and differential cells in BALF were counted by a hemocytometer. Cell differentials were performed on cytospin preparations stained with the protocol Hema3 stain (Fisher Healthcare). Evans blue concentration in BALF was determined by an ELISA reader under 405 nm for lung permeability analysis. Total protein level was determined by bichinonic acid protein assay.

RNA analysis

RNA was isolated from cells with TRizol reagent (Invitrogen) or the mirVana miRNA Isolation Kit (Ambion). The expression of miR-127 was normalized to sn0202 and analyzed using TaqMan miRNA assays (Applied Biosystems).

Luciferase assay

Construct generation. The full-length FcγRI 3' UTR was amplified by PCR from mouse genomic DNA with the following primers: FcγRI Forward, 5'-GGT GAC TAG TGG ACC GCA GCA GCC-3' and FcγRI-Reverse, 5'-AAG TGA CAG GTC CTC TGG AAG-3'. The amplified 1404-bp product was inserted into the Spel/Sacl sites of the luciferase expression vector pmIR-REPORT (Applied Biosystems) immediately downstream of the luciferase gene and termed pmIR-REPORT [FcγRI-3' UTR]. A mutant construct was generated with two primers: FcγRI-3m-Forward, 5'-AGC TGG CCG CTA GGA CAA CAA GAG GGA AGC CGA GCA GCC-3' and FcγRI-3m-Reverse, 5'-GGC GGG ACC GTG TTA ATT TGG TTT ATT TAA GAG GTG CAT GCC-3' cloned into pmIR-REPORT, resulting in pmIR-REPORT [mFcγRI-3' UTR].
ciferase pRL-TK (Promega) in 24-well plates by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. For each well, 10 nM miR-127 precursor and miR-127 inhibitor (Ambion) were used. For luciferase assay, Firefly and Renilla luciferase activities were measured consecutively by using dual luciferase assays (Promega) 48 h after transfection according to the manufacturer’s suggestions.

Cytokine and complement production

TNF-α, IL-6, IL-1β, MIP-1β, MIP-2, MCP-1, KC, and complement 5 fragment (C5a) protein levels in supernatant collected from cell culture in BALF of injured mice were measured immunologically using commercial ELISA kits (R&D Systems). Complement C5a in BAL of injured mice were determined with ELISA with Abs from BD Pharmingen (capture Ab: purified rat anti-mouse C5a, catalog number 558028; standard: purified recombinant mouse C5a, catalog number 622597; detect Ab: biotin rat anti-mouse C5a, catalog number 558028).

Protein analysis

Protein extracts were isolated from cells or lung tissues. CD64, STAT3, and phospho-Stat3 (Tyr705) levels were determined using goat polyclonal anti-CD64 (R&D Systems), mouse monoclonal anti-Stat3 (Cell Signaling Technology), or rabbit monoclonal anti-phospho-Stat3 (Tyr705) (Cell Signaling Technology) and normalized to GAPDH (Cell Signaling Technology).

Microarray analysis

Microarray analysis was performed using the Affymetrix Mouse Genome 430 2.0 Array (Affymetrix). Gene chips were scanned by the GeneChip 4000B scanner (Molecular Devices, Sunnyvale, CA). Data and images were obtained using GenePix Pro 6.1 (Molecular Devices, Sunnyvale, CA) according to the manufacturer’s instructions. RNAs extracted from MH-S-Sico cells were hybridized on Cy3, and RNAs extracted from MH-S–miR-127 cells were hybridized on Cy5. Data were background subtracted and normalized within the array using the LOESS normalization by Genespring GX 11 software. The complete microarray data set is available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE27642.

Flow cytometry

RAW264.7 and MH-S cells were stained with PE-conjugated anti-mouse CD64 Ab (catalog 558455; BD Pharmingen), and flow cytometry was used to determine the CD64 staining-positive cells in MH-S-Sico and MH-S–miR-127 cells, to determine the CD64 staining-positive cells in miR-127 precursor, inhibitor, and control probe-transfected Raw 264.7 cells, and to select the top 1% of highest CD64-expressing cells as CD64hi and the bottom 1% of lowest CD64-expressing cells as CD64lo.

Synthetic 2′-OME–, cholesterol-modified, and Fam (5′)-labeled miR-127 oligonucleotide or control oligonucleotide without Fam labeling (100 μg in 40 μl PBS) was intranasally administered to C57BL/6J mice. BAL cells were isolated 2 h later and stained with CD64-PE (catalog number 558352; BD Biosciences) and F4/80-allophycocyanin (catalog number MCA497APC; AbD Serotec) Abs. Macrophages uptaking Fam-labeled miR-127 oligonucleotide and CD64 expression on BAL cells isolated from miR-127 and control oligonucleotide-treated mice were analyzed with flow cytometry.

Histology

To morphologically assess lung injury 4 h after IgG IC deposition, the lungs were inflated with 10% formalin via a tracheal cannula and removed from the thoracic cavity. Tissue was fixed overnight, embedded in paraffin, and sectioned for staining with H&E.

Statistics

Data are expressed as the mean ± SEM where applicable. We assessed differences in measured variables using the unpaired two-sided Student t test or Wilcoxon rank-sum test. Differences between multiple groups were calculated using one-way ANOVA with Tukey-Kramer posttest or two-way ANOVA with Bonferroni multiple comparisons. Statistical difference was accepted at p < 0.05. Prism 5.0 software (GraphPad) was used to perform statistical analysis.

Results

Mature miR-127 is regulated during inflammation

There are limited reports suggesting that miR-127 may play a role in development (36) and tumor progression (37). However, there is no report on miR-127 targets or on its role in inflammation. We recently analyzed miRNA expression after bleomycin-induced lung injury with an miRNA array study (29) and identified that miR-127 expression was regulated after injury. Bleomycin treatment led to downregulation of miR-127 at day 1 after bleomycin (Fig. 1A). We also found that miR-127 was downregulated after IgG IC-induced lung injury (Fig. 1B). We reasoned that miR-127 may play a role in regulating lung injury and inflammatory response. When we measured the expression of miR-127 in murine RAW264.7 macrophages and MH-S solven macrophages upon LPS stimulation, we found that overnight treatment with LPS showed a marginal downregulation of miR-127 expression in both MH-S (Supplemental Fig. 1A) and RAW264.7 macrophages (Supplemental Fig. 1B).

Overexpression of miR-127 decreased cytokine expression

We next determined the ability of miR-127 to modulate the inflammatory response in macrophages in vitro. To accomplish this, we transduced MH-S cells with a lentivirus encoding miR-127 (pSico–miR-127) or an empty lentivirus (pSico) as control (31). Pre–miR-127 gene was cloned into a pSico vector in which the CMV-GFP cassette is located downstream of the U6 promoter and is flanked by loxP sites such that Cre-mediated recombination is expected to result in the loss of GFP expression and activation of miRNA expression (Supplemental Fig. 2A). PCR confirmation of Cre-mediated recombination was performed to amplify the recombinant and unrecbined viral DNA (Supplemental Fig. 2B). High-efficiency transduction by the vector was achieved as indicated by uniform GFP expression in infected cells (Supplemental Fig. 2C). After superinfection with a Cre-expressing adenovirus, near complete recombination with concomitant loss of GFP fluorescence was observed (Supplemental Fig. 2C). One week after Cre expression, >2-fold higher level of miR-127 expression was detected in cells infected with pSico–miR-127 versus control cells (Supplemental Fig. 2D). This suggested we had successfully established the miR-127 overexpressed MH-S cell line.

To test the hypothesis that the gain of miR-127 function plays a role in the release of cytokines during inflammation, MH-S–miR-127 and control cells were treated with LPS. Overexpression of miR-127 in MH-S cells significantly decreased production of proinflammatory cytokines induced by LPS, such as TNF-α, IL-1β, and IL-6 (Fig. 2A–C). These data suggest that miR-127 functions as a negative regulator of inflammatory response. Given the ability of miR-127 to downregulate TLR4-induced inflammatory responses, we next investigated if miR-127 also modulates activation of PGN, hyaluronan fragments, and IgG IC-stimulated cells. As shown in Fig. 2, miR-127 significantly attenuated TNF-α and IL-1β production induced by PGN and hyaluronan fragments (Fig. 2A, 2B), as well as IL-6 production induced by PGN and IgG IC (Fig. 2C).

![FIGURE 1. Lung injury regulated miR-127 expression. (A) Total RNA was isolated from normal lungs and lungs 1 d after intratracheal bleomycin instillation (2.5 U/kg). miR-127 expression was determined with TaqMan microRNA assay. (B) miR-127 was decreased after IgG IC-induced lung injury. p < 0.05.](http://www.jimmunol.org/)

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miR-127 INHIBITS LUNG INFLAMMATION

FIGURE 2. Stable expression of miR-127 in MH-S cells attenuated inflammatory response. (A–C) MH-S–miR-127 and control cells were treated with LPS, HA, PGN, and IgG IC for 4 h. The levels of TNF-α (A), IL-1β (B), and IL-6 (C) in the supernatants were determined by ELISA. (D and E) miR-127 precursor attenuated IgG IC-induced inflammatory response in RAW264.7 cells and MH-S cells. RAW264.7 cells (D) and MH-S cells (E) were transiently transfected with 10 nM control probes, miR-127 precursor, and miR-127 inhibitor. At 48 h after transfection, the cells were cultured with or without IgG IC for 4 h. The levels of IL-6 in the supernatants were then determined. (F) Human monocyte U937 cells were activated with PMA at 100 nM for 24 h, and the cells were washed and then cultured in a regular medium for 24 h before transfection. The cells were transiently transfected with 10 nM control probes, miR-127 precursor, and miR-127 inhibitor. At 48 h after transfection, the cells were cultured without or with IgG IC for 4 h. The levels of IL-6 in the supernatants were then determined. miR-127 precursor attenuated IgG IC-induced TNF-α production of human U937 cells. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with control group.

To further address this question, RAW264.7 cells, MH-S cells, and PMA-treated human monocyte U937 cells were transiently transfected with a specific miR-127 precursor, miR-127 inhibitor, or control probes. miR-127 precursor markedly inhibited IgG IC-induced IL-6 by RAW264.7 cells (Fig. 2D), TNF-α by MH-S cells (Fig. 2E), and human TNF-α by U937 cells (Fig. 2F). However, miR-127 precursor, miR-127 inhibitor, or the control probes alone did not alter cytokine production by RAW264.7, MH-S cells, or U937 cells (Fig. 2D–F). These results demonstrated that miR-127 possesses the ability to suppress cytokine releases in various inflammation models in vitro.

CD64 is a target of miR-127

We next focused our study on miR-127 regulation in IgG IC-induced inflammation because putative mRNA targets predicted in silico (MicroCosm Targets Version 5 [http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/]) were members of pathways implicated in FcγR-mediated immune response in macrophages, including FcγRI, P13K category class IA, VAV-1, PIP5KI, and myosin II. Notably, CD64 was predicted to have a strong miR-127 binding site (Fig. 3A) and showed a minimum free energy of −26.24 kcal/M.

We then performed cDNA microarray analysis to determine if CD64 expression is different between MH-S–miR-127 and control MH-S–Sico cells. The microarray analysis of RNA isolated from MH-S–miR-127 and MH-S–Sico cells revealed a downregulation of FcγRI transcripts in MH-S–miR-127 cells compared with MH-S–Sico cells (Supplemental Fig. 3). These data display an inverse relationship between CD64 expression and miR-127 expression, indicating that CD64 is a putative target of miR-127.

Next, Western blot analysis of CD64 was performed in MH-S–miR-127 and control cells with and without LPS treatment. CD64 protein expression was induced by LPS in control cells, but protein expression of CD64 in MH-S–miR-127 cells was significantly inhibited in response to LPS stimulation (Fig. 3B). Because CD64 is an integral membrane glycoprotein, we performed FACS (9) to investigate if membrane CD64 is regulated by miR-127. As expected, flow cytometry analysis showed a decrease in CD64-positive staining on MH-S–miR-127 cells when compared with control cells (Fig. 3C). We also found decreased CD64-positive staining in miR-127 precursor-transfected RAW 264.7 cells compared with the transfection with miR-127 inhibitor or control probe (Fig. 3D). Together, these data demonstrate that CD64 is a potential target of miR-127 and is downregulated by miR-127 overexpression.

To further obtain direct evidence that CD64 is a target of miR-127 and determine whether miR-127 targets and represses CD64 directly through a 3′-UTR interaction, we constructed a luciferase reporter vector containing the full-length CD64 3′-UTR (pMIR-REPORT [FcγRI-3′-UTR]) and analyzed the effect of miR-127 on luciferase expression. As anticipated, cotransfection of miR-127 attenuated expression of luciferase activity from the pMIR-REPORT [FcγRI-3′-UTR] reporter (Fig. 3E), whereas the inhibition was not observed when either control miRNA or miR-127 inhibitor were used (Fig. 3F). Correspondingly, we also generated a mutant reporter (pMIR-REPORT [mFcγRI-3′-UTR]) in which the seed binding sequence in 3′-UTR of CD64 was mutated. The luciferase activity in pMIR-REPORT [mFcγRI-3′-UTR] and miR-127–cotransfected cells showed no differences when compared with pMIR-REPORT [mFcγRI-3′-UTR] and control miRNA or miR-127 inhibitor cotransfection (Fig. 3G). These suggest that miR-127 reduced the luciferase activity by binding to CD64–3′-UTR, but had no effect on CD64 mutant 3′-UTR. Therefore, we concluded that CD64 is a bona fide target of miR-127.

Expression of CD64 is positively correlated with IgG IC-induced inflammatory response

To test whether CD64 functionally regulates IgG IC-induced cytokine expression, RAW264.7 and MH-S cells were stained with anti-CD64 Ab, and flow cytometry analysis was used to sort out the top 1% of the highest CD64-expressing cells as CD64hi and the bottom 1% of the lowest CD64-expressing cells as CD64lo. Next, the highest and lowest CD64-expressed RAW264.7 and MH-S cells were treated with IgG IC, and cytokine expression in the conditioned media was measured. RAW264.7-CD64hi and CD64lo cells showed a decrease in TNF-α, IL-1β, and IL-6 release after IgG IC stimulation as compared with RAW264.7-CD64lo cells (Fig. 4A). Similarly, MH-S–CD64hi cells showed a decrease in proinflammatory cytokines such as IL-6, after IgG IC stimulation, as compared with MH-S–CD64lo cells (Fig. 4B). RAW264.7-CD64lo cells also showed a decrease in IL-1β release after LPS stimulation as compared with RAW264.7-CD64hi cells (Fig. 4C). These data demonstrate that expression of CD64 is closely associated with the inflammatory response of macrophages activated by IgG IC.

miR-127 attenuates IgG IC-induced lung injury in vivo

We have shown that the IgG IC-induced inflammation is inhibited by miR-127 in vitro. We next investigated whether miR-127...
mediates regulation of inflammatory response in complement-
mediated ALI in vivo. We administered miR-127 probes or con-
trol (CEMR67 or PBS) intranasally 2 h before onset of the IgG IC
and then analyzed the extent of lung inflammation 4 h after onset of
the IgG IC model. Neither CEMR67 nor PBS had any effect. Flow
cytometry revealed that more than half of macrophages had
taken up Fam-labeled miR-127 (Supplemental Fig. 4A). RT-PCR
revealed an accumulation of miR-127 in the lungs of the miR-127
 treatment group (Supplemental Fig. 4B). miR-127–treated mac-
rophages expressed less cell-surface CD64 when compared with
control oligonucleotide CEMR67 (or PBS)-treated macrophages
(Supplemental Fig. 4C). IgG IC induced a typical lung pathol-
gy (30, 33) characterized by lung hemorrhage, edema, fibrin
deposition, and accumulation of inflammatory cells. However,
treatment with miR-127 displayed a significant reduction in
pathological changes (Fig. 5A). We collected bronchoalveolar
lavage fluid (BALF) from mice of control, IgG IC model, and
miR-127 treatment groups. Examination of the inflammatory re-
sponse to IgG IC revealed a significant attenuation in total protein
level (Fig. 5B) and Evans blue concentration (Fig. 5C). Further-
more, miR-127 also markedly reduced inflammatory cell infiltra-
tion (Fig. 5D, 5E) as well as neutrophil influx (Fig. 5F). These
data suggest that miR-127 significantly attenuated IgG IC-induced
lung injury.

miR-127 inhibits IgG IC-induced lung inflammation in vivo

Next we examined the role of miR-127 in the effect on cytokine
release. Consistent with our in vitro observation, miR-127 sig-
nificantly reduced cytokine release into the alveolar space. All
major cytokines and chemokines (IL-6, TNF-α, MIP-1β, MIP-2,
MCP-1, and KC) were reduced by miR-127 in IgG IC-induced
lung injury (Fig. 6A).

Complement activation is an important component of IC-
mediated tissue injury (38). C5a has been demonstrated to be
required for the full development of injury and neutrophil accu-
mulation in an IgG IC model of lung injury (33, 39). Anti-C5a Ab
reduced lung vascular permeability and lung myeloperoxidase and
suppressed upregulation of ICAM-1 and TNF-α in the IgG IC
model of lung injury (33). Anti-C5a Ab attenuated ARDS and
some of the systemic manifestations of sepsis in nonhuman pri-

FIGURE 3. FcyR1 (CD64) is a target of miR-127. (A) Predicted binding site for miR-127 in 3′-UTR of CD64. miR-127 seed sequence was in blue. (B) Western blot analysis was performed to determine the levels of CD64 protein in MH-S-miR-127 and control cells without or with LPS (100 ng/ml) treatment for 4 h. (C) Flow cytometry was performed to determine the percent of CD64 staining positive cells in MH-S-miR-127 and control cells. (D) RAW264.7 cells were transfected with 10 nM control probes, LNA–miR-127, and LNA-anti–miR-127. Forty-eight hours after transfection, the cells were stained with CD64 Ab. The percent of CD64 staining positive cells were then determined. Luciferase activity of miR-127 precursor (E), miR-127 inhibitor (F), or control probe and pMIR-REPORT [FcyR1-3′-UTR] cotransfection. (G) Luciferase activity of miR-127 precursor, miR-127 inhibitor, or control probe and pMIR-REPORT [mFcyR1-3′-UTR] (containing a mutated 3′-UTR of FcyR1) cotransfection (n = 3 to 4). The experiments were repeated three times. *p < 0.05, **p < 0.01, ****p < 0.0001.

FIGURE 4. Expression of CD64 correlated with IgG IC-induced inflammatory response. (A) RAW264.7 cells were sorted for the highest or lowest 1% cells expressing CD64. CD64hi and CD64lo cells were treated without or with IgG IC for 4 h. The levels of IL-1β, IL-6, and TNF-α in the supernatants were then determined by ELISA. (B) MH-S cells were sorted for CD64 highest or lowest 1% expressing cells. The levels of IL-6 in IgG IC-treated supernatant of CD64hi and CD64lo MH-S cells were then determined. (C) CD64hi and CD64lo RAW264.7 cells were treated with or without LPS for 4 h. The levels of IL-1β in the supernatants were then determined by ELISA. Values are presented as mean ± SEM. n = 3–6. The experiments were repeated three times. ****p < 0.0001 compared with control group.
the targets of miR-127 have not been identified. immune response and inflammation has not been investigated, and a role in innate immunity (43). However, the role of miR-127 in Th2 responses in the lung (25). miR-146a has been shown to play inflammation in circulating leukocytes (42). miR-126 promotes Let-7g was reported to be downregulated in LPS-induced acute pulmonary fibrosis and let-7d targets HMGA2 in epithelial cells (41). The downregulation of let-7d was found in the patients with idiopathic pulmonary fibrosis and that miR-21 may regulate lung injury and fibrosis through targeting Smad7 (24). The downregulation of miR-21 was upregulated in the lungs of mice with bleomycin-induced injury and also in the lungs of patients with idiopathic pulmonary fibrosis (30). Therefore, we wanted to determine whether complement C5a was affected. IgG IC markedly induced C5a in mouse BAL. miR-127 treatment significantly reduced C5a protein levels in the BAL (Fig. 6B).

STAT3 is a transcription factor transducing signals in response to cytokines and growth factors. STAT3 is activated in the lung during IgG IC-induced ALI (30). Therefore, we wanted to determine if miR-127 treatment influences STAT3 activation. STAT3 and p-STAT3 protein levels were examined in total lung proteins. p-STAT3 protein levels were elevated in the IgG IC onset lungs, but diminished by miR-127 treatment (Fig. 6C). Thus, miR-127 appears to function in attenuating IgG IC-induced inflammatory response in vivo through the regulation of CD64.

Discussion
In this study, we found that miR-127 expression was regulated during inflammation both in vitro and in vivo. Functionally, miR-127 regulated cytokine release by macrophages in vitro and attenuated lung injury in an IgG IC-induced lung injury model in vivo. Mechanistically, miR-127 directly targeted the 3′-UTR of CD64, resulting in the downregulation of CD64.

Several studies have demonstrated that miRNAs regulate inflammation and the immune response. For example, a recent study showed that miR-21 was upregulated in the lungs of mice with bleomycin-induced injury and also in the lungs of patients with idiopathic pulmonary fibrosis and that miR-21 may regulate lung injury and fibrosis through targeting Smad7 (24). The downregulation of let-7d was found in the patients with idiopathic pulmonary fibrosis and let-7d targets HMGA2 in epithelial cells (41). Let-7g was reported to be downregulated in LPS-induced acute inflammation in circulating leukocytes (42). miR-126 promotes Th2 responses in the lung (25). miR-146a has been shown to play a role in innate immunity (43). However, the role of miR-127 in immune response and inflammation has not been investigated, and the targets of miR-127 have not been identified.

There are limited reports suggesting that miR-127 may play a role in development (36) and tumor progression (37, 44, 45). We recently analyzed miRNA expression after bleomycin-induced lung injury with a miRNA array study (29) and identified that miR-127 expression was decreased at 1 d after bleomycin treatment. In this study, we showed that miR-127 expression is marginally downregulated in IgG IC-induced lung injury in vivo and decreased in macrophages when stimulated with inflammatory stimuli in vitro, suggesting miR-127 may play a role during inflammatory response. We focused our study on miR-127 regulation in IgG IC-induced inflammation because putative miRNA targets predicted in silico were members of pathways implicated in FcγRI-mediated immune response in macrophages, including FcγRI, P13K cat class IA, VAV-1, PIP5KI, and myosin II.

We provide several lines of evidence suggesting that FcγRI is a genuine target of miR-127. First, using bioinformatics tools, targets of miR-127 were identified, and CD64 was selected for experimental validation, given its known role in the inflammatory response (8). cDNA array analysis identified that CD64 was downregulated in miR-127–overexpressing macrophages. We further showed that overexpression of miR-127 resulted in a decrease in mRNA, protein, and cell-surface expression of CD64. Lastly, reduced luciferase activity was observed in a reporter system containing the full length of 3′-UTR of CD64, demonstrating that miR-127 directly targets CD64. However, we did not detect any effect of miR-127 inhibitor on IgG IC-induced cytokine release, cell-surface CD64 expression, or CD64-luciferase activity in macrophages. It is possible that the endogenous miR-127 expression is low in lung tissue (36) as well as in macrophages, whereas CD64 expression is abundant on macrophages. Introduction of anti–miR-127 into macrophages may not be able to further reduce endogenous miR-127 levels, so that anti–miR-127 may not upregulate CD64 expression in the cells. On the contrary, introduction of miR-127 mimics into cells can readily increase

**FIGURE 5.** miR-127 attenuated IgG IC-induced ALI in mice. Mice received either PBS or miR-127 intranasally 2 h before onset of IgG IC model (10 μg/mouse anti-BSA in 40 μl PBS intranasal instillation and 5 mg/kg BSA together with 0.25% Evans blue in 200 μl PBS intravascular injection). At 4 h after IgG IC onset, mice were sacrificed, and BALF was collected. Lung tissues were fixed, embedded in paraffin, and sectioned for staining with H&E. (A) miR-127 decreases lung inflammation and alveolar hemorrhage on lung sections stained by H&E. Scale bar, 100 μm. (B) Total protein levels in BALF were performed by bicinchoninic acid assay. (C) Evans blue concentrations in BALF were measured. (D) Total cell counts in BALF were performed to determine inflammatory cell infiltration. (E) Representative cytospin preparations stained with Protocol Hema3 stain set. Original magnification ×400. (F) Differential cell counts were performed on cytospin preparations of BAL. Values are presented as mean ± SEM. n = 3–6 in each group. The experiments were repeated three times. *p < 0.05, **p < 0.01 compared with IgG IC group. LYM, lymphocytes; MAC, macrophages; PMN, neutrophils.
miR-127 levels in the cells and lead to significantly downregulated CD64 expression.

By establishing stable or transient miR-127 overexpression in macrophage cell lines, we observed decreased TNF-α, IL-1β, IL-6 release correlated with overexpression of miR-127 when stimulated with LPS, HA, PGN, or IgG IC. We also present a functional role for the miR-127–CD64 axis in the signaling pathways induced in response to IgG IC. IgG IC or LPS treatment of sorted highest and lowest CD64 protein levels in lung tissues, *p < 0.05, **p < 0.01, ***p < 0.001 compared with IgG IC group.

In summary, we demonstrate that an miRNA was involved in IgG IC-induced ALI model in vivo. Mice received either PBS or miR-127 probes intranasally 2 h before onset of IgG IC model. At 4 h after IgG IC onset, mice were sacrificed, and BALF or lung tissue was collected. (A) Protein levels of IL-6, TNF-α, IL-1β, MIP-1β, MIP-2, MIP-1, and KC in BALF were measured with ELISA. (B) Complement C5a protein in BALF was measured with ELISA. Values are presented as mean ± SEM. n = 3–6 in each group. The experiments were repeated three times. (C) Western blot was performed to determine STAT3 and p-STAT3 protein levels in lung tissues. *p < 0.05, **p < 0.01, ***p < 0.001 compared with IgG IC group.
Disclosures
The authors have no financial conflicts of interest.

References