LPS induces KH-type splicing regulatory protein-dependent processing of microRNA-155 precursors in macrophages

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ABSTRACT **The importance of post-transcriptional mechanisms for the regulation of the homoeostasis of the immune system and the response to challenge by microorganisms is becoming increasingly appreciated. We investigated the contribution of microRNAs (miRNAs) to macrophage activation induced by lipopolysaccharide (LPS). We first observed that Dicer knockout in bone marrow-derived macrophages (BMDMs) increases the LPS-induced expression of some inflammation mediators. miRNA microarray analysis in BMDMs revealed that LPS significantly induces the expression of a single miRNA, miR-155, and this induction depends on enhanced miR-155 maturation from its precursors. The single-strand RNA-binding protein KH-type splicing regulatory protein (KSRP) binds to the terminal loop of miR-155 precursors and promotes their maturation. Both inhibition of miR-155 and KSRP knockdown enhance the LPS-induced expression of select inflammation mediators, and the effect of KSRP knockdown is reverted by mature miR-155. Our studies unveil the existence of an LPS-dependent post-transcriptional regulation of miR-155 biogenesis. Once induced, miR-155 finely tunes the expression of select inflammation mediators in response to LPS.—Ruggiero, T., Trabucchi, M., De Santa, F., Zupo, S., Harfe, B. D., McManus, M. T., Rosenfeld, M. G., Briata, P., Gherzi, R. LPS induces KH-type splicing regulatory protein-dependent processing of microRNA-155 precursors in macrophages. *FASEB J.* 23, 2898–2908 (2009). www.fasebj.org**

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INNATE AND ADAPTIVE IMMUNE SYSTEMS act in concert in the elimination of invading pathogenic microorganisms in vertebrates. Innate immune and inflammatory responses to microbial infections are critically mediated by binding of pathogen-associated molecular patterns to evolutionary conserved Toll-like receptors (TLRs), which are expressed at high levels on macrophages and dendritic cells. The best characterized TLR is TLR4, which recognizes the gram-negative product lipopolysaccharide (LPS). In B cells and macrophages, signals originated by the LPS-triggered TLR4 activate the transcription factor NF-κB and the three classes of MAP kinases (ERK, p38, and JNK) (1–3). On activation, these pathways coordinate the up-regulation of several functionally distinct gene subsets through both transcriptional and post-transcriptional mechanisms (4, 5). It has been proposed that LPS transcriptionally activates inflammatory mediators with a two-wave mode (6). Proteins encoded by LPS-induced genes, such as cytokines and chemokines, are intended to initiate microbial clearance. However, during pathological situations, the same mediators and the pathways they activate can also play a role in such conditions as cancer and autoimmunity (7–9). Furthermore, an acute and excessive production of cytokines in response to LPS is regarded as the cause of septic shock (10). LPS is also a potent inducer of anti-inflammatory cytokines (*e.g.*, IL-10 and transforming growth factor β). It has been postulated that such anti-inflammatory cytokines secondarily regulate the overwhelming production of inflammatory cytokines (11). This supports the requirement of a delicate balance between proinflammatory and anti-inflammatory mediators in both development and resolution of the inflammatory response.

microRNAs (miRNAs) are a large family of short regulatory RNAs that post-transcriptionally control the expression of target mRNAs (12). It is generally accepted that

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miRNAs control gene expression by regulating mRNA translation or stability in the cytoplasm (12). Functional studies indicate that miRNAs participate in the regulation of almost every cellular process investigated and that changes in their expression are observed in—and might underlie—human pathologies, including cancer (12). Early studies have indicated that miRNAs have a role in the regulation of hematopoiesis, immune response, and inflammation (13). Consistent with their multiple essential roles, miRNA expression needs to be tightly regulated. Regulation of miRNA biogenesis can be achieved at either transcriptional or post-transcriptional levels. Long primary miRNAs (pri-miRNAs) are processed first to precursor miRNAs (pre-miRNAs) and then to mature miRNAs by the multiprotein Drosha and Dicer complexes, respectively (12). However, the regulatory mechanisms by which miRNA processing is controlled in specific cells and tissues remain largely unknown. We have recently demonstrated that the single-strand RNA-binding protein KH-type splicing regulatory protein (KSRP) is a required component of the molecular machines that process a cohort of miRNA precursors (unpublished results).

While the role of miRNAs in cell fate decisions was recognized early on, the importance of these small RNAs on immune system development and function has only recently become evident. Genetic approaches have recently shown that miR-155-knockout mice are immunodeficient due to a defect in B cells that fail to respond to antigen immunization (14, 15). Furthermore, miR-155-deficient T cells show a tendency to differentiate into Th2 cells under a variety of conditions (14). Recently, Baltimore's group (16, 17), screening an array of ~200 miRNAs, found that LPS up-regulated the expression of 3 miRNAs, including miR-132, miR-146, and miR-155, in human THP-1 monocytes. miR-155 overexpression in mouse hematopoietic stem cells resulted in myeloproliferative disorders in bone marrow (18). Similarly, Croce and co-workers (19) reported modulation of miR-155 and miR-125b by LPS/TNF-α in the RAW 264.7 macrophage cell line. However, the expression regulation, as well as the exact role of miR-155 in the course of the innate immune response in macrophages, has yet to be clarified.

Here, we report that LPS-induced miR-155 up-regulation in bone marrow-derived macrophages (BMDMs) and RAW 264.7 macrophages is dependent on its maturation from precursors. miR-155 inhibition causes a strong increase in the LPS-induced expression of several inflammation mediators. This recapitulates the results obtained in BMDMs from conditional Dicer-knockout mice. KSRP is a required factor in miR-155 maturation and controls, in a miR-155-dependent way, the expression of the same set of mediators.

MATERIALS AND METHODS

Reagents

LPS, interferon-γ (IFN-γ), and polyinosinic:polycytidylic acid [p(I:C)] were purchased from Sigma (St. Louis, MO, USA). TNF-α was purchased from Roche (Mannheim, Germany).

Recombinant proteins and antibodies

Production and purification of recombinant KSRP and GST-p37AUF1 have been described previously by Gherzi *et al.* (20). Affinity-purified rabbit polyclonal anti-KSRP antibody was previously described (20–22). Mouse monoclonal anti-α-tubulin was from Sigma. Affinity-purified rabbit polyclonal anti-nitric oxide synthase II (NOS2A; cat. no. AB16311), anti-IKKβ (05-535), and anti-IKKε (07-580) antibodies were from Millipore (Billerica, MA, USA). Affinity-purified rabbit polyclonal anti-tristetraprolin (TTP) antibody (H-120) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Dicer-null conditional allele mice

Dicer-null conditional allele mice were described in detail previously (23). The Dicer conditional allele (Dicerflox) was created by inserting loxP sites around an exon that encodes most of the second RNaseIII domain (23). An adenovirus-expressing CRE recombinase (Ad-CMV-CRE) was purchased from Vector Biolab (Eagleville, PA, USA) and used to infect BMDMs derived from either Dicer-null conditional allele mice or wild-type mice, according to the manufacturer's instructions. Cre-mediated recombination resulted in the removal of 90 aa from Dicer (23).

Primary BMDMs

Bone marrow cells were isolated from either wild-type or Dicer-null conditional allele mice. Bone marrow cells (10^6) were plated in 10-cm plates in 5 ml of BM medium (DMEM supplemented with 20% low-endotoxin fetal bovine serum, 30% L929-cell conditioned medium, 1% L-glutamine, 1% Pen/Strep, 0.5% Na pyruvate, and 0.1% β-mercaptoethanol), and fed with 2.5 ml of fresh medium every 2 d, according to a previously published method (24).

Cell transfection and immunoblotting

BMDMs and RAW 264.7 macrophages (purchased from American Type Culture Collection, Sesto San Giovanni, Italy) were electroporated using the Nucleofector II (Amaxa, Walkersville, MD, USA), according to manufacturer's instructions. Gel electrophoresis and immunoblotting were performed as described previously (25).

siRNA- and shRNA-mediated KSRP knock down miR-155 and anti-miR-155 expression

To knock down mouse KSRP, the 5′-GGACAGUUUCACGACAACG-3′ siRNA was used, while control luciferase siRNA (siControl) was 5′-CGUACGCGGAAUACUUC GAUU-3′ (synthesized by TIB MolBiol, Genoa, Italy). To stably knock down mouse KSRP, the oligonucleotide 5′-GGACAGTTTCACGACAACG-3′ was cloned into pSUPER-Puro (Oligoengine, Seattle, WA, USA). RAW 264.7 macrophages were transfected as described above. Transfectant pools were kept under selection in medium containing 3 μg/ml puromycin (Sigma). Negative control anti-miR neg and anti-miR-200 (used as negative controls), as well as anti-miR-155, were purchased from Ambion (Austin, TX, USA). Double-stranded murine mature miR-155 was purchased from Qiagen (Milan, Italy). The RNAs were transfected as described above.

miRNA profiling

MicroRNA microarray assay was provided by LC Sciences (Houston, TX, USA). BMDMs were treated with either LPS (100 ng/ml) or normal medium for 8 h, and total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA), enriched with RNA <40 nt using PureLink miRNA Isolation Kit (Invitrogen), and used to interrogate a human/mouse/rat miRNA array (LC Sciences) comprising a total of 1256 unique mature miRNAs (837 human, 599 mouse, and 350 rat, based on Sanger miRBase 11.0; Sanger Institute, Hinxton, UK). In a different set of experiments, RAW 264.7 macrophages were transfected with either siControl or siKSRP. RNA was extracted and utilized for MicroRNA microarray assay (LC Sciences), as detailed above. Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (locally weighted regression). The ratio of the two sets of detected signals (log 2 transformed, balanced), and P values of the t test were calculated; differentially detected signals were those with values of $P < 0.01$.

Ribonucleoprotein complex immunoprecipitation (RIP) and gel mobility shift assays

RIP assays were performed as described by Chen *et al.* (25), with some modifications. Briefly, cell lysates were immunoprecipitated with either protein A- or proteinA/proteinG-sepharose-coupled antibodies at 4°C overnight. Pellets were sequentially washed with the following buffers: buffer I (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1; and 150 mM NaCl), buffer II (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1; and 500 mM NaCl), and buffer III (0.25 M LiCl; 1% Nonidet P-40; 1% deoxycholate; 1 mM EDTA; and 10 mM Tris-HCl, pH 8.1). Total RNA was prepared using TRIzol (Invitrogen), retrotranscribed using random primers, and amplified by PCR. The primer sequences are detailed in Supplemental Table 1. Gel mobility shift assays were performed as described previously (ref. 21 and unpublished results).

Northern blot analysis

Total RNA (10 μg/lane) was resolved on 15% polyacrylamide-urea gels and electroblotted onto HyBond N+ membranes (GE Healthcare, Little Chalfont, UK). Membranes were hybridized overnight with radiolabeled antisense miRNAs in ExpressHyb solution (Clontech, Mountain View, CA, USA). After hybridization, membranes were washed 3 times with 2× SSC and 0.05% SDS, and twice with 0.1× SSC and 0.1% SDS, exposed overnight to imaging screens, and analyzed using a Storm 860 PhosphorImager (GE Healthcare). Signals were quantitated using Imagequant V1.2 (GE Healthcare). The same blot was hybridized (on stripping in boiling 0.1% SDS) with 3 distinct probes, including control U6 RNA.

Semiquantitative and quantitative RT-PCR

Total RNA was isolated using the miRNeasy mini kit (Qiagen), treated with DNAseI (Promega, Madison, WI, USA), and retrotranscribed using SuperScript III (Invitrogen), according to manufacturers' instructions. Semiquantitative RT-PCR was performed exactly as described by Gherzi *et al.* (20). For quantitative RT-PCR (qPCR), 100 ng of DNase I-treated total RNA prepared with either miRNeasy mini kit (Qiagen) or PureLink Isolation Kit (Invitrogen) was retrotranscribed, and PCR reactions were performed using either the IQ SYBR Green Mix Super (Bio-Rad, Hercules, CA, USA) or the Real Mastermix (5 Prime, Hamburg, Germany) and the Realplex II Mastercycler (Eppendorf, Milan,

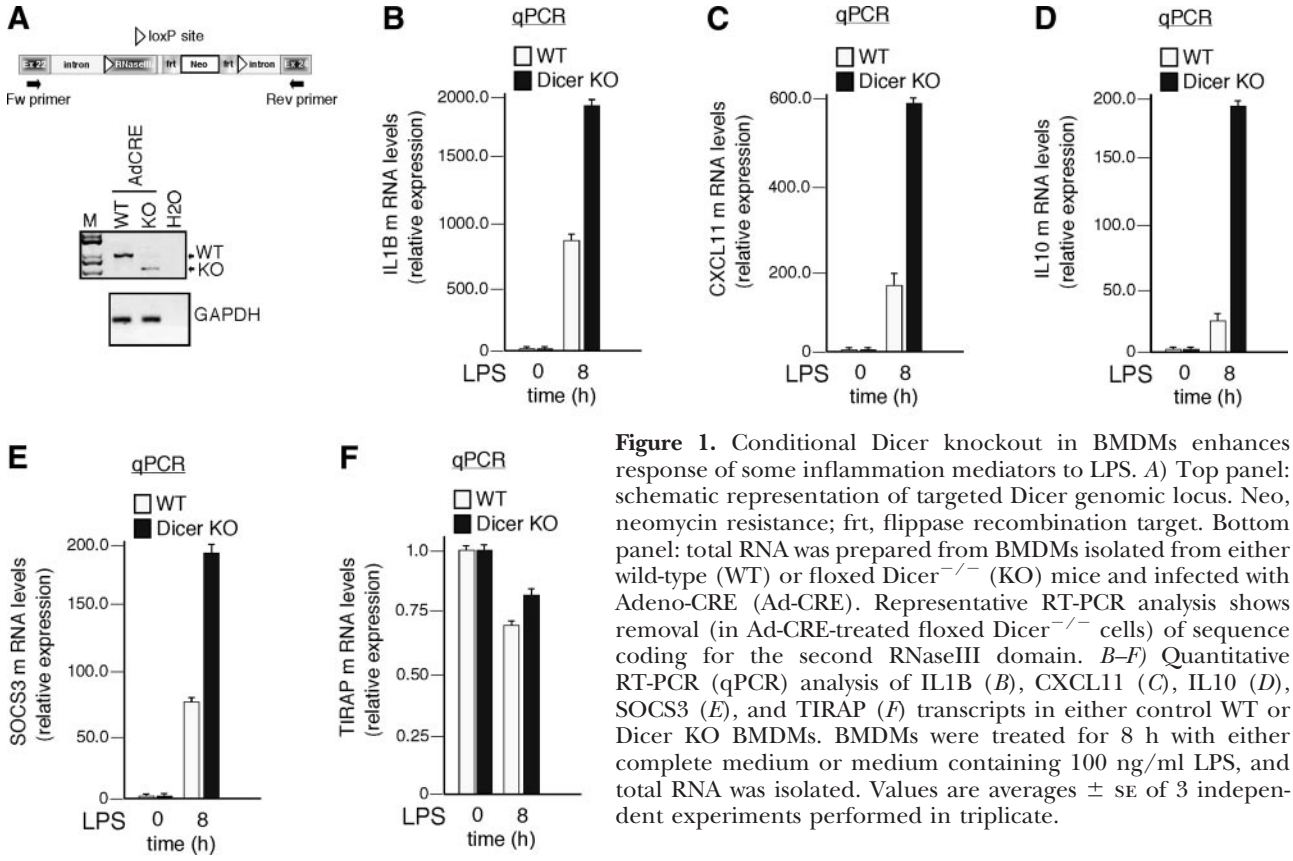

Figure 1. Conditional Dicer knockout in BMDMs enhances response of some inflammation mediators to LPS. *A*) Top panel: schematic representation of targeted Dicer genomic locus. Neo, neomycin resistance; frt, flippase recombination target. Bottom panel: total RNA was prepared from BMDMs isolated from either wild-type (WT) or floxed Dicer$^{-/-}$ (KO) mice and infected with Adeno-CRE (Ad-CRE). Representative RT-PCR analysis shows removal (in Ad-CRE-treated floxed Dicer$^{-/-}$ cells) of sequence coding for the second RNaseIII domain. *B–F)* Quantitative RT-PCR (qPCR) analysis of IL1B (*B*), CXCL11 (*C*), IL10 (*D*), SOCS3 (*E*), and TIRAP (*F*) transcripts in either control WT or Dicer KO BMDMs. BMDMs were treated for 8 h with either complete medium or medium containing 100 ng/ml LPS, and total RNA was isolated. Values are averages ± SE of 3 independent experiments performed in triplicate.

Italy). The sequence-specific primers used for PCR reactions are listed in Supplemental Table 1. Quantitative PCR analysis of mature miRNAs (qmiR-PCR) was performed using the NCode miRNA first-strand cDNA synthesis kit (Invitrogen), according to manufacturer's instructions, and the IQ SYBR Green Mix Super (Bio-Rad).

Pathway-specific qPCR array analysis

StellArray mouse Toll-like receptor signaling and mouse NF-κB signaling qPCR arrays were purchased from Lonza (Walkersville, MD, USA) and used according to the manufacturer's instructions. Data were analyzed using Global Pattern Recognition (GPR) analytical software (Lonza), which employs a global normalization algorithm in which the expression data from each gene are normalized against that of every other gene, thus eliminating the reliance on single-gene normalization (26). Analysis of the results was performed at Bar Harbor BioTechnology (Trenton, ME, USA).

RESULTS

LPS induces miR-155 maturation in mouse macrophages

It is well established that LPS activates macrophages to produce a wide range of inflammation mediators (Supplemental Fig. 1). To investigate the role of miRNAs in LPS-induced macrophage activation, we produced Dicer knockout in BMDMs derived from mice bearing floxed conditional Dicer alleles (23) by infection with a replication-deficient recombinant adenovirus vector producing Cre recombinase (Ad-CRE; **Fig. 1*A***). Dicer knockout increased the LPS-induced expression of some inflammation mediators (IL1B, CXCL11, and IL10) and of the JAK/STAT signaling negative regulator SOCS3, while it did not affect the mRNA levels of the signaling molecule TIRAP (Fig. 1*B–F*). These results suggested a role for miRNAs in LPS-induced macrophage activation.

To find miRNAs whose expression changes in BMDMs in response to LPS, extending and completing previous partial analyses (16, 19), we performed a miRNA microarray screening using a system based on miRBase 11.0, including a total number of 1256 mature miRNAs (599 mouse, 837 human, and 350 rat). Despite the increased complexity of the microarray platform that we used in comparison to those utilized in previous studies (16, 19), the number of LPS-regulated miRNAs was relatively small. Eight hours of LPS treatment up-regulated miR-155 and miR-132 while down-regulating miR-320 and miR-92b (**Figure 2*A***). Down-regulation of miR-320 and miR-92b was not confirmed by qmiR-PCR analysis, while the up-regulation of miR-132 was small and transient (Fig. 2*B* and data not shown). Instead, as shown by both Northern blot and qmiR-PCR analysis, LPS induced a strong and prolonged increase of miR-155 expression in BMDMs (Fig. 2*C, D*). Interestingly, mature miR-155 up-regulation was not accompanied by a parallel increase in pri-miR-155 levels,

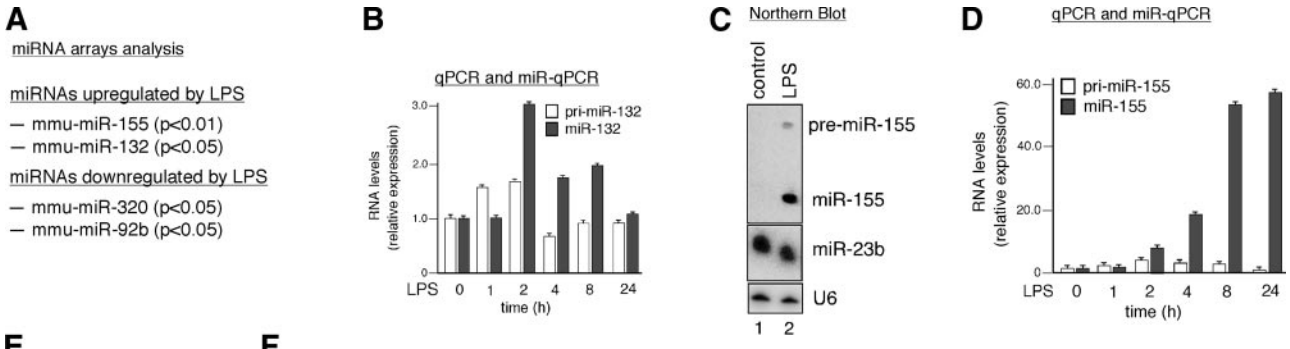

Figure 2. Expression of mature miR-155 is induced by LPS in macrophages without a concomitant increase of pri-miR-155. *A*) BMDMs were treated for 8 h with either complete medium or complete medium containing 100 ng/ml LPS. Total RNA was prepared, and miRNA microarray analysis (based on Sanger miRBase 11.0) was performed by LC Sciences (Houston, TX, USA). miRNAs whose expression was significantly ($P \leq 0.05$) changed by LPS are displayed. *B*) qPCR analysis and quantitative miR-PCR (qmiR-PCR) analysis performed on total RNA prepared from either untreated or LPS-treated (100 ng/ml, for indicated times) BMDMs. *C*) Northern blot analysis of total RNA purified from either untreated or LPS-treated (8 h) BMDMs. Representative blot sequentially hybridized with miR-155, miR-23b, and U6 probes is displayed. *D*) qPCR analysis and qmiR-PCR analysis performed on total RNA prepared from either untreated or LPS-treated (100 ng/ml, for indicated times) BMDMs. *E*) Northern blot analysis of total RNA purified from either untreated or LPS-treated (8 h) RAW 264.7 macrophages. Representative blot sequentially hybridized with miR-155, let-7, and U6 probes is displayed. *F*) qPCR and qmiR-PCR analysis performed on total RNA prepared from either untreated or LPS-treated (100 ng/ml, for the indicated times) RAW 264.7 macrophages. Values are averages ± SE of 3 independent experiments performed in triplicate.

suggesting that LPS mainly modulates miR-155 maturation (Fig. 2*D*). Similarly, in the RAW 264.7 murine macrophage cell line, LPS treatment induced maturation of miR-155 (Supplemental Fig. 2*A–E*), leaving unaffected the expression of both let-7a and miR-23b (negative controls) (Fig. 2*E, F* and Supplemental Fig. 2*F*). LPS did not significantly enhance the basal transcriptional levels of the BIC gene, which encodes pri-miR-155, as detected by the combination of chromatin immunoprecipitation and sequencing (ChIP-seq) (unpublished results).

Additional stimuli known to induce inflammatory response in macrophages were evaluated (Supplemental Fig. 3*A, B, D, E, G, H*). Either IFN-γ or, to a lesser extent, TNF-α treatment, similarly to LPS, induced maturation of miR-155 (Supplemental Fig. 3*C, F*, respectively), while the synthetic viral intermediate poly(I:C) (double-stranded RNA) caused a strong increase of pri-miR-155 expression that preceded the increase of mature miR-155 (Supplemental Fig. 3*I* and ref. 17).

Altogether, these results indicate that LPS treatment induces in macrophages the sustained expression of miR-155 due to increased maturation from its precursors.

RNA-binding protein KSRP interacts with the terminal loop (TL) of miR-155 precursors and favors its maturation

We have recently demonstrated that the single-strand RNA-binding protein KSRP interacts through its K-homology (KH) domains with the TL of a class of miRNA precursors and favors their maturation affecting both Drosha and Dicer enzymatic complexes (unpublished results). miRNA array analysis in RAW 264.7 macrophages depleted of KSRP revealed a reduction of miR-155 expression (Supplemental Table 2). Therefore, we performed RIP assays to evaluate the interaction of KSRP with pri-miR-155 in RAW 264.7 macrophages. We observed that KSRP binds to pri-miR-155 in a time-dependent way (**Fig. 3*A***), and specificity was established because an antibody directed to tristetraprolin (TTP), an RNA-binding protein expressed in RAW 264.7 cells and able to immunoprecipitate TNF-α mRNA, failed to immunoprecipitate pri-miR-155 (Fig. 3*A* and Supplemental Fig. 4*A*). Anti-KSRP antibody did not immunoprecipitate pri-miR-23b (Supplemental Fig. 4*B*). Furthermore, recombinant KSRP bound with high affinity to the TL of miR-155 precursors, while it did not interact with the TL of miR-23b in mobility shift assays (Fig. 3*B*). Recombinant RNA-binding protein p37AUF1 did not interact with the TL of miR-155 precursors (data not shown).

To investigate whether KSRP affects miR-155 maturation, we knocked KSRP down in both BMDMs and RAW 264.7 macrophages. Transient KSRP knockdown reduced the expression of mature miR-155 and up-regulated both pre- and pri- miR-155, as evaluated by a combination of Northern blot and RT-PCR (Fig. 3*C, D* and Supplemental Fig. 4*C–E*). A kinetic analysis demonstrated that KSRP stable knockdown in RAW 264.7 macrophages caused a time-dependent up-regulation

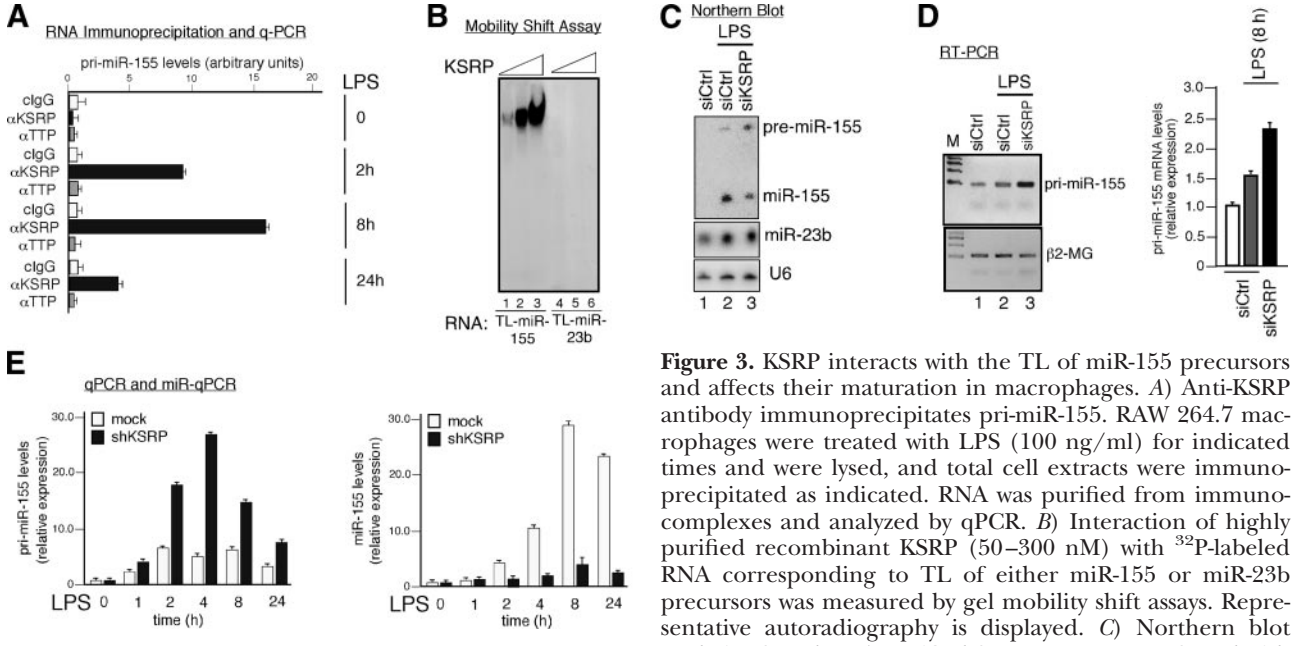

Figure 3. KSRP interacts with the TL of miR-155 precursors and affects their maturation in macrophages. *A*) Anti-KSRP antibody immunoprecipitates pri-miR-155. RAW 264.7 macrophages were treated with LPS (100 ng/ml) for indicated times and were lysed, and total cell extracts were immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by qPCR. *B*) Interaction of highly purified recombinant KSRP (50–300 nM) with ^{32}P-labeled RNA corresponding to TL of either miR-155 or miR-23b precursors was measured by gel mobility shift assays. Representative autoradiography is displayed. *C*) Northern blot analysis of total RNA purified from BMDMs transfected with either luciferase siRNA (siCtrl) or KSRP siRNA (siKSRP) and either untreated or treated with LPS (100 ng/ml, 8 h). Representative blot sequentially hybridized with miR-155, miR-23b, and U6 probes is displayed. *D*) Left panel: RT-PCR analysis of total RNA purified from BMDMs transfected with either luciferase siRNA (siCtrl) or KSRP siRNA (siKSRP) and either untreated or treated with LPS (100 ng/ml, 8 h). Right panel: intensity of gel bands from 3 independent experiments was quantitated. *E*) qPCR (left panel) and qmiR-PCR (right panel) analysis performed on total RNA prepared from either untreated or LPS-treated (100 ng/ml, for indicated times) RAW 264.7 macrophages. Values are averages ± SE of 3 independent experiments performed in triplicate.

of pri-miR-155, while mature miR-155 failed to accumulate (Fig. 3*E*).

Our results indicate that KSRP promotes miR-155 precursor maturation in LPS-stimulated macrophages.

miR-155 affects the expression of inflammation mediators in macrophages

The results that we obtained in Dicer knockout (Fig. 1) prompted us to investigate the role of miR-155 in LPS-induced inflammatory response in macrophages. First, we interrogated TLR and NF-κB signaling pathway-specific microarrays using mRNA prepared from RAW 264.7 cells transfected with either negative control anti-miR or anti-miR-155. As presented in **Table 1**, cells transfected with anti-miR-155 displayed an enhanced LPS-dependent overexpression of several inflammation mediators. These results were validated by qPCR analysis in RAW 264.7 cells treated for different times with LPS. As shown in **Fig. 4**, anti-miR-155 transfection strongly enhanced the expression of IL1B, CCL5, CXCL11, CXCL10, IL10, SOCS3, and NOS2A (NOS2A was not present in the arrays). Neither anti-miR-200 (a miRNA that is not expressed in macrophages) nor anti-miR neg from Ambion (both used as negative controls) affected the LPS-induced expression of inflammation mediators (data not shown), and we decided to use anti-miR-200 as negative control in all of the experiments. Notably, the same inflammation mediators (and, in addition, IL12B) were consistently up-regulated on anti-miR-155 transfection in BMDMs (**Fig. 5** and data not shown). None of these mRNAs

TABLE 1. *Regulation of inflammation-related genes in RAW 264.7 macrophages transfected with either anti-miR (control) or anti-miR-155 and untreated or treated with LPS (100 ng/ml for 8 h)*

Gene symbol	Fold change (control *vs.* LPS) (anti-miR control transfection)	*P* value	Fold change (control *vs.* LPS) (anti-miR-155 transfection)	*P* value
CXCL11[a]	94.5	0.007	777.2	1.3×10^{-5}
CCL5[a]	61.1	0.0005	141	1.8×10^{-5}
IL1B[a]	144	0.005	328.1	2.6×10^{-5}
TNFAIP	7.6	0.0002	9.6	0.0001
FOS	−3.4	0.0007	−3.5	0.0005
CXCL10[a]	9	0.006	13.5	0.0008
CCL3	18	0.001	29	7.6×10^{-5}
TREM2	−6.8	0.005	−5.5	0.0006
SOCS3[a]	3.5	0.001	6.8	0.001
IRF7	2.5	0.005	7	0.0007
TNF	3.9	0.001	5.2	0.001
NOX4	7.5	0.005	−3.6	0.001
CCL4	32	0.005	28.3	0.001
IL10[a]	5.2	0.005	10.8	0.005
CASP1	2.5	0.005	2.9	0.005
BCL3	7.6	0.005	2.7	0.005
MYD88	1.3	0.005	2.1	0.005
AKT1	−2.4	0.005	−2.3	0.005
TIRAP	−1.6	0.005	−21	0.005
HSP90B1	−3.1	0.005	−1.8	0.005
RELA	−2.1	0.005	−1.9	0.005
CARD9	−3.1	0.005	−2.1	0.005
IKKE[a]	−3.2	0.005	−1.1	0.005
TLR4	−7	0.005	−2.4	0.005
CYLD	−4.8	0.005	−2.4	0.005
TLR3	−5	0.005	−2.4	0.005
CSK	−2.9	0.005	−1.3	0.005
RAC1	−8.1	0.005	−2.4	0.005
TRAF3	3.1	0.005	−1.2	0.005
NOD2	4.6	0.005	4.2	0.005
STAT1	3.8	0.005	5	0.005
JUN	−3.3	0.005	−1.2	0.005
NFKB1	2.4	0.005	2.4	0.005
NFKB2	2.3	0.005	2.4	0.005
RIPK2	2	0.005	2	0.005
NFKBIA	3	0.005	3	0.005
IRAK1BP1	−4.9	0.005	−3.4	0.005
MAP3K7	−2.6	0.005	−2.8	0.005

All indicated fold changes (only changes >2-fold are presented) were derived from StellArray (Lonza, Walkersville, MD, USA) microarray experiments. Fold changes are a mean of gene expression values derived from 4 independent microarray experiments, each performed in triplicate. Data analysis was performed using the Microarray Global Pattern Recognition Analysis Tool (Lonza). Only genes that yielded values of $P \leq 0.005$ are listed. [a]Gene whose expression was analyzed in detail.

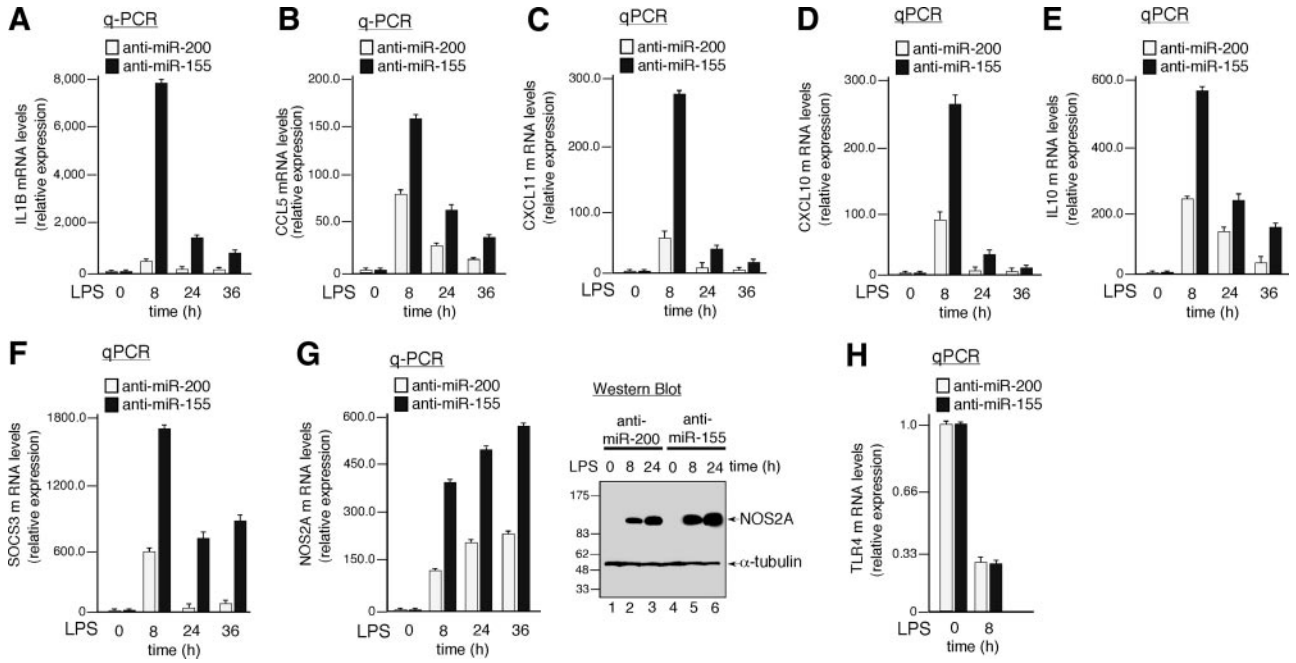

Figure 4. Anti-miR-155 enhances the LPS-induced expression of inflammation mediators in RAW 264.7 macrophages. *A–F*) qPCR analysis of IL1B (*A*), CCL5 (*B*), CXCL11 (*C*), CXCL10 (*D*), IL10 (*E*), and SOCS3 (*F*) transcripts in RAW 264.7 macrophages transfected with either anti-miR-200 (control) or anti-miR-155. Cells were treated with 100 ng/ml LPS for indicated times, and total RNA was isolated and analyzed. *G*) qPCR analysis (left) and immunoblot analysis (right) of NOS2A expression in RAW 264.7 macrophages transfected with either anti-miR-200 (control) or anti-miR-155 and treated with 100 ng/ml LPS for indicated times. *H*) qPCR analysis of a transcript (TLR4) whose expression was not affected by anti-miR-155 in our microarray experiments (see Table 1). Values are averages ± SE of 3 independent experiments performed in triplicate.

displayed canonical consensus sequences for miR-155 binding on a bioinformatic basis (miRANDA, TargetScan, and PicTar analysis). The only transcript identified in our microarray screening that contains putative miR-155 recognition sites in its 3′ untranslated region (UTR) encodes the noncanonical IκB kinase IKK-ε (IKBKE). IKBKE has been previously reported as a direct target of miR-155 in HEK-293 and lymphoblastoid B cells (19, 27), and we confirmed this finding in both BMDMs and RAW 264.7 macrophages (**Fig. 6*A***). Accordingly, the expression of IKBKE was also increased in BMDMs on Dicer knockout (Fig. 6*B*). Furthermore, LPS treatment of BMDMs and RAW 264.7 down-regulated IKK-ε mRNA and protein levels at time points that correspond to the peak of miR-155 expression (8 and 24 h; Fig. 6*C, D*) and anti-miR-155 prevented this effect (Fig. 6*D*).

KSRP regulates, in a miR-155-dependent way, the expression of inflammation mediators

The evidence that KSRP promotes miR-155 precursor maturation prompted us to investigate whether KSRP knockdown affects the expression of miR-155-regulated transcripts, including IKBKE and the inflammation mediators. KSRP knockdown strongly increased the LPS-induced expression of NOS2A, IL1B, IL12B, CXCL10,

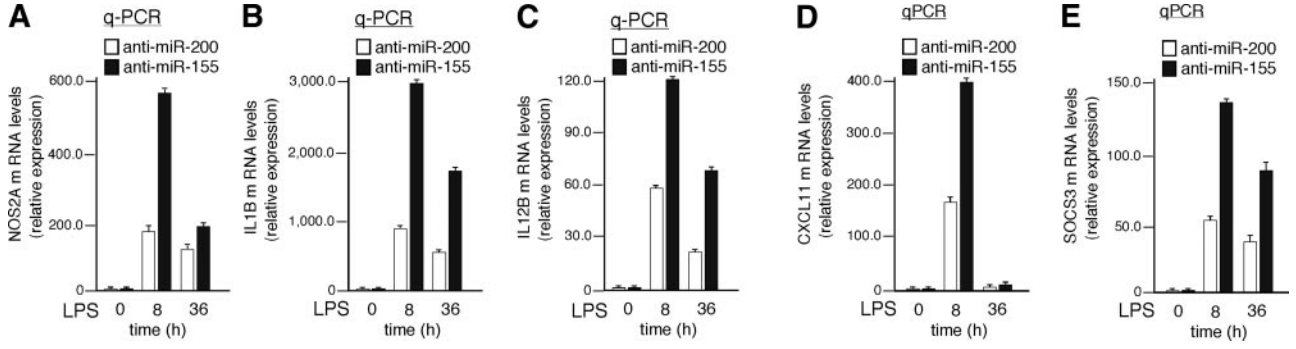

Figure 5. Anti-miR-155 enhances LPS-induced expression of inflammation mediators in BMDMs. *A–E*) qPCR analysis of NOS2A (*A*), IL1B (*B*), IL12B (*C*), CXCL11 (*D*), and SOCS3 (*E*) transcripts in BMDMs transfected with either anti-miR-200 (control) or anti-miR-155. Cells were treated with 100 ng/ml LPS for indicated times; total RNA was isolated and analyzed. Values are averages ± SE of 3 independent experiments performed in triplicate.

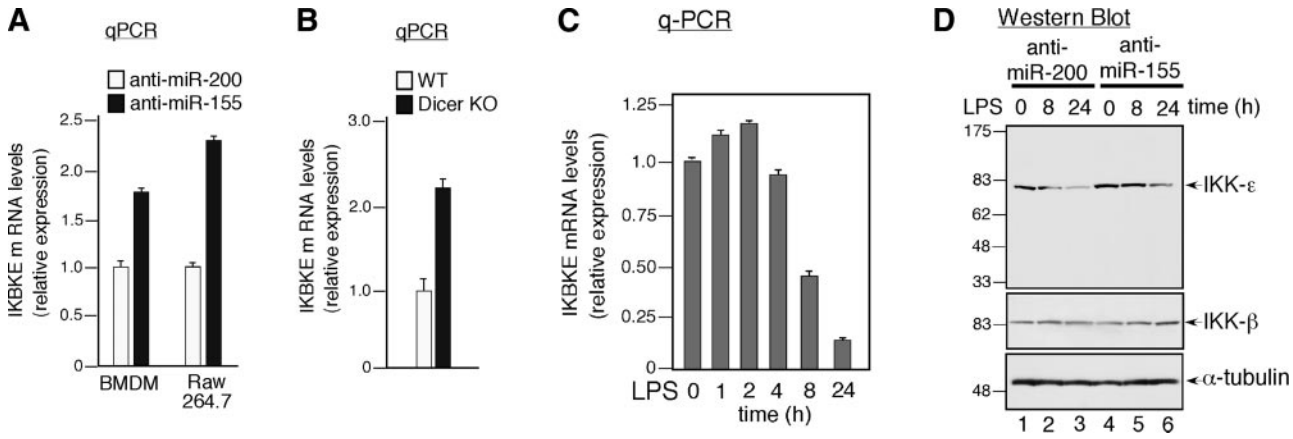

Figure 6. miR-155 is responsible for LPS-induced down-regulation of IKK-ε expression in macrophages. *A*) Transfection of anti-miR-155 in either BMDMs or RAW 264.7 macrophages enhances expression of IKBKE gene encoding IKK-ε kinase. *B*) IKBKE expression is increased in Dicer KO BMDMs. *C*) LPS treatment (100 ng/ml for indicated times) down-regulates IKBKE expression in BMDMs. Total RNA was prepared and analyzed by qPCR (*A–C*). Values are averages ± SE of 3 independent experiments performed in triplicate. *D*) Representative immunoblot analysis of IKK-ε (top panel), IKK-β (middle panel), and α-tubulin (bottom panel) expression in RAW 264.7 macrophages transfected with either anti-miR-200 (control) or anti-miR-155 and treated with 100 ng/ml LPS for indicated times.

CXCL11, IL10, SOCS3, and CCL5 in both BMDMs and RAW264.7 macrophages (**Fig. 7*A–E***, Supplemental Fig. 5*A*, B, and data not shown). As expected, IKBKE expression was increased on KSRP knockdown (Fig. 7*F*). Most important, the effect of KSRP knockdown was abrogated by simultaneous transfection of mature miR-155, while transfection of mature miR-23b was ineffective (Fig. 7*G, H*, Supplemental Fig. 5*C–F*, and data not shown).

Altogether, these results indicate that KSRP, regulating miR-155 expression, participates in the control of its function in LPS-stimulated macrophages.

DISCUSSION

In this report, we show that LPS-induced expression of miR-155 in mouse macrophages depends on its maturation from precursors. KSRP, a multifunctional single-strand RNA-binding protein, is a component of the process that governs miR-155 maturation. miR-155 regulates, likely indirectly, the extent of the response to LPS of several inflammation mediators, and, likely as a consequence of its function on miR-155 biogenesis, KSRP participates in the expression control of the same factors.

We have recently demonstrated that KSRP modulates the expression of a select group of miRNAs (unpublished results). Interacting with the TL of miRNA precursors and being an integral component of both Drosha- and Dicer-containing multiprotein complexes, KSRP greatly enhances the processing of a class of miRNA precursors (unpublished results). Here, we show that KSRP depletion in macrophages severely impairs the induction of miR-155 expression by LPS, while it induces a concomitant accumulation of pri-miR-155. It has become recently evident that individual miRNAs are subjected to post-transcriptional regulation both at the level of Drosha and/or Dicer processing (28–30). However, how physiological and pathological conditions control these events remains largely obscure. LPS-activated macrophages represent an informative model to study how activation of signaling pathways, targeting KSRP and possibly other proteins, affect the maturation and, in turn, the expression of miR-155.

Besides its involvement in the maturation of a set of miRNA precursors (unpublished results and the present report), KSRP is a decay-promoting factor for a variety of AU-rich element (ARE)-containing labile mRNAs (20–22, 31, 32). We observed that most of the mRNAs encoding the inflammation mediators whose expression is regulated by miR-155 contain AREs in their 3′ UTR (data not shown), and one of them, NOS2A, has been reported to interact with and to be regulated by KSRP (31). Indeed, we observed, by RIP analysis, a specific binding of KSRP to IL1B, CXCL10, CXCL11, IL10, SOCS3, and NOS2A mRNAs (Supplemental Fig. 6*A*). However, KSRP knockdown did not affect the decay rate of these transcripts on 24 h LPS stimulation (Supplemental Fig. 6*B* and data not shown). Together with the evidence that mature miR-155 is able to revert the effects of KSRP knockdown on the expression of inflammation mediators, these data allow us to propose the hypothesis that KSRP's effect on the expression of miR-155-regulated inflammation mediators is dependent on its ability to promote miR-155 maturation. Obviously, LPS-induced modulation of the decay rates of some labile mRNAs, including those coding for inflammation mediators, plays an important role in their accumulation, especially at early time points (see below).

Our data underlie the complexity of the regulation of miR-155 expression. LPS enhances miR-155 levels in macrophages, increasing precursor maturation, while it

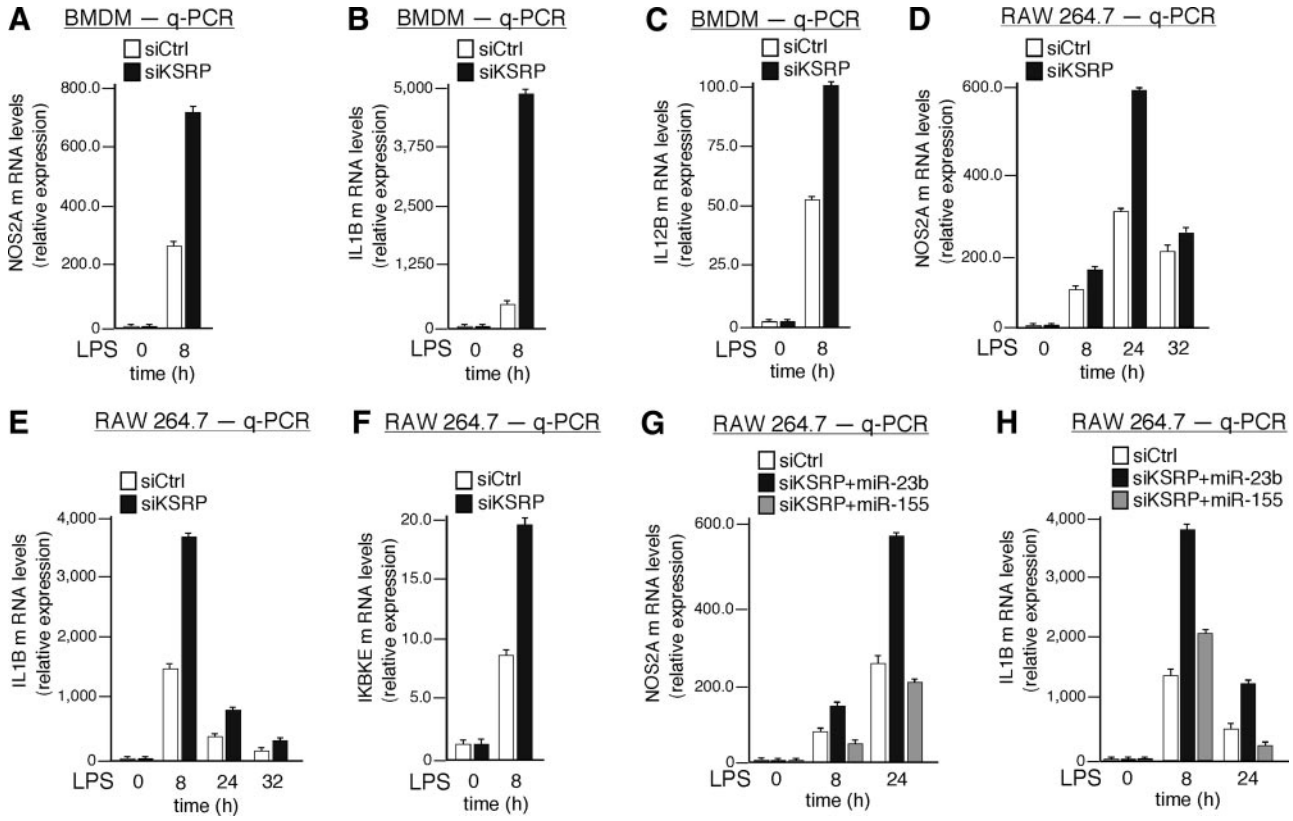

Figure 7. KSRP knockdown enhances, in a miR-155-dependent way, the LPS-induced expression of inflammation mediators and IKBKE in macrophages. *A–C*) qPCR analysis of NOS2A (*A*), IL1B (*B*), and IL12B (*C*) transcripts in BMDMs transfected with either luciferase (siCtrl) or KSRP siRNA (siKSRP). Cells were treated with 100 ng/ml LPS for 8 h, and total RNA was isolated and analyzed. *D–F*) qPCR analysis of NOS2A (*D*), IL1B (*E*), and IKBKE (*F*) transcripts in RAW 264.7 macrophages transfected with either luciferase (siCtrl) or KSRP siRNA (siKSRP). Cells were treated with 100 ng/ml LPS for indicated times; total RNA was isolated and analyzed. *G, H*) qPCR analysis of NOS2A (*G*) and IL1B (*H*) transcripts in either siCtrl-transfected or siKSRP-transfected RAW 264.7 macrophages cotransfected with either mature miR-23b or mature miR-155. Cells were treated with 100 ng/ml LPS for indicated times; total RNA was isolated and analyzed. Values are averages ± SE of 3 independent experiments performed in triplicate.

induces transcriptional activation of the pri-miR-155 gene in B-lymphocytes purified from human germinal center without affecting its maturation (present report and unpublished results). Furthermore, p(I:C) induces the expression of miR-155, enhancing pri-miR-155 transcription in RAW 264.7 macrophages (Supplemental Fig. 3). Altogether, these results indicate that both signal- and cell-specific mechanisms that control miR-155 expression appear to exist. Interestingly, enhanced miR-155 precursor maturation has been suggested as a potential cause of miR-155 overexpression detected in several types of B cell lymphomas (33), while a blockade of miR-155 maturation has been described in Burkitt lymphoma cell lines (34), thus allowing us to hypothesize that deregulated processing of a miRNA with paramount importance in cancer initiation and progression, such as miR-155, may contribute to the pathogenesis of diseases (18, 33, 34).

Exposure of macrophages in culture to inflammatory stimuli induces, with different kinetics, both proinflammatory and anti-inflammatory mediators (6, 35–37). Some genes are activated early and transiently, while others are induced at later time points, up to 24 h poststimulation (6, 35–37). In agreement with the kinetics of its maturation from precursors, we found that miR-155 controls the expression of "late" transcripts (that peak at or after 4 h of LPS) but does not affect the mRNA levels of TNF, CXCL2, SOD2, and other "early" and transiently expressed genes (Figs. 4 and 5, Supplemental Fig. 6*C, D*, and data not shown) (6, 37).

LPS induces a robust transcriptional induction of a large number of genes that is accompanied by the increase of the half-life of a more restricted number of transcripts generally containing ARE-destabilizing sequences in their 3′ UTR (5, 32, 37, 38). Our results point to an additional regulatory level, mediated by miR-155, by which LPS controls the expression of inflammation mediators. We failed to identify miR-155 target sequences by bioinformatic analysis (using miRANDA, TargetScan, and PicTar software) of the sequence of the vast majority of transcripts regulated by miR-155. This observation favors the hypothesis that miR-155 affects the expression of some regulatory molecules that, secondarily, regulate the expression of cytokines and chemokines. Among the transcripts we identified in our microarray analysis, the only one containing putative miR-155 recognition sites is IKBKE,

which encodes the noncanonical IκB kinase IKK-ε. IKBKE has been previously identified as a miR-155 target in different cells (14, 19, 27). On activation in response to Toll-like receptor agonists and viral infections, IKK-ε, through phosphorylation of IRF-3 and IRF-7, up-regulates type I interferon expression (39). IKK-ε is also known to activate the NF-κB signaling pathway by inducing phosphorylation and nuclear accumulation of cRel, an NF-κB component (40). Further studies will be required to understand whether the effects of miR-155 on inflammation mediators are totally dependent on IKK-ε regulation or additional regulatory factors are involved.

Although inflammation protects against pathogenic stimuli and promotes healing, it may cause more damage than the inciting event if its magnitude and duration are not strictly controlled by intrinsic negative regulators. Unbalanced and unrestrained inflammatory responses underlie diverse forms of chronic inflammatory diseases, regardless of the pathogenic mechanisms involved. Our data suggest that miR-155 controls the expression of both proinflammatory mediators (IL1B, IL12, CCL5, CXCL10, CXCL11) and molecules with anti-inflammatory function (IL10, SOCS3), thus allowing us to propose that miR-155, which modulates the expression of transcripts with divergent functions, can coordinate pro- and anti-inflammatory responses in macrophages.

While this manuscript was in preparation, Pierre and co-workers (41) showed that miR-155 modulates the interleukin-1 signaling pathway in LPS-treated human dendritic cells. These data strengthen our hypothesis that miR-155 is critical for the fine-tuning of the inflammatory response. FJ

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