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MicroRNA-125b Potentiates Macrophage Activation

Aadel A. Chaudhuri,^{*1} Alex Yick-Lun So,^{*1} Nikita Sinha,^{*} William S. J. Gibson,^{*} Konstantin D. Taganov,[†] Ryan M. O'Connell,[‡] and David Baltimore^{*}

MicroRNA (miR)-125b expression is modulated in macrophages in response to stimulatory cues. In this study, we report a functional role of miR-125b in macrophages. We found that miR-125b is enriched in macrophages compared with lymphoid cells and whole immune tissues. Enforced expression of miR-125b drives macrophages to adapt an activated morphology that is accompanied by increased costimulatory factor expression and elevated responsiveness to IFN- γ , whereas anti-miR-125b treatment decreases CD80 surface expression. To determine whether these alterations in cell signaling, gene expression, and morphology have functional consequences, we examined the ability of macrophages with enhanced miR-125b expression to present Ags and found that they better stimulate T cell activation than control macrophages. Further indicating increased function, these macrophages were more effective at killing EL4 tumor cells in vitro and in vivo. Moreover, miR-125b repressed IFN regulatory factor 4 (IRF4), and IRF4 knockdown in macrophages mimicked the miR-125b overexpression phenotype. In summary, our evidence suggests that miR-125b is at least partly responsible for generating the activated nature of macrophages, at least partially by reducing IRF4 levels, and potentiates the functional role of macrophages in inducing immune responses. *The Journal of Immunology*, 2011, 187: 5062–5068.

The mammalian innate immune system provides a critical first line of defense against pathogens. Macrophages are key components of this system, acting to release cytokines, kill pathogens directly, and present Ags to the adaptive immune system. The macrophage surface contains sensing proteins, like TLRs and IFN- γ R that, when engaged, lead to a rapid differentiation event termed activation, in which the cell transforms from relative quiescence to an effector state characterized by far-heightened microbicidal ability. Macrophages also carry costimulatory proteins such as CD80 and CD86 for interacting with T cells, thus bridging innate immunity to adaptive immunity (1, 2).

Recently, microRNAs have been shown to be important mediators of the macrophage activation process. miR-155, -146, -147, -9, and -21 are induced by ligands of the TLRs (3, 4). These microRNAs, in turn, inhibit expression of signaling proteins in the inflammatory signaling cascade, thus modulating immunity through feedback regulation (3, 4). miR-125b, a homolog of the *Caenorhabditis elegans* microRNA lin-4, has been shown to be decreased in macrophages in response to TLR4 signaling (5–7).

We and others found that miR-125b is enriched in hematopoietic stem cells and that increased miR-125b enhances hematopoietic engraftment (8, 9). Further increased levels of miR-125b cause an aggressive myeloproliferative disorder that leads to leukemia (8). In this study, we examine the role miR-125b plays in regulating macrophage activation. We find that macrophages express a particularly high concentration of miR-125b. When miR-125b is overexpressed in macrophages, it enhances surface activation markers both basally and in response to IFN- γ . By contrast, treatment of RAW264.7 macrophages with anti-miR-125b causes them to express decreased surface CD80 both basally and in response to IFN- γ . We demonstrate that macrophages expressing increased miR-125b become more potent stimulators of immune responses as shown by increased Ag-specific T cell activation and antitumor immunity. Lastly, we find that IFN regulatory factor 4 (IRF4) is an important target of miR-125b in macrophages and that IRF4 knockdown mimics the miR-125b overexpression phenotype.

Materials and Methods

Cell culture

293T cells, RAW264.7 cells, and BMMs were cultured at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. For IFN- γ treatment, cells were treated overnight with 200 U/ml recombinant mouse IFN- γ (eBioscience).

Mice

C57BL/6 and OTI OVA TCR-transgenic BALB/c mice were bred in the Caltech Office of Laboratory Animal Resources facility or purchased from The Jackson Laboratory. The Caltech Institutional Animal Care and Use Committee approved all mouse experimental protocols.

Isolation of immune cells and tissues

T cells and B cells were purified from the spleens of C57BL/6 mice using magnetic beads (Miltenyi Biotec). Peritoneal macrophages were isolated 4 d after injecting mice with 3% thyoglycollate.

DNA constructs

The murine stem cell virus GFP (MG), murine stem cell virus puromycin GFP (MGP), MG-125b-1, and MGP-125b-1 vector systems have been described previously (8, 10, 11). The human miR-125b-1 sequence was

^{*}Division of Biology, California Institute of Technology, Pasadena, CA 91125; [†]Regulus Therapeutics, San Diego, CA 92121; and [‡]Department of Pathology, University of Utah, Salt Lake City, UT 84112

¹A.A.C. and A.Y.S. contributed equally to this work.

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Address correspondence and reprint requests to Dr. David Baltimore, Department of Biology, 330 Braun Building, California Institute of Technology, Pasadena, CA 91125. E-mail address: baltimo@caltech.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: BMM, bone marrow-derived macrophage; IRF4, IFN regulatory factor 4; MG, murine stem cell virus GFP; MGP, murine stem cell virus GFP puromycin; MHC II, MHC class II; miR, microRNA; qPCR, quantitative real-time PCR; shRNA, short hairpin RNA; UTR, untranslated region.

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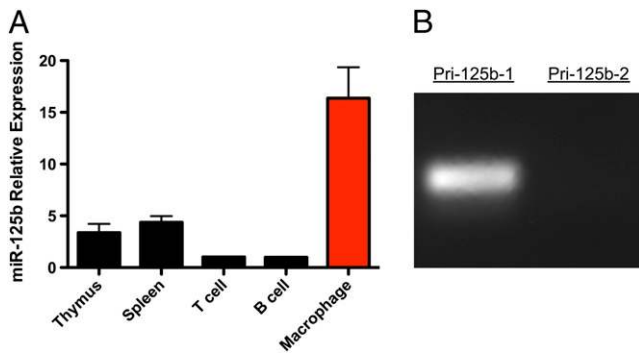


FIGURE 1. miR-125b expression is enriched in macrophages. *A*, Relative expression of miR-125b in immune tissues and cells assessed by quantitative PCR. Data represent the mean with SEM of three biological replicates per group. *B*, Expression of the miR-125b primary transcripts, pri-125b-1 and pri-125b-2, in BMMs. Data are representative of two independent experiments.

also cloned into the pcDNA3 vector downstream of the CMV promoter. The IRF4 short hairpin RNA (shRNA) sequence was predicted and cloned into MGP as described previously (11, 12). NC1 is a negative control shRNA sequence predicted not to target any protein coding genes in the mouse genome (Invitrogen). For reporter assays, pMIR-REPORT vector (Ambion) containing Picalm and Cut1 3' untranslated regions (UTRs) were constructed previously (10). A 3-kb region of the human IRF4 3' UTR, which includes the miR-125b putative binding site, was cloned into pMIR-REPORT downstream of luciferase. A positive control 2-mer containing two tandem sites complementary to miR-125b was also cloned. Primer sequences are listed in Supplemental Table I.

Retrovirally transduced bone marrow-derived macrophages

To generate retrovirus for infecting bone marrow, 293T cells were transfected with pCL-Eco and MG, MGP, MG-125b-1, or MGP-125b-1 vectors. After 36 h, 10 μg/ml polybrene (Millipore) was added to retrovirus-containing culture supernatant, which was used to spin-infect bone marrow from C57BL/6 mice. Cells were counted, and 1 million were plated per well in a six-well plate with 10 ng/ml M-CSF (eBioscience) and differentiated for 6 d to yield retrovirally transduced BMMs (8).

Stable cell lines

RAW264.7 cells were stably transduced with vesicular stomatitis virus-G-pseudotyped MGP or MGP-125b-1 retrovirus, and puromycin selection was subsequently performed as described previously (11).

Electroporation of anti-miRs

RAW264.7 cells were coelectroporated with anti-miR-125b or a mismatched control (Regulus Therapeutics) and pmxGFP vector (Lonza) using an Amaxa Nucleofector (Amaxa). Thirty-six hours postelectroporation, GFP-positive cells were analyzed by FACS. Anti-miR-125b or mismatched control compound were chimeric 2'-fluoro/2'-O-methoxyethyl-modified oligonucleotides with a completely modified phosphorothioate backbone (13) (Regulus Therapeutics). The exact chemistry is available on request.

T cell macrophage coculture

A total of 50,000 BMMs stably expressing either MG or MG-125b-1 were cocultured with 150,000 T cells harvested from OTI OVA TCR-transgenic BALB/c mouse in a 48-well flat-bottom plate in the absence or presence of OVA protein. Flow cytometry and ELISAs to assess T cell activation were performed 72 h later. ELISAs were performed with an IL-2 detection kit from eBioscience and carried out according to the manufacturer's instructions.

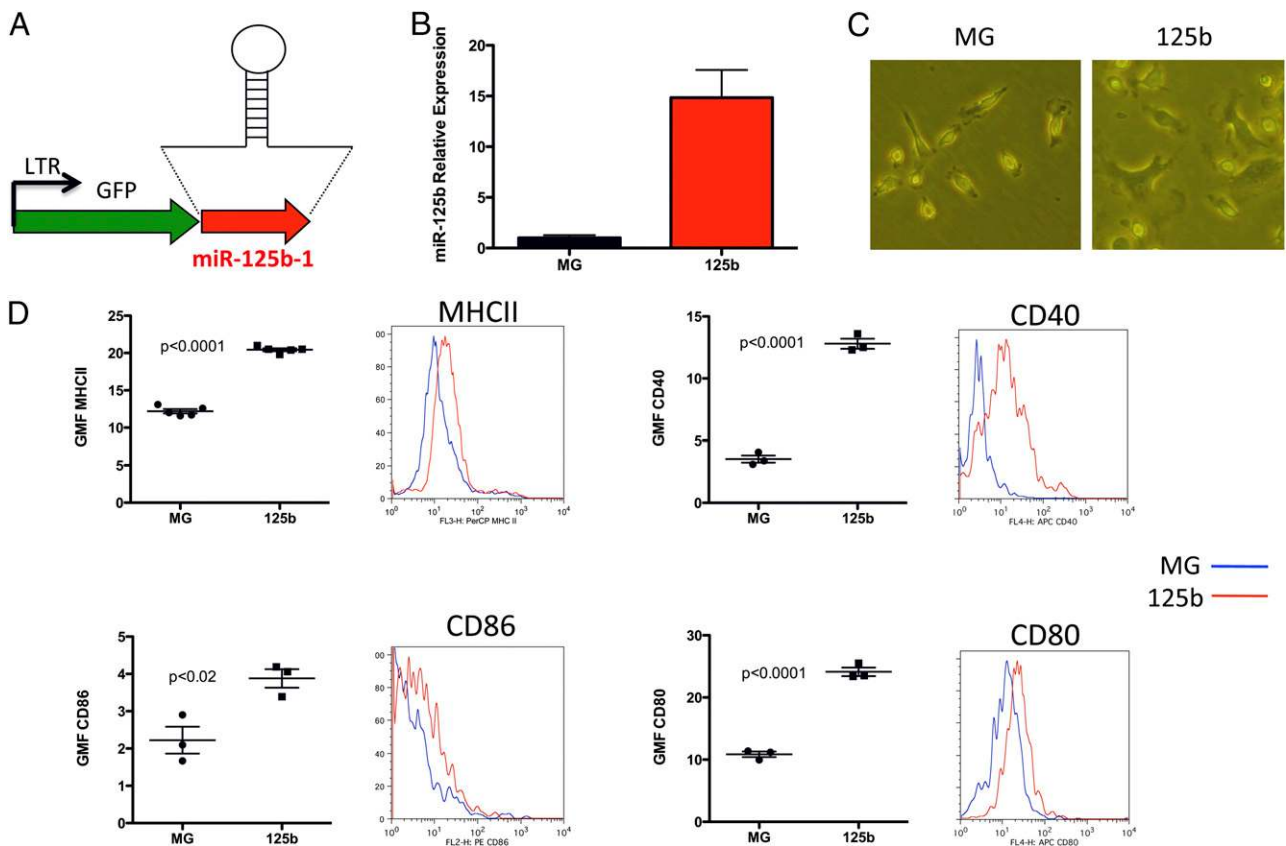


FIGURE 2. miR-125b enhances basal macrophage activation. *A*, Retroviral vector design for overexpression of miR-125b-1. *B*, Relative expression of miR-125b in BMMs after transduction with MG or MG-miR-125b-expressing vector. *C*, Morphology of control MG or miR-125b overexpressing BMMs. Data represent five independent experiments. *D*, Geometric mean fluorescence (GMF) of MHC II, CD40, CD86, and CD80 are shown. Representative plots obtained from flow cytometric analyses are also shown for each marker. Data are the mean with SEM of three to five samples per group and represent two independent experiments.

EL4 tumor cell experiments

A total of 500,000 BMMs stably expressing either MG or MG-125b-1 were generated per well in six-well plates. One million EL4-Fluc cells were added to each well supplemented with 20 ng/ml LPS. EL4-Fluc apoptosis was measured 94 h later by staining cells in suspension with Annexin V Ab (BD Pharmingen). For the in vivo experiments, 2 million EL4-Fluc cells were coinjected with 400,000 BMMs s.c. into albino C57BL/6 mice. Mice were closely monitored over the next 12 d. Tumor luminescence was measured using a Xenogen imager (Xenogen). At the experimental endpoint, animals were euthanized, and tumors were removed and weighed. Tumor surface area was assessed using a caliper; tumor length and width were measured in centimeters, and the product was taken to determine surface area.

Sequence alignment

The miR-125b seed region and IRF4 3' UTR sequences from human (*Homo sapien*), mouse (*Mus musculus*), cat (*Felis catus*), and armadillo (*Dasypus novemcinctus*) were obtained and aligned using Targetscan (14–16).

Luciferase reporter assay

293T cells were cotransfected with pcDNA-125b or pcDNA, as well as a β -gal expression vector, and the pMIR-REPORT vectors containing 3' UTRs of Cutl1, Picalm, IRF4, or 2-mer. The luciferase activity was quantified 48 h later and normalized to β -gal activity as previously described (11, 12).

RNA preparation and quantification

RNA was isolated using TRIzol (Invitrogen), RNEasy (Qiagen), or miRNEasy (Qiagen) as per the manufacturer's instructions. Quantitative real-time PCR (qPCR) was conducted using a 7300 Real-time PCR machine (Applied Biosystems) or a Realplex Real-time PCR machine (Eppendorf). SYBR Green was used to assay IRF4 and L32 expression. PCR with previously published primer sequences for mouse pri-miR-125b-1 and pri-miR-125b-2 were used to assay levels of miR-125b primary transcripts (17). TaqMan-based qPCR was conducted to assay miR-125b, miR-125a, and snoRNA-202 (Applied Biosystems). Primer sequences are listed in Supplemental Table I.

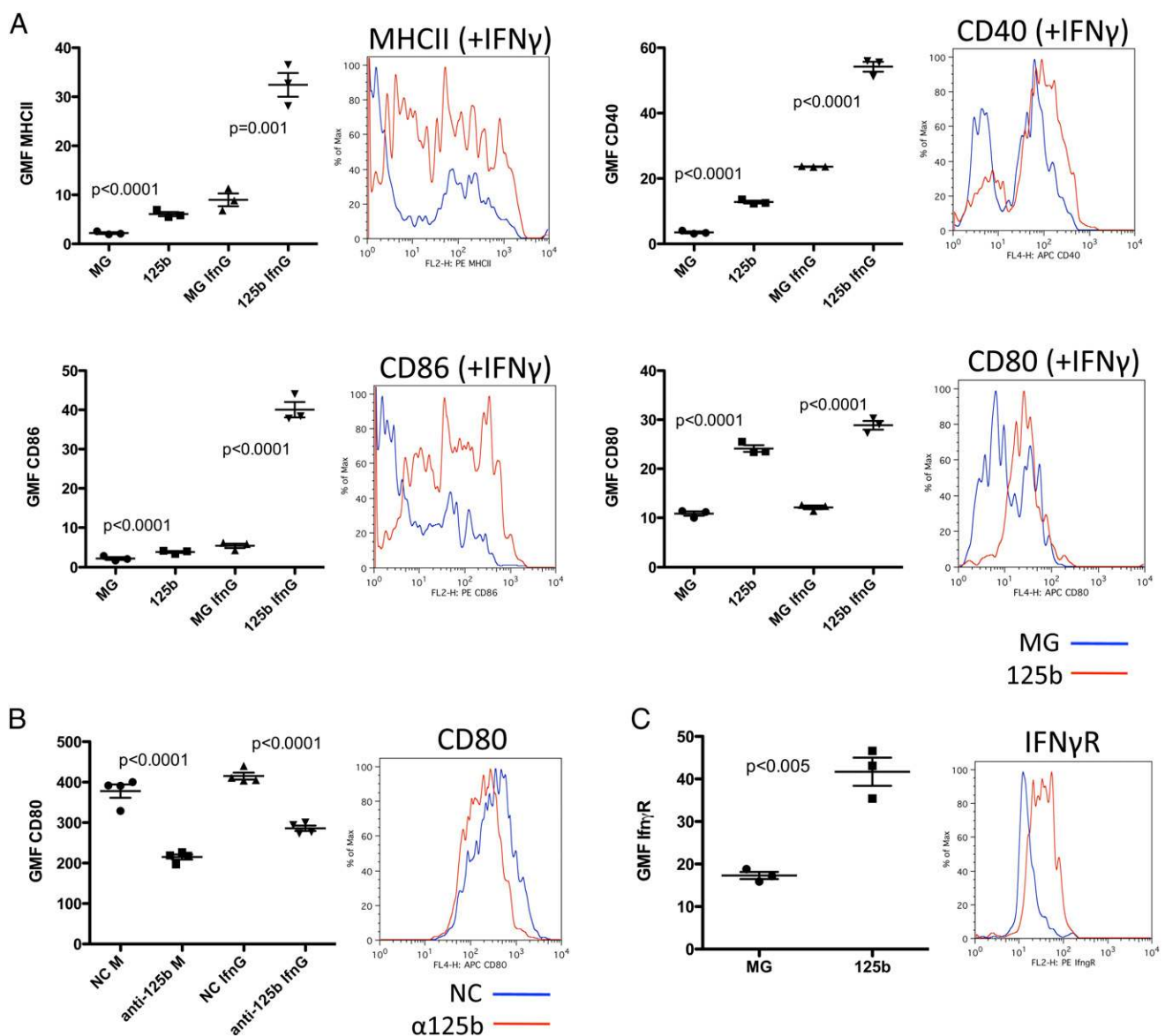


FIGURE 3. miR-125b increases macrophage response to IFN- γ . **A**, Surface expression of MHC II, CD40, CD86, and CD80 in response to media alone or IFN- γ . A representative flow cytometric plot of the IFN- γ -treated samples is shown for each factor. **B**, Raw264.7 macrophages electroporated with negative control (NC) or anti-miR-125b (α 125b) were subjected to flow cytometry for the surface expression of CD80. A representative FACS plot of the media-treated samples is shown. **C**, Surface expression of IFN- γ R in control (MG) versus miR-125b-overexpressing macrophages. A representative FACS plot is shown. All data shown represent the mean expressed with SEM of three samples per group and are representative of two independent experiments.

Flow cytometry

Cells were stained with the following fluorophore-conjugated Abs: CD80, CD86, CD40 (BioLegend), MHC class II (MHC II; eBioscience), and Annexin V (BD Pharmingen). Cell-surface receptors were measured using a FACSCalibur (BD Biosciences), and all data were analyzed with FlowJo (Tree Star). Data were gated on GFP-positive events when retrovirally transduced cells were analyzed.

Results

miR-125b expression is enriched in macrophages

To investigate the expression of miR-125b in different immune cells and tissues, we harvested RNA from total splenocytes, thymocytes, splenic T cells, splenic B cells, and peritoneal macrophages from C57BL/6 mice. Levels of miR-125b were assessed by reverse transcription followed by quantitative PCR. The expression of miR-125b was much higher in macrophages compared with the other immune cells and tissues (Fig. 1A). Within macrophages, miR-125b levels were also significantly higher than its homolog, miR-125a, indicating that miR-125b is the dominant isoform in these cells (Supplemental Fig. 1). Also, miR-125b is expressed from two loci in the mouse genome, each encoding a different primary transcript. We performed RT-PCR for each of these primary transcripts and determined that macrophages express primarily miR-125b-1 (Fig. 1B). Because miR-125b-1 is enriched in macrophages, we set out to determine the functional role of miR-125b-1 (referred from here on as miR-125b) in these cells.

Enforced expression of miR-125b enhances macrophage activation status

To examine the response of macrophages to miR-125b, we used a miR-125b overexpression system based on the MG vector (MG-miR-125b), which was originally derived from the murine stem cell virus (10) (Fig. 2A). Bone marrow cells isolated from C57BL/6 mice were spin-infected with either MG-miR-125b or MG control vector. These cells were then differentiated into bone marrow-derived macrophages (BMMs) by treatment with M-CSF. Using this system, miR-125b was overexpressed 15-fold above endogenous levels in BMMs (Fig. 2B). Interestingly, miR-125b overexpressing BMMs acquired a spread morphology with extensive pseudopods that resembled activated macrophages (Fig. 2C). We performed flow cytometric analyses and observed increased expression of MHC II and the costimulatory molecules CD40, CD86, and CD80 in these macrophages, indicating that these cells were indeed more activated (Fig. 2D). Ectopic expression of miR-125b in RAW264.7 macrophages gave similar results (Supplemental Fig. 2A), further emphasizing that this microRNA promotes activation of macrophages.

miR-125b increases macrophage response to IFN-γ

Next, we assessed the effect of miR-125b on the responsiveness of macrophages by stimulating these cells with IFN-γ. IFN-γ treatment increased the expression of MHC II, CD40, CD86, and CD80 activation markers in control macrophages (Fig. 3A), whereas miR-125b overexpressing macrophages expressed significantly higher levels of these markers (Fig. 3A). Similar results

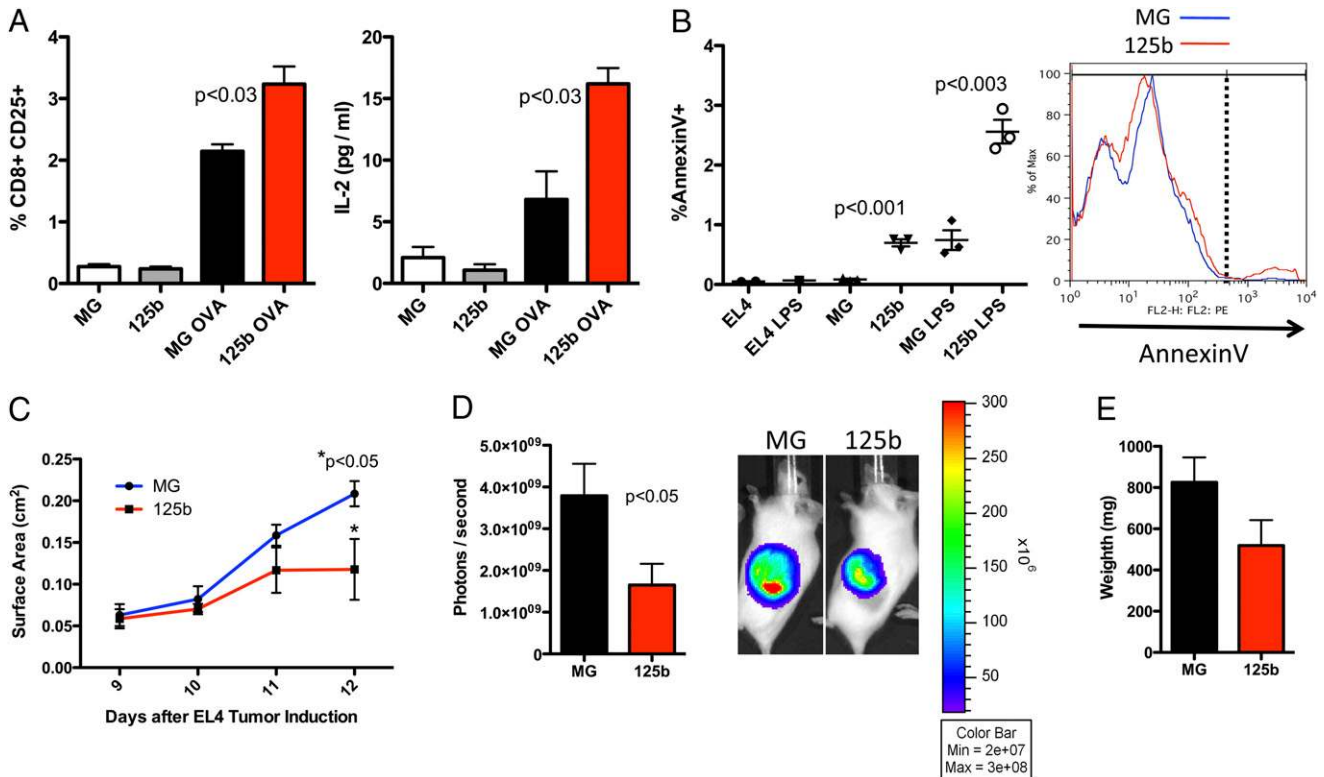


FIGURE 4. miR-125b enhances macrophage function. *A*, BMMs expressing the vectors MG or MG-125b were cocultured with OVA-specific OT1 T cells with or without OVA for 72 h. The percent CD8⁺CD25⁺ T cells are shown in the left panel. Concentration of IL-2 (pg/ml) produced by the T cells in the supernatant is shown in the right panel. Data represent the mean with SEM of three biological replicates per group. *B*, The percent AnnexinV⁺ EL4-Fluc cells after 94 h of coculture with control or miR-125-overexpressing macrophages in the presence of media alone or LPS. A representative flow cytometric plot of the LPS-treated group is shown. Data expressed as mean with SEM of one to three experimental samples per group. *C–E*, EL4-Fluc cells were s.c. coinjected with LPS-activated control or miR-125b-overexpressing macrophages into albino C57BL/6 mice. Tumor surface area in cm² was monitored from days 9–12 (*C*). The relative intensity of luminescence (*D*) and weight (*E*) of the EL4 tumors was measured on day 12. Data represent the mean plotted with SEM of eight mice per group. Representative of two independent experiments.

were obtained in RAW264.7 macrophages with enforced miR-125b expression (Supplemental Fig. 2A).

To examine whether reducing the concentration of miR-125b had an effect inverse to that of overexpression, RAW264.7 macrophages were treated with synthetic antisense oligonucleotides (anti-miRs), and surface CD80 levels were monitored as an indication of the cells' activation status. Anti-miR-125b caused a reduction of both basal and IFN- γ -induced levels of CD80 compared with cells treated with a control anti-miR (Fig. 3B). Thus, miR-125b appears to control CD80 expression in macrophages under normal, physiological conditions.

A likely reason for the heightened response to IFN- γ in miR-125b-treated cells could be increased expression of the IFN- γ R. Indeed, miR-125b-overexpressing BMMs (Fig. 3C) and RAW264.7 macrophages (Supplemental Fig. 2B) expressed significantly higher levels of surface IFN- γ R. Thus, in addition to potentiating macrophage activation, miR-125b promotes enhanced macrophage responsiveness to IFN- γ and increases surface expression of its cognate receptor.

miR-125b enhances macrophage-mediated function

Because we found that miR-125b drove macrophages to adopt an elevated activation status and become more responsive to stimu-

latory cues, we examined whether miR-125b would also potentiate macrophage-mediated immune function. To this end, we investigated whether miR-125b would increase the ability of macrophages to present Ags and induce activation of T cells. miR-125b-overexpressing macrophages were cocultured with transgenic T cells that express a chicken OVA-specific TCR (OT1) in the presence of OVA. Indeed, compared with control macrophages, miR-125b-overexpressing cells were more effective at inducing T cell activation, which was indicated by increased CD25 expression and IL-2 secretion by the T cells in response to OVA (Fig. 4A). Thus, enforced expression of miR-125b led to an elevated ability of macrophages to act as effective APC for stimulation of T cell responses.

In addition to serving as APC, another major function of macrophages is to eliminate aberrant cells, such as tumor cells. We therefore assessed whether miR-125b-stimulated macrophages were more effective at killing tumor cells. We used the EL4-Fluc thymoma tumor line, which was engineered to express luciferase, and cocultured these cells with either control macrophages or macrophages overexpressing miR-125b (18). Consistent with augmented function, miR-125b-expressing macrophages were better at inducing apoptosis of EL4-Fluc cells (Fig. 4B). Macrophages exposed to LPS gained the ability to induce apoptosis

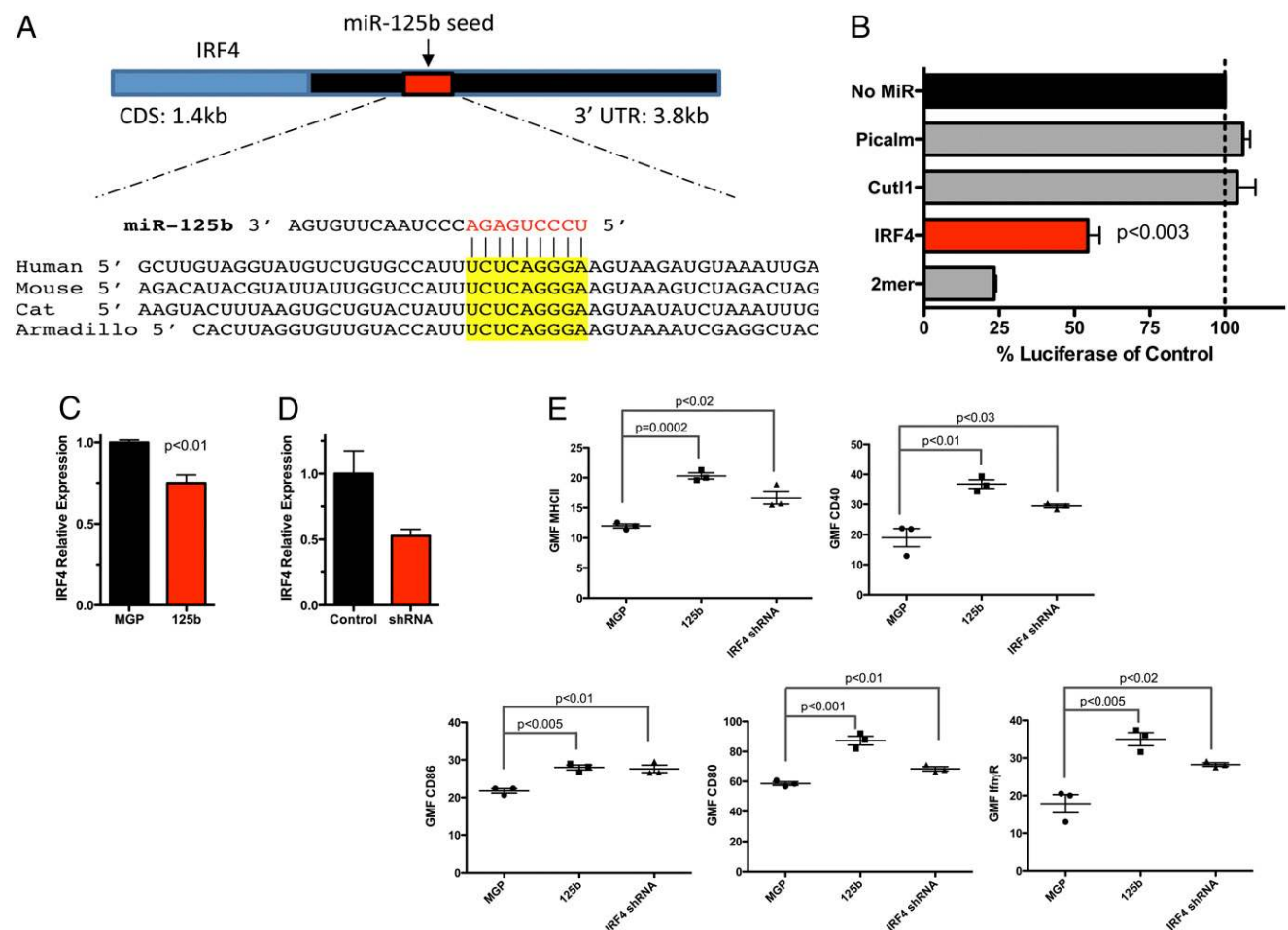


FIGURE 5. IRF4 is a target of miR-125b in macrophages. *A*, IRF4 contains a conserved miR-125b target site. *B*, Luciferase reporters carrying the 3' UTR of IRF4, Picalm (negative control), Cutl1 (negative control), or 2-mer (positive control) were cotransfected into 293T cells with β -gal reporter and \pm miR-125b. The relative luciferase activity of each reporter in the presence of miR-125b is shown relative to the no miR control. RAW264.7 macrophages were transduced with either a control (MGP) or miR-125b-expressing vector (*C*) or with control (NC1) or IRF4 shRNA-expressing vector (*D*). RNA was harvested, and L32-normalized IRF4 levels were determined by qPCR. *E*, BMMs expressing MGP, MGP-125b, or shRNA against IRF4 were measured for surface expression of the activation markers MHC II, CD40, CD86, CD80, and IFN- γ R. Geometric mean fluorescence (GMF) measured by flow cytometry is shown. All data represent the mean with SEM of three samples per group and are representative of two independent experiments.

of EL4-Fluc cells, with miR-125b–overexpressing macrophages having superior effectiveness (Fig. 4B). To test whether miR-125b levels in macrophages affect tumor killing in vivo, we s.c. coinjected into mice equal numbers of LPS-activated control or LPS-activated miR-125b–overexpressing macrophages with EL4-Fluc cells and tracked growth of the resulting tumor by measuring tumor surface area over time. Because EL4-Fluc cells were engineered to express luciferase, we also monitored tumor growth by measuring luminescence in vivo. Consistent with our in vitro data, macrophages with miR-125b ectopic expression suppressed the ability of EL4 cells to expand in vivo (Fig. 4C). At the endpoint of the experiment on day 12, animals injected with MG-125b macrophages had smaller EL4-derived tumors that were significantly less luminescent than those injected with control macrophages (Fig. 4D, 4E). Thus, miR-125b expression in macrophages appears to aid them in preventing the expansion of tumorigenic cells, further demonstrating that miR-125b enhances macrophage function.

IRF4 is a miR-125b target in macrophages

To identify targets regulated by miR-125b that modulate macrophage activation, we used TargetScan 5.1 to identify transcripts in the mouse genome that contain conserved putative miR-125b binding sites in their 3' UTRs. Among these genes, the 3' UTR of IRF4 harbored a conserved miR-125b binding site (Fig. 5A) and had been previously validated as a miR-125b target in B cell lines (19–22). We found that miR-125b indeed represses via the 3' UTR of IRF4 (Fig. 5B) and that miR-125b inhibits IRF4 expression in macrophages (Fig. 5C). Next, using the MGP retroviral vector system (11), we knocked down the expression of IRF4 using RNA interference (Fig. 5D) and examined the effect in macrophages. MGP-125b led to a 6-fold increase in miR-125b in BMMs. Similar to miR-125b overexpression, decreased IRF4 expression resulted in increased surface expression of MHC II, CD40, CD86, CD80, and IFN- γ R (Fig. 5E). Thus, IRF4 knockdown in macrophages enhances activation, mimicking the miR-125b overexpression phenotype. These data are consistent with previous reports demonstrating that IRF4 is a negative regulator of macrophage proinflammatory pathways (20, 21). Collectively, our data suggest that IRF4 is a primary target of miR-125b in regulating macrophage activation.

Discussion

In this study, we demonstrate that miR-125b is enriched in macrophages and that further elevation of miR-125b promotes greater activation, IFN- γ response, and immune function in these cells. We also performed loss-of-function studies using synthetic antisense oligonucleotides designed to inhibit miR-125b and found that this anti-miR-125b compound effectively decreased CD80 levels, supporting a physiological role for miR-125b in macrophage activation. Other groups have reported that miR-125b levels decrease in macrophages 3 h postinflammatory stimulation (5, 7). Thus, decreasing miR-125b may serve as a natural mechanism to limit the inflammatory response. In our studies, miR-125b overexpression also promoted the ability of macrophages to present Ag and induce T cell activation, demonstrating that miR-125b can enhance the macrophage's role in mediating adaptive immunity. The increase in activated T cells would in theory result in more secretion of IFN- γ , which in turn would further magnify the activation status of miR-125b–expressing macrophages. In this way, by affecting macrophage function alone, miR-125b could amplify both innate and adaptive immune responses by orchestrating positive-feedback loops between macrophages and T cells.

In B cells, miR-125b inhibits differentiation of germinal center B cells into plasma cells and does so via repression of the transcription factors IRF4 and BLIMP1 (19, 22). Recently, IRF4 was shown in B cell lines to regulate levels of BIC/miR-155, an interaction that might be important in leukemic transformation (23). IRF4, a member of the IFN response factor family of transcription factors, also has important functions in macrophages where it acts as an inhibitor of the inflammatory response (20, 21). We demonstrate in this study that IRF4 is a target of miR-125b and that IRF4 knockdown mimics the miR-125b–mediated activation phenotype in macrophages. Thus, miR-125b's ability to potentiate macrophage activation is consistent with previously demonstrated roles of its target, IRF4, to inhibit macrophage proinflammatory pathways (24).

miR-125b is upregulated in certain leukemias and downregulated in many nonhematopoietic solid cancers (12, 25–35). We have shown in this study that increased miR-125b expression in tumor macrophages slows tumor growth. Our data suggest that supplementing tumor macrophages with miR-125b may be a useful strategy for treating certain cancers. Future studies should aim to better understand the physiological and pathological mechanisms underlying control of miR-125b expression in macrophages.

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Disclosures

K.D.T. is an employee at Regulus Therapeutics. D.B. is a scientific advisor to Regulus Therapeutics. The other authors have no financial conflicts of interest.

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