Mammalian hematopoiesis involves the generation of blood cells from a common hematopoietic stem cell (HSC) through many intermediate stages, each of which can give rise to various types of malignancies upon their dysregulation. However, the molecular mechanisms that govern this process are incompletely understood. In particular, the quantitative decisions regarding how many cells take which pathway of maturation remain obscure. Most studies of this process have focused on cytokines and transcription factors, which can control cellular proliferation and differentiation decisions (1). MicroRNAs (miRNAs) are a novel class of small, regulatory RNA molecules that play evolutionarily conserved roles in cellular development and function, and mediate target gene repression through 3′ untranslated region (UTR) interactions (2–4). Recently, a growing body of evidence has implicated specific miRNAs in the modulation of mammalian hematopoiesis during both physiological and pathological conditions (5, 6).

Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder

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Mammalian microRNAs are emerging as key regulators of the development and function of the immune system. Here, we report a strong but transient induction of miR-155 in mouse bone marrow after injection of bacterial lipopolysaccharide (LPS) correlated with granulocyte/monocyte (GM) expansion. Demonstrating the sufficiency of miR-155 to drive GM expansion, enforced expression in mouse bone marrow cells caused GM proliferation in a manner reminiscent of LPS treatment. However, the miR-155–induced GM populations displayed pathological features characteristic of myeloid neoplasia. Of possible relevance to human disease, miR-155 was found to be overexpressed in the bone marrow of patients with certain subtypes of acute myeloid leukemia (AML). Furthermore, miR-155 repressed a subset of genes implicated in hematopoietic development and disease. These data implicate miR-155 as a contributor to physiological GM expansion during inflammation and to certain pathological features associated with AML, emphasizing the importance of proper miR-155 regulation in developing myeloid cells during times of inflammatory stress.

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Abbreviations used: AML, acute myeloid leukemia; FSC, forward scatter; GM, granulocyte/monocyte; HSC, hematopoietic stem cell; miRNA, microRNA; SSC, side scatter; UTR, untranslated region.
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B cell (B220+), and erythroid precursor (Ter-119+) populations by 24 h after LPS treatment (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072108/DC1), substantial expansion of GM cells and reductions in B cells and erythroid precursors were evident by 72 h (Fig. 1 C), consistent with a previous study (17). Histological analyses also showed myeloid preponderance and hyperplasia, with relative...
erythroid hypoplasia, after 72 h of LPS treatment (Fig. 1 D). Collectively, these data indicate that LPS-induced miR-155 expression in the bone marrow precedes GM cell expansion.

**Enforced expression of miR-155 in HSCs causes a myeloproliferative disorder in the bone marrow**

We next investigated whether miR-155 is sufficient to mediate GM expansion in the mouse bone marrow in vivo. Retroviral-mediated gene transfer was used to force expression of GFP and miR-155 in HSCs (Fig. 2 A), followed by engraftment of these cells into lethally irradiated C57BL6 mouse recipients. By 2 mo after reconstitution, mice were killed and coexpression of miR-155 and GFP was detected in various hematopoietic tissues, including the bone marrow (Fig. 2 B), thymus, spleen, and lymph nodes (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072108/DC1). Control mice only expressed GFP but not miR-155.

Gross analyses of femurs and tibias from mice expressing miR-155 revealed a white-tan bone marrow coloration unlike the vibrant red seen in controls (Fig. 2 C). Upon microscopic inspection of hematoxylin and eosin–stained bone marrow sections and Wright-stained bone marrow smears (Fig. 2 D), miR-155–expressing bone marrow was dominated by GM cells at a variety of either normal or abnormal developmental stages based upon their morphology. Indeed, many of the cells that appeared to be granulocytic precursors showed irregular segmentation of their nuclei and lacked condensation of nuclear chromatin (Fig. 2 E). Conversely, miR-155 expression also led to a reduction in erythrocytes, megakaryocytes, and lymphocytes in the bone marrow (Fig. 2 D).

Flow cytometry identified approximately twice as many Mac1⁺ GR1⁺ GM cells, very few Ter-119⁺ erythroid precursors, and a reduction in B220⁺ B cells in the bone marrow of mice expressing miR-155 versus the control vector (Fig. 2 F). When gated on GFP⁺ cells (expressing miR-155), there was a dramatic increase in large granular cells, as defined by having high forward scatter (FSC) and side scatter (SSC), respectively (Fig. 2 G). Back-gating confirmed that these cells were Mac1⁺, with a majority also positive for Gr1. Furthermore, the cells responsible for the overall GM, B, and erythroid precursor differences were largely GFP⁺ (Fig. 2 H). These observations reveal profound myeloid proliferation with dysplastic changes in the bone marrow of mice expressing miR-155 compared with controls.

**MiR-155 expression in HSCs causes splenomegaly and extramedullary hematopoiesis**

Splenomegaly was observed in miR-155–expressing compared with control mice (Fig. 3 A). Hematoxylin and eosin staining of splenic sections from miR-155–expressing mice revealed expanded interfollicular regions containing various hematopoietic elements, as well as constricted and disrupted B cell follicles compared with control spleens (Fig. 3 B). Upon analyses of Wright-stained splenic touch preparations, we observed a large number of erythroid precursors, megakaryocytes, and some developing GM cells in the spleens of
miR-155–expressing mice, whereas very few of these cell types were observed in control spleens (Fig. 3 B).

FACS analyses corroborated these observations. We saw elevated numbers of Mac1+ Gr1+ myeloid cells and Ter-119+ erythroid cells, with little change in CD4+ T cells and B220+ B cells in miR-155–expressing compared with control spleens (Fig. 3 C). When gated on GFP+ cells (expressing miR-155), there were elevated numbers of large granular cells, as defined by having high FSC and SSC, respectively, with a majority co-expressing Mac1 and Gr1 (Fig. 3 D). Furthermore, miR-155–expressing splenocytes contained overall higher numbers of Mac1+ cells that expressed GFP compared with controls (Fig. 3 E). Conversely, the Ter-119+ cell population from miR-155–expressing spleens was largely negative for GFP, possibly arising from nontransduced HSCs. These results clearly demonstrate the presence of splenic extramedullary hematopoiesis in miR-155–expressing mice, likely compensating for the bone marrow defects.

Expression of miR-155 in HSCs perturbs peripheral blood cell populations

Consistent with the disrupted hematopoiesis observed in miR-155–expressing mice, their peripheral blood demonstrated several distinct abnormalities compared with controls. By 2 mo after reconstitution, FACS detected significantly elevated numbers of Mac1+ cells (Fig. 4 A), and Wright-stained blood smears revealed the presence of morphologically abnormal GM cells in miR-155–expressing mice (Fig. 4 B). Complete blood cell counts showed a significant reduction in red blood cell, hemoglobin, and platelet levels (Fig. 4 C), FACS found
AML patients show decreased B220+ B cells and CD4+ T lymphocytes (Fig. 4 C), indicating a reduced immune response. In contrast, no significant difference in the average expression levels of miR-155 was observed between normal subjects and AML patients of the FAB subtypes M4 and M5. Group differences were considered statistically significant when the p-value (*) was <0.05.

decreased B220+ B cells and CD4+ T lymphocytes (Fig. 4 C), and Wright staining identified several immature erythrocytes demonstrating polychromatophilia in miR-155–expressing animals (Fig. 4 D).

A subset of human AML patients overexpress miR-155

Several of the pathological features observed in miR-155–expressing mice are associated with human myeloid malignancies, including AML. Therefore, bone marrow samples from 24 AML patients and 6 healthy donors were assayed for miR-155 and 5S RNA expression levels by quantitative PCR. On average, the AML samples significantly overexpressed miR-155 compared with healthy donors, with a level approximately four and a half times higher (Fig. 5 A). A few AML samples had miR-155 levels that were lower than the normal samples, whereas the overall AML sample distribution had a wide variance. In contrast, no significant difference in the average expression levels of 5S RNA was observed between the groups (Fig. 5 A). MiR-155 levels in different subtypes of AML were next ascertained using the World Health Organization (WHO) classification system. Patients with acute myelomonocytic leukemia and acute monocytic leukemia, corresponding to FAB-AML-M4 and FAB-AML-M5, respectively, exhibited significant overexpression of miR-155 compared with normal samples (Fig. 5 B). These observations demonstrate that miR-155 expression in the bone marrow is significantly elevated in a subset of patients suffering from AML.

MiR-155 can directly repress genes implicated in hematopoietic development and disease

MiRNAs exert their biological functions through the degradation and/or translational repression of target mRNAs. To identify miR-155 target genes that may be involved in the observed myeloproliferative phenotype, we first transduced RAW 264.7 myeloid cells with a miR-155–expressing retrovirus that increased mature miR-155 cellular levels within twofold of those observed after LPS stimulation (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20072108/DC1). A miRNA microarray analysis was next performed on RNA samples collected from miR-155–expressing and control cells to identify genes regulated by miR-155 (Fig. S5). Some 1,080 transcripts were down-regulated >1.2-fold with a p-value of <0.08, and 89 of the repressed mRNAs contained conserved (human and mouse) miR-155 binding sites with 7–or 8-mer seeds in their 3’ UTRs according to published lists of computationally predicted target genes found on the Targetscan 4.0 website (19, 20). Finally, genes with reported roles in myeloid hyperplasia and/or hematopoiesis were identified through literature searching. Using these criteria, our attention was drawn to 10 candidate targets: Bach1, Sla, Cutl1, Csf1r, Jarid2, Cebpβ, PU.1, Amtl, Hif1α, and Picalm (Fig. 6 A). To confirm the microarray results, quantitative PCR was performed using gene-specific primers. It showed that the miRNAs encoding these proteins were down-regulated ∼20–70% in RAW 264.7 cells expressing miR-155 versus empty vector control (Fig. 6 A). We also observed repression of Cebpβ, PU.1, Cutl1, and Picalm protein levels in RAW 264.7 cells expressing miR-155 (Fig. 6 B), albeit to somewhat varying degrees between experiments (not depicted).

Next, we tested whether miR-155 could directly repress the identified miRNA targets through 3’ UTR interactions. Each full-length 3’ UTR, or in two cases (Bach1 and Cebpβ) the region of the UTR containing the miR-155 binding site(s), was cloned into a reporter vector downstream from luciferase. These vectors were then used to assess whether miR-155 could repress luciferase gene expression in 293T cells. Luciferase expression was repressed between 35 and 78% depending on the 3’ UTR tested (Fig. 7). There was even a rough correlation between the quantitative PCR results in RAW 264.7 cells and the luciferase repression in 293T cells. To demonstrate a direct interaction between miR-155 and the 3’ UTRs tested, we systematically mutated each conserved miR-155 7–or 8-mer seed region and found that a majority of the miR-155–mediated repression was abolished (Fig. 7).

As a control, miR-155 repressed a reporter construct containing tandem miR-155 sites ∼80%. However, luciferase levels were relatively unaffected when the Traf6 or Irak1 3’ UTRs were tested, consistent with their lack of miR-155 binding sites (Fig. 7). These results provide strong evidence that miR-155 can directly regulate several genes with relevance to hematopoiesis and the myeloproliferative phenotype.

DISCUSSION

As cells of the innate immune system combat infectious pathogens, their numbers are often depleted and must be replenished.
miR-155 expression in human CD34+ cells, a population containing HSCs and early progenitors of lymphoid and myeloid lineages (5). However, defective myeloid populations in miR-155–deficient mice were not observed when analyzed under steady-state (non-inflammatory) conditions (8).

Whether miR-155 is only sufficient or in fact required for increased myelopoiesis during inflammation, our findings demonstrate that its unregulated expression triggers a myeloproliferative disorder, exhibiting many preleukemic aspects. Because frank myeloid leukemia was not observed in any of...
our mice analyzed within 2 mo of reconstitution with miR-155–expressing HSCs, such a transition may require additional mutations. However, the relevance of our observed phenotype in mice is substantiated by the elevated expression levels of miR-155 seen in human patients with AML. Interestingly, the two AML subgroups found to be overexpressing miR-155 are characterized as myelomonocytic (M4) and monocytic (M5), both thought to be derived from cells demonstrating aspects of GM cell differentiation, similar to the expanded GM cells in our miR-155–expressing mice. It is of note that miR-155 may also be elevated in other subtypes of AML where we did not have enough samples to make such a conclusion. If miR-155 does prove to be dysregulated in specific subtypes of AML, it might complement the recent finding that miR-181 expression positively correlates with M1 and M2 subtypes of AML, but not M4 or M5 (22). Furthermore, as the full spectrum of miRNAs that become dysregulated during AML is defined, these small RNAs may prove to have utility as diagnostic indicators of AML subtypes.

Despite the similarities mentioned above, certain aspects of the miR-155–induced myeloproliferative phenotype were not observed during the 3-d analysis period after LPS-mediated acute inflammation, such as GM dysplasia, peripheral blood leukopenia and polychromatophilic RBCs, and reduced macrokaryocyte and platelet levels (not depicted). These observations may reflect a differential effect of sustained miR-155 expression in cell types that require strictly regulated levels of this miRNA. However, chronic inflammation (which may also sustain high miR-155 expression levels) might trigger some of these pathological events given enough time. For instance, after months of polymicrobial sepsis in mice, there is reported extramedullary hematopoiesis in the spleen and significantly increased numbers of morphologically heterogeneous GM cells in both the spleen and bone marrow compartments (23). The inflammatory response in the bone marrow involves profound myeloid proliferation and, through factors such as miR-155, may prove to create a microenvironment suitable for cancer formation and development if not resolved in a timely manner.

Unlike a previous report, which found that B cell–restricted transgenic expression of miR-155 triggers B cell lymphoma in mice (7), we did not observe a B cell malignancy in our model. This may be explained by differences in the systems used because our model allows for miR-155 expression beginning in adult HSCs, which precedes formation of pro-B cells during hematopoietic development (1). These observations suggest that miR-155 may trigger unique phenotypes when expressed at different stages or in distinct cell type(s) during hematopoiesis. There is also evidence that developing B cells and GMs may occupy an overlapping bone marrow niche. Based upon our current findings, miR-155 expression may allow for GM progenitors to dominate this compartment and inhibit B cell development, which has been proposed to occur during inflammation (17). This mechanism might also block events required for miR-155–dependent B cell transformation.

In an effort to explore the mechanistic basis for the myeloproliferative phenotype caused by HSC expression of miR-155, we identified several mRNA targets that were directly repressed by miR-155 according to 3′ UTR reporter assays. Of note, the reduced expression or altered function of some of these targets has been linked to AML, as in the case of PU.1 and Picalm (24, 25), or myeloproliferative conditions, as is true for Cutl1 and Csf1r (26, 27). Other identified targets have been implicated in control of various aspects of hematopoiesis involving many of the cell types that are perturbed in mice expressing miR-155 in HSCs. These include Cebpb (28), Bach1 (29), Arml (30), Sla (31), Jarid2 (32), and Hif1α (33). Thus, miR-155 could mediate its overall biological affects, both physiological and pathological, through the combinatorial repression of a broad range of targets in a variety of cell types. Such a multi-target regulation has recently been described for T cell receptor signaling (34). Therefore, it is possible that complete rescue of this phenotype will not be achieved through replacing any one of the specific miR-155 target genes. However, the specific spatial and temporal contributions of individual targets to the myeloproliferative phenotype and AML in the context of miR-155 repression remain an area for future investigation.

There is emerging evidence that individual miRNAs are part of a more complex regulatory network involving other miRNAs and transcriptional regulators that cooperate to govern hematopoiesis. For example, Csf1r is important for monocyte development and has recently been reported to be regulated indirectly by miRNAs 17-5p-20a-106a (26). miRNAs 17-5p-20a-106a repress the transcriptional regulator AML1 required for Csf1r transcription. Therefore, both miRNAs 17-5p-20a-106a and miR-155 can influence Csf1r expression through different mechanisms. In the case of miR-155 targets PU.1 and Cebpb, they have been shown to transcriptionally regulate expression of myeloid-specific miR–223 (35). miR–223 is subsequently involved in unleashing CEBPβ function, a central transcription factor in hematopoiesis, through the direct repression of its inhibitor, NFI-A (36). Such dynamic systems require appropriate miRNA expression levels and kinetics to carefully orchestrate hematopoietic development, as has been recently described during T cell development in the thymus (37). However, it is easy to see how this delicate process would be vulnerable to dysregulated miRNA expression leading to pathological outcomes. This concept is exemplified by the dysplastic features observed in many GM cells from our miR-155–expressing mice. It is possible that although initial miR-155 expression expands GM numbers, its timely down-regulation is necessary for these cells to complete their developmental programs. Such a model would be consistent with the transient expression of miR-155 that precedes expansion of morphologically normal GM populations in the bone marrow after LPS treatment.

Based upon our current study, miR-155 appears to play a role in promoting GM cell expansion during inflammatory responses while initiating pathological processes under forced expression. Due to the enhanced expression of miR-155 in a
subset of AML patients, and its ability to repress several genes relevant to myeloid malignancies, therapeutic targeting of miR-155 with such agents as antagonirs may provide a beneficial option (38). Because miR-155 knockout mice display few detrimental phenotypes in the absence of infection (8), decreasing miR-155 function in human patients suffering from myeloid, lymphoid, or other malignancies correlated with enhanced miR-155 expression may provide more benefits than harm.

MATERIALS AND METHODS

Cell culture and reagents. RAW 264.7 and 293T cells were both cultured in complete DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin with 5% CO₂. Mouse bone marrow-derived macrophages were made using M-CSF−containing media. LPS from Escherichia coli strain 055:B5 was purchased from Sigma-Aldrich, and recombinant mouse GM-CSF was purchased from ebioscience.

DNA constructs. A miR-155 expression cassette containing the human miR-155 hairpin sequence and flanking regions was cloned from a B cell cDNA library into pcDNA3 as described previously (12). This cassette was subcloned into pMCSVpuro, FUW, or pMG. pMG155 is a modified MSCP vector whereby GFP was placed downstream from the 5′ LTR, and the miR-155 expression cassette was cloned downstream from the GFP stop codon (detailed cloning strategy available upon request). For reporter assays, the 3′UTRs of the respective mRNAs were cloned into pmiReport (Ambion) after amplification from a mouse macrophage cDNA library. Primer sequences are described in Table S1, which is available at http://www.jem.org/cgi/content/full/jem.20072108/DC1. The Bach1 3′UTR region was amplified from a human B cell library. Site-directed mutagenesis was used to change specific nucleotides found within the miR-155 seed regions (Table S2). The 2-mer control insert consists of a tandem repeat of the complimentary sequence to the mature mouse miR-155 sequence. Cloning of the TRAF6 and IRAK1 3′UTR into pmiReport was described previously (13).

Mice. WT C57BL/6 mice were purchased from The Jackson Laboratory, and Rag1−/− mice were bred in-house. All experiments involved female mice and were performed according to IACUC-approved protocols.

Retroviral infections, stable cell lines, and bone marrow reconstitution.

To generate VSVg-pseudotyped retroviruses containing the miR-155 expression cassette, 2 × 10⁶ 293T cells were transfected with pMSCVpuro−miR-155, pGag-Pol, and pVsVg using a standard calcium phosphate protocol. After 48 h, viral supernatant was harvested and used to infect 5 × 10⁶ RAW 264.7 cells for 8 h in the presence of polybrene at 10 μg/ml. After 48 h, stably transduced cells were selected using puromycin at 7 μg/ml for 7–10 d, and miR-155 expression was assessed at the same time as experiments were performed by Northern blotting or quantitative PCR for all batches made.

To obtain HSC-enriched bone marrow cells, mice were injected i.p. with 5 μg 5-fluorouracil for 5 d before bone marrow harvest (39). Cells were collected from the bone marrow, and RBCs were removed using an RBC lysis solution (Invitrogen). Cells were cultured for 24 h in 10% IL-3, 50 ng/ml IL-6, and 50 ng/ml SCF (all from ebioscience) containing complete RPMI (10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 50 μM β-macroporphthanol) before initial retroviral infection. To generate retoviruses for infecting HSC-enriched bone marrow cells, 293T cells were transfected with pMGI55 and pCL-Eco. After 48 h, 8 μg/ml polybrene was added to culture supernatant−containing retroviruses, and this was used to spin-infect 10⁶ HSC-enriched cells per donor for 1.5 h at 2,500 RPM and 30°C. This procedure was repeated three times once daily, followed by infection of 10⁶ retrovirally infected HSC-enriched cells per lethally irradiated (1,100 rads from Cesium 137 source at 50 rads/minute) recipient. Recipients were maintained on Septra throughout the reconstitution period.

RNA quantification. Northern blotting and quantitative PCR were used to assay miR-155 and other miRNAs as described previously (14). Gene-specific primer sequences used for quantitative PCR are shown in Table S3. For the microarray study, total RNA was collected from five RAW 264.7 stably infected clones expressing miR-155 or empty vector using the RNeasy Mini kit per the manufacturer’s instructions (QIAGEN). The Affymetrix Mouse Genome 430 2.0 microarray analysis was then performed using pooled RNA from each group by the Millard and Muriel Jacobs Genetics and Genomics Laboratory at Caltech according to detailed protocols (http://affymetrix.com/products/arrays/specific/mouse430_2a.affx). Data were analyzed using Rosetta Resolver Software. Microarray data were deposited in the GEO database under accession number GSE10467.

Western blotting. Western blotting was performed using standard protocols and the following antibody clones from Santa Cruz Biotechnology, Inc.: Cebpb (C-19), PU.1 (T-21), Cull1 (M-222), Pcia1 (C-18), and αTubulin (AA12). Protein expression differences were determined using Scion Image software.

Flow cytometry and cell separation. Fluorophor-conjugated monoclonal antibodies specific for either Mac-1, Gr1, Ter-119, B220, and CD4 (all from ebioscience) were used in various combinations to stain RBC-depleted splenocytes, bone marrow, or peripheral blood mononuclear cells that were fixed after washing using paraformaldehyde (1% final). Stained cells were assayed using a BD FACS caliber flow cytometer and further analyzed with Flojo software. Cell separation was performed using biotinylated monoclonal antibodies against Mac-1, Ter-119, and B220 (ebioscience), streptavidin-conjugated magnetic beads (Miltenyi Biotec), and MACS LS Separation Columns (Miltenyi Biotec).

Luciferase reporter assays. 8 × 10⁴ 293T cells were plated in DMEM containing 5% FBS for 18 h, followed by transfection of relevant plasmids using lipofectamine (Invitrogen) per the manufacturer’s instructions. Luciferase assays were performed 48 h later using a dual luciferase kit (Promega). A β-gal expression plasmid was cotransfected and β-gal levels were assayed and used to normalize the luciferase values.

Human AML sample collection and analysis. Bone marrow biopsy samples collected from patients with AML were flash-frozen and stored at −80°C after the completion of diagnostic work in a tissue bank at University of California, Los Angeles. For this study, 24 samples were rapidly thawed and subjected to TRizol purification of RNA. In addition, six RNA samples were isolated from healthy donors. AML cases were categorized according to the WHO “Classification of Tumors” using anonymous clinical reports. All work performed on these tissues was approved by the Institutional Review Board at UCLA.

Morphological assessment of hematolymphoid tissues. For histological sectioning, organs were placed into 10% neutral-buffered formalin immediately after necropsy, fixed for 12–18 h, washed, and transferred to 70% ethanol before standard paraffin embedding, sectioning, and staining with hematoxylin and eosin. Bones were also decalcified. For cytological assessment, touch preparations of the cut surface of the spleen were performed. Peripheral blood smears were obtained from tail vein bleeds or from the heart at necropsy. Bone marrow smears were prepared from extracted bone marrow of reconstituted mice. All cytological preparations were air-dried and stained with Wright’s stain. Both histological and cytological preparations were examined on an Olympus BX-51 microscope and photographed using a Spot Digital Camera and software. Complete blood cell counts were performed at UCLA’s Department of Laboratory Animal Medicine.

Statistical tests. All statistical analyses were performed using Microsoft Excel statistical software module. For patient samples, an F-test determined that the distributions of miR-155 expression in normal samples versus AML samples were heteroscedastic (P = 4.5 × 10⁻¹⁰ for F-test). Similarly, the distributions of miR-155 expression in normal versus AML-M4 was determined to be
Online supplemental material. Fig. S1 demonstrates that both immature and mature cell-enriched bone marrow populations up-regulate miR-155 in response to LPS. Fig. S2 provides FACS data showing the percentage of Mac1<sup>+</sup>, Gr1<sup>+</sup>, B220<sup>+</sup>, Ter-119<sup>+</sup>, or CD4<sup>+</sup> cells in the bone marrow after 24 h of LPS versus PBS treatment. Fig. S3 presents the coexpression of miR-155 and GFP in several different immune organs from reconstituted mice. Fig. S4 compares miR-155 expression in Raw 264.7 cells and primary macrophages mediated by retroviral overexpression versus LPS stimulation. Fig. S5 outlines the scheme used to identify miR-155 targets with relevance to hematopoiesis. Table S1 provides the primer sequences used for cloning different 3' UTRs into pmirReport. Table S2 provides sequence information regarding mutations introduced into the 3' UTRs used for reporter assays. Table S3 provides the primer sequences used to assay miR-155 target miRNAs by quantitative PCR. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20072108/DC1.

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REFERENCES


