Forced Activation of Notch in Macrophages Represses Tumor Growth by Upregulating miR-125a and Disabling Tumor-Associated Macrophages

Jun-Long Zhao\textsuperscript{1}, Fei Huang\textsuperscript{1}, Fei He\textsuperscript{2}, Chun-Chen Gao\textsuperscript{1}, Shi-Qian Liang\textsuperscript{1}, Peng-Fei Ma\textsuperscript{2}, Guang-Ying Dong\textsuperscript{1}, Hua Han\textsuperscript{1,2}, Hong-Yan Qin\textsuperscript{1}

\textsuperscript{1}State Key Laboratory of Cancer Biology, Department of Medical Genetics and Developmental Biology, \textsuperscript{2}Department of Hepatic Surgery, Xijing Hospital, Fourth Military Medical University, Xi’an 710032, China

Corresponding Authors:

Hong-Yan Qin: Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Chang-Le Xi Street #169, Xi’an 710032, China; Tel.: +86 29 84774487; Fax: +86 29 83246270; Email: hyqin@fmmu.edu.cn

Hua Han: Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Chang-Le Xi Street #169, Xi’an 710032, China; Tel.: +86 29 84774513, Fax: +86 29 83246270; Email: huahan@fmmu.edu.cn

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

J-L. Zhao and F. Huang contributed equally to this study.

Running title: Notch regulates TAMs through miR-125a

Disclosure of Potential Conflicts of Interest: The authors disclose no potential conflicts of interest.
Abstract

Tumor-associated macrophages (TAM) contribute greatly to hallmarks of cancer. Notch blockade was shown to arrest TAM differentiation but the precise role and underlying mechanisms require elucidation. In this study, we employed a transgenic mouse model in which the Notch1 intracellular domain (NIC) is activated conditionally to define the effects of active Notch1 signaling in macrophages. NIC overexpression had no effect on TAM differentiation, but it abrogated TAM function leading to repressed growth of transplanted tumors. Macrophage miRNA profiling identified a novel downstream mediator of Notch signaling, miR-125a, which was upregulated through an RBP-J-binding site at the first intronic enhancer of the host gene Spaca6A. miR-125a functioned downstream of Notch signaling to reciprocally influence polarization of M1 and M2 macrophages by regulating FIH1 and IRF4, respectively. Notably, macrophages transfected with miR-125a mimetics increased phagocytic activity and repressed tumor growth by remodeling the immune microenvironment. We also identified a positive feedback loop for miR-125a expression mediated by RYBP and YY1. Taken together, our results showed that Notch signaling not only supported the differentiation of TAM but also antagonized their protumorigenic function through miR-125a. Targeting this miRNA may re-program macrophages in the tumor microenvironment and restore their antitumor potential.

Key words: Notch signal, miR-125a, TAMs, macrophage polarization, FIH1, IRF4
Introduction

Tumor-associated macrophages (TAMs) play pivotal roles in tumor microenvironment to facilitate tumor growth and metastasis (1-3). TAMs inhibit anti-tumor immunity by recruiting myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs), and by repressing CD8⁺ cytotoxic T-cells (1-3). TAMs are characterized by a molecular signature reminiscent of alternatively activated (M2) macrophages. These macrophages, with interleukin (IL)-4-activated macrophages as a prototype, express higher levels of immunosuppressive cytokines such as transforming growth factor (TGF)-β and IL-10, together with arginase-1 (Arg-1), mannose receptor (MR) and other molecules involved in anti-inflammatory and/or tissue remodeling (4-6). TAMs also secret epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) and other growth factors to promote cancer cell proliferation and tumor vascularization, respectively. In contrast to TAMs or M2 macrophages, lipopolysaccharide (LPS)- and interferon-γ (IFN-γ)-stimulated macrophages or M1 macrophages upregulate IL-12, inducible nitric oxide synthase (iNOS), and tumor necrosis factor (TNF)-α, accompanied by increased antigen presentation capacity. Macrophages with the M1 phenotype repress tumor growth through phagocytosis and enhanced anti-tumor immunity (1-3). Therefore, it is possible to re-educate TAMs to elicit anti-tumor activities, given that the regulation and mechanisms of macrophage polarization are established (7-9).

The Notch-RBP-J (recombination signal-binding protein Jκ) signaling pathway plays critical roles in cell fate specification and cell plasticity (10, 11). Notch
signaling is involved in macrophage activation and polarization (12-19). Recently, Franklin et al have demonstrated that Notch signal is required for TAMs differentiation in a mouse mammary tumor model (20). However, the role and mechanisms of Notch in TAMs after differentiation remain to be elucidated, although NF-κB, MAPK, STAT3, interferon regulatory factor (IRF) 8, and cylindromatosis (CYLD), as well as pyruvate dehydrogenase phosphatase 1 (Pdp1)-mediated mitochondrial metabolism reprogramming, have been implicated (17, 19, 21, 22).

miRNAs participate in myeloid differentiation and macrophage activation (23-25). miR-125a is enriched in myeloid progenitors but at low level in monocytes (25, 26), and is upregulated in M1 and downregulated in M2 macrophages (23, 24). miR-125a is involved in differential activation of macrophages and other immune cells, as well as in myeloproliferative neoplasm (27-30). In this study, we show that forced Notch activation in macrophages by conditional overexpressing Notch intracellular domain (NIC) was sufficient to support TAM differentiation but abrogate TAM functions, most likely through miR-125a, leading to repressed tumor growth in mice.

Materials and Methods

Mice

Mice were maintained on C57BL/6 background in a specific pathogen-free (SPF) facility. RBP-J-floxed (RBP-Jf) mice (31) or ROSA-Stopf-NIC transgenic mice (a gift from HL Li) were mated with Lyz2-Cre (#019096, Jackson Laboratory, Bar Harbor, ME) or Mx1-Cre (provided by K Rajewsky) mice to obtain mice with appropriate genotypes. Mx1-Cre-RBP-Jf mice were further injected intraperitoneally (i.p) with
poly(I)-poly(C) (Sigma, St. Louis, MO) to induce RBP-J gene disruption in bone marrow (BM) (inducible conditional RBP-J knockout, RBP-J<sup>icKO</sup>) (31). The ROSA-Stop<sup>f</sup>-NIC transgenic mice contain a sequence encoding NIC (amino acids 1749-2293, lacking the C-terminal PEST domain) of the mouse Notch1 followed by a IRES-GFP cassette, which is inserted into the GT(ROSA)26Sor locus. Mice were genotyped with tail DNA by PCR using the primers listed in supplemental Table S1. All animal experiments were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University.

Lewis lung carcinoma (LLC) cells and B16F melanoma cells were purchased from the authenticated ATCC (American Type Culture Collection) repository in 2014, and cell lines were tested by PCR for the absence of mycoplasma contamination and C57BL derivation during the experiment and injected subcutaneously (s.c) on rear back of C57BL/6 mice. Tumor growth was monitored by measuring tumor length (L) and short (S) with a sliding caliper (tumor size = L × S<sup>2</sup> × 0.51). Mice were sacrificed 2 (B16F) or 4 (LLC) weeks after inoculation.

**Cell culture**

BM-derived macrophages (BMDMs) were cultured from BM monocytes as described (17). BMDMs were stimulated with LPS (50 ng/ml, Sigma) and IFN-γ (20 ng/ml) or IL-4 (20 ng/ml, PeproTech) for 24 h. In some experiments, γ-secretase inhibitor IX (GSI, 30 μM, Sigma) was included in the medium, with DMSO as a control. The transfection of BMDMs with synthetic miR-125a mimics or antisense oligonucleotides (ASO, Ribio, Guangzhou, China) and small interference RNAs...
(siRNAs) was performed by using Lipofectamine LTX™ (Invitrogen), according to the recommended protocol. The sequences of siRNAs for murine factor inhibiting hypoxia-inducible factor 1 alpha (FIH1), IRF4, Yin and Yang 1 (YY1), and Notch1 were shown in supplemental Table S1. RAW264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 2 mM L-glutamine and transfected with plasmids using Lipofectamine 2000™ (Invitrogen).

Recombinant mD1R, which is composed of the DSL domain of mouse Dll1 and an arginine-glycine-aspartic acid (RGD) motif, was manufactured in E. coli (32). Wells of 12-well dishes were coated with 400 µl of mD1R (50 µg/ml) at 4 °C overnight, with PBS as a control. BMDMs (5 × 10⁵) were then seeded in the wells and cultured in the presence of LPS and IFN-γ for 24 h.

Allogenic T-cell stimulation assay was performed as described (17).

**Immunofluorescence and flow cytometry**

Cells were stained with antibodies listed in supplemental Table S2, and observed under a laser scanning confocal microscope (FV-1000, Olympus, Tokyo, Japan). Fluorescence-activated cell sorter (FACS) analysis was performed using a FACSCalibur™ and FACSAriaII flow cytometer (BD Immunocytometry Systems). Data were analyzed with the FlowJo vX.0.6 software (FlowJo, LLC, Ashland, OR). Dead cells were excluded by propidium iodide (PI) staining.

**Phagocytosis**

L1210 murine leukemia cells (ATCC) (1 × 10⁶ cells/ml) were labeled with carboxyfluorescein succinimidyl amino ester (CFSE) (Dojindo Molecular
Technologies, Inc., Kumamoto, Japan), and incubated with BMDMs (1 × 10^5 cells/ml) on coverslips at 37 °C for 2 h. After washing, samples were stained with anti-F4/80, and visualized under a fluorescence microscope (BX51, Olympus, Tokyo, Japan). The average number of engulfed L1210 cells per macrophage was calculated. The engulfment of bacteria by BMDMs was determined in a similar way by using *E. coli* BL21 transformed with an EGFP-expressing plasmid.

**miRNA profiling**

BMDMs from RBP-J^icKO^ and control mice were stimulated with LPS for 24 h and used for miRNA expression profiling with an Agilent miRNA chip (Sanger miRBase v.12.0, 627 mouse miRNAs and 39 mouse viral miRNAs were represented) conducted by a commercial service (ShanghaiBio, Shanghai, China). The data were uploaded to Gene Expression Omnibus database (accession number GSE67364).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA preparation, reverse transcription and real time PCR were performed as described, with β-actin or U6 RNA (for miRNAs) as internal controls. Rapid amplification of cDNA ends (RACE) was performed using a SMARTer RACE cDNA Amplification Kit (Clontech, Shiga, Japan). The PCR primers are shown in supplemental Table S1.

**Plasmids**

The 3’-untranslated regions (UTRs) of IRF4, FIH1, and RING1- and YY1-binding protein (RYBP) were amplified by PCR with a mouse cDNA library as a template. Point mutations were generated by PCR. Wild-type or mutant 3’-UTR
fragments were inserted into pGL3-promoter (Promega, Fitchburg, WI) at the 3’ end of the luciferase gene to generate reporters (pGL3-IRF4, pGL3-FIH1 and pGL3-RYBP, respectively). The enhancer fragments of pri-miR-125a and MR genes were amplified by PCR with mouse genomic DNA as a template. These fragments and different truncations were inserted into pGL3-promoter to construct reporters (reporters 1–6 in Figure 3A; pGL3-MR). A DNA fragment containing 4 × hypoxia responsive elements (HREs) was synthesized and inserted into pGL3-promoter to construct pGL3-HRE. pEFBOS-NIC was as described (33). Full-length PU.1, IRF4 and RYBP cDNAs were amplified from a mouse cDNA library and inserted into pFlag-CMV2 to construct pFlag-PU.1, pFlag-IRF4, and pFlag-RYBP, respectively.

**Reporter assay**

HeLa cells were transfected with different combinations of reporters, expression vectors, and miRNA using Lipofectamine 2000™, with a Renilla luciferase vector (phRL-TK, Promega) as an internal control. Cells were harvested 24 or 48 h after the transfection, and luciferase activity was measured with Dual Luciferase Reporter Assay using a Gloma X™ 20/20 Luminometer (Promega).

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed by using a kit (Merck Millipore, Billerica, MA) according to the manufacturer’s instructions using anti-RBP-J or anti-NIC antibody. Collected immune complexes were extracted and analyzed by PCR using the primers listed in supplemental Table S1.

**Western blot**
Whole cell lysates were extracted with the RIPA buffer (Beyotime, Haimen, China). Protein concentration was determined with a BCA Protein Assay kit (Pierce, Waltham, MA). Samples were separated by SDS-PAGE, blotted onto polyvinylidene fluoride (PVDF) membranes and probed with primary and secondary antibodies (supplemental Table S2). Membranes were developed with chemo-luminescent reagents (Pierce).

**NO production**

BMDMs were cultured, and 50 μl culture supernatants were added to 50 μl Griess reagent I and 50 μl Griess reagent II (Beyotime). Absorbance was measured at 540 nm with a microplate reader.

**Statistics**

Images were imported into Image Pro Plus 5.1 software (Media Cybernetics Inc., Bethesda, MA) to quantify the densities of electrophoretic bands. Data were analyzed with Graph Pad Prism 5 software. Comparisons between groups were performed with unpaired Student’s T-test or the paired T-test. The results are expressed as the mean ± SD. *P* < 0.05 was considered significant.

**Results**

**Forced activation of Notch signaling in macrophages abrogated TAM phenotypes**

Notch1 was the dominant type of Notch receptors expressed in macrophages (supplemental Figure S1). Blocking Notch signaling by RBP-J disruption arrests TAM differentiation (20). To investigate the role of Notch signaling in TAMs after differentiation, we employed mice with a NIC transgene controlled by Lyz2-Cre.
(hereafter named as NICcA), which was expected to have sufficient TAM differentiation. No obvious abnormal myeloid development was noticed in NICcA mice (data not shown). In NICcA mice, the growth of subcutaneously inoculated LLC and B16F tumors was delayed significantly (Figure 1A, supplemental Figure S2A). The number of TAMs showed no significant difference between the NICcA and control mice (supplemental Figure S2B, S3A, S3B).

Although TAMs in NICcA mice expressed comparable levels of MHC II and VCAM-1, they expressed significantly lower level of MR (supplemental Figure S3A). qRT-PCR confirmed that several M1 markers increased and M2 markers decreased significantly in sorted TAMs from LLC tumors on NICcA mice (Figure 1B), suggesting a loss of TAMs phenotypes. Consistently, CD11b+Ly6G+ MDSCs decreased while CD8+ cytotoxic T-cells increased in LLC tumors on NICcA mice (Figure 1C; supplemental Figure S3C, S3D). Tumor vasculature also decreased remarkably (Figure 1D). These results suggested that forced Notch activation in macrophages subverted TAMs phenotypes.

**Identification of miR-125a as a downstream molecule of Notch signaling in macrophages**

To identify molecules downstream to Notch signaling in regulating TAMs, we compared miRNA profiles between LPS-activated BMDMs derived from RBP-J^ic^KO and control mice. Thirteen miRNAs exhibited differential expression between RBP-J-deficient and control macrophages (Figure 2A). miR-125a was chosen for
further investigation because several recent reports have highlighted its role(s) in macrophages (26-29).

We examined miR-125a expression in RBP-J deficient (RBP-J<sup>cKO</sup>) and Notch-activated (NIC<sup>cA</sup>) macrophages stimulated with PBS (M0), LPS+IFN-γ (M1), or IL-4 (M2). The results showed that M1 polarization led to a significant upregulation of miR-125a while RBP-J deficiency led to its downregulation in macrophages (Figure 2B). Constitutive Notch activation upregulated miR-125a, even in unstimulated macrophages (Figure 2B). During the <i>in vitro</i> differentiation of monocytes into macrophages, the level of miR-125a increased steadily, in correlation with RBP-J and Hey1 expression (Figure 2C). Blocking Notch signaling by disrupting RBP-J or with GSI significantly suppressed miR-125a upregulation during macrophage differentiation <i>in vitro</i> (Figure 2D; supplemental Figure S4).

To access the regulation of miR-125a expression by Notch signaling more specifically, we knocked down Notch1 expression in BMDMs using siRNA (supplemental Figure S5A). Notch1 knockdown resulted in attenuated M1 and strengthened M2 polarization of BMDMs (supplemental Figure S5B) (17). Notch1 knockdown also reduced miR-125a expression during <i>in vitro</i> macrophage differentiation and activation (Figure 2E; supplemental Figure S5C). Moreover, we activated Notch signaling in BMDMs with immobilized Notch ligand mD1R, and found that Notch activation resulted in upregulation of miR-125a (Figure 2F; supplemental Figure S6). These data suggest that miR-125a is a downstream molecule of Notch signaling in macrophages.
Notch signaling directly transactivated the enhancer of \( \text{pri-miR-125a} \) through an RBP-J-binding site

The \( \text{pri-miR-125a} \) gene is located upstream to the \text{sperm acrosome-associated protein} (Spaca) 6 gene (NC_000083.6) (30). We performed 5′-RACE to determine the transcription starting site of \( \text{Spaca6/pri-miR-125a} \) using cDNA generated from BMDMs (supplemental Figure S7A). Sequencing the 5′-RACE-amplified fragment identified a novel exon (exon 1′) located 3.6 kb upstream to exon 1 of \( \text{Spaca6} \) (GenBank accession number KP893886), and an alternative splicing acceptor within exon 1 (supplemental Figure S7B, S7C). The first intron of \( \text{Spaca6A} \) contains three pri-miRNA genes including \( \text{pri-miR-99b, pri-let-7e, and pri-miR-125a} \), and a putative enhancer element containing recognition sites for YY1, MYB, Smad3 and RBP-J (supplemental Figure S8).

The full-length and truncated \( \text{pri-miR-125a} \) enhancer fragments were inserted into a pGL3-promoter to construct serial reporter genes (Figure 3A, left). HeLa cells were co-transfected with pEFBOS-NIC and different reporter constructs. All constructs with the RBP-J-binding site (reporters 1, 3, 4, and 5) were transactivated by NIC, and disruption of this site by mutagenesis (reporter 6) prevented NIC-mediated transactivation (Figure 3A, right). ChIP assay indicated that, consistent with the miR-125a expression pattern, occupation of the RBP-J-binding site by RBP-J and NIC increased significantly on day 3 of BMDM differentiation \textit{in vitro} (Figure 3B). miR-99b was regulated coordinately by Notch signaling in BMDMs (Figure 2E, 2F,
3C; supplemental Figure S5C). These data indicated that Notch signaling directly transactivated the enhancer of pri-miR-125a through the RBP-J-binding site.

**miR-125a functioned downstream to Notch signaling to promote M1 and suppress M2 polarization**

BMDMs from normal mice were transfected with miR-125a mimics or control oligonucleotides and stimulated with PBS, LPS+IFN-γ or IL-4 for 24 h. qRT-PCR showed that miR-125a upregulated the M1 markers iNOS, IL-12 and TNF-α, and downregulated the M2 marker MR in BMDMs (Figure 4A). Moreover, transfection of miR-125a into BMDMs enhanced NO production (Figure 4B). We also co-cultured miR-125a-transfected BMDMs with allogenic naïve T-cells, and found that miR-125a-overexpressing BMDMs promoted stronger T-cell proliferation (Figure 4C). In addition, miR-125a-transfected BMDMs exhibited enhanced bacterial phagocytosis, consistent with enhanced M1 polarization (supplemental Figure S9). These results suggested that miR-125a promoted M1 and suppressed M2 polarization. In line with this finding, transfection of an ASO of miR-125a downregulated M1 markers and upregulated MR (Figure 4D).

In cultured BMDMs, Notch signaling enhanced M1 while suppressed M2 polarization (supplemental Figure S5B, S10) (17). BMDMs were prepared from RBP-J^KO and control mice and transfected with miR-125a mimics or control oligonucleotides, and stimulated with PBS, LPS+IFN-γ or IL-4. While RBP-J disruption downregulated iNOS and TNF-α and upregulated MR, transfection of
miR-125a reversed these changes (Figure 4E). These results indicated that miR-125a acted downstream to Notch signaling to regulate macrophage polarization.

**miR-125a targeted FIH1 and IRF4 to enhance M1 and attenuate M2 polarization simultaneously**

The 3′-UTRs of FIH1 and IRF4 are potential miR-125a targets (supplemental Figure S11A, S12A). BMDMs were transfected with miR-125a, and the expression of FIH1 and IRF4 was determined with Western blot. FIH1 and IRF4 expression decreased significantly in BMDMs transfected with miR-125a (Figure 5A). Reporter assay showed that miR-125a reduced luciferase activity in cells transfected with reporters containing the wild-type 3′-UTR of FIH1 or IRF4, and disruption of the proximal seed sequence (302-309 bp) in the FIH1 3′-UTR or the unique seed sequence in the IRF4 3′-UTR abrogated this effect (Figure 5B, 5C). These data suggested that miR-125a downregulated FIH1 and IRF4 in macrophages through their 3′-UTRs.

FIH1 suppresses Hif-1α activity, which promotes M1 polarization through glycolysis and iNOS (34). Culturing BMDMs under hypoxia or knockdown of FIH1 with siRNA upregulated iNOS in BMDMs (supplemental Figure S11B, S11C). Transfection with miR-125a enhanced pGL3-HRE transactivation mildly in BMDMs, suggesting that miR-125a enhanced Hif-1α activity (supplemental Figure S11D). On the other hand, IRF4 enhances M2 polarization (35, 36). IRF4 knockdown downregulated the M2 marker MR (supplemental Figure S12B). IRF4 binds to PU.1, and there are four PU.1-binding sites in or near the MR promoter (37, 38).
(supplemental Figure S12C). ChIP assay confirmed that IRF4 could be recruited to the PU.1 site in the first intron of the MR gene (supplemental Figure S12C), and transactivated the MR enhancer reporter (pGL3-MR) in RAW264.7 cells (supplemental Figure S12D), which was dependent on PU.1 in NIH3T3 cells (supplemental Figure S12E). Consistently, transfection of miR-125a suppressed pGL3-MR transactivation in RAW264.7 cells (supplemental Figure S12F).

To further evaluate the contribution of FIH1 and IRF4 to miR-125a-mediated macrophage polarization, we co-transfected BMDMs with a miR-125a ASO and siRNA targeting FIH1 or IRF4. The miR-125a ASO downregulated M1 markers IL-12, iNOS and TNF-α, and upregulated the M2 marker MR. FIH1 knockdown partially rescued the effect of the miR-125a ASO by increasing IL-12, iNOS and TNF-α expression (Figure 5D). On the other hand, IRF4 knockdown reduced MR expression and nearly reversed miR-125a ASO-mediated MR upregulation (Figure 5E). These results suggested that miR-125a promoted M1 and suppressed M2 polarization simultaneously through FIH1-HIF-1α pathway and IRF4, respectively.

**Macrophages overexpressing miR-125a exhibited strong anti-tumor activity**

The expression of miR-125a was upregulated in sorted TAMs overexpressing NIC (Figure 6A). We transduced BMDMs with a lentivirus overexpressing miR-125a and EGFP, with lentivirus expressing EGFP only as a control. These BMDMs were mixed with LLC cells and inoculated in normal mice. miR-125a-overexpressing macrophages strongly repressed tumor growth (Figure 6B). FACS analysis of tumoral macrophages indicated that in the EGFP+ compartment, macrophages overexpressing
miR-125a expressed a higher level of iNOS and a lower level of MR, suggesting that they were M1-polarized (Figure 6C). Interestingly, in the EGFP− compartment most, if not all, host-derived macrophages were also M1-polarized (Figure 6C, right). The expression of pri-miR-125a, iNOS, TNF-α, and IL-12 increased while the expression of MR, IL-10 and TGF-β decreased significantly in tumors containing miR-125a-overexpressing macrophages (Figure 6D). In addition, the number of CD11b+Ly6G+ MDSCs decreased in tumors and spleens, while CD8+ T-cells increased in tumors and lymph nodes, of mice bearing tumors containing miR-125a-overexpressing macrophages (Figure 6E). These results suggested that macrophages overexpressing miR-125a skewed the immune microenvironment of the tumors into an anti-tumor state. Moreover, BMDMs transfected with miR-125a exhibited stronger phagocytic activity to L1210 leukemia cells in vitro, suggesting that miR-125a enhanced direct anti-tumor activities of macrophages (Figure 6F).

**miR-125a amplified its own expression through RYBP**

RYBP mRNA was another predicted potential target of miR-125a (supplemental Figure S13A). RYBP binds YY1 to suppress transcription (39, 40). Two YY1 recognition sites exist in the pri-miR-125a enhancer (supplemental Figure S7, S8). Therefore it is likely that miR-125a augments its own expression in macrophages through a negative feedback loop composed of miR-125a - RYBP/YY1 - pri-miR-125a enhancer. BMDMs were transfected with miR-125a. Western blot showed that miR-125a efficiently downregulated RYBP expression (Figure 7A). Consistently, reporter assay indicated that miR-125a suppressed a reporter encoding
the 3’-UTR of RYBP mRNA (Figure 7B). RYBP overexpression significantly downregulated miR-125a and miR-99b in RAW264.7 cells (supplemental Figure S13B), and knockdown of YY1 with siRNA reversed the downregulation of miR-125a resulted from ectopic RYBP overexpression (Figure 7C). Reporter assay showed that RYBP overexpression suppressed transactivation of a reporter containing the full length pri-miR-125a enhancer fragment (reporter 1 in Figure 3A), and this suppression was reversed by knockdown of YY1 with siRNA (Figure 7D), suggesting that RYBP inhibited miR-125a expression through YY1. In BMDMs transfected with miR-125a, the expression of both pri-miR-125a and miR-99b was enhanced (Figure 7E). These data suggested that miR-125a could positively regulate its enhancer to augment its expression in macrophages.

Discussion

A novel mechanism mediating the regulation of TAMs by Notch signaling. Conditional deletion of RBP-J abrogated CD11c⁺ TAMs while maintained MHCII⁺CD11b⁺ TAMs in the MMTV-PyMT mammary tumor model, suggesting that Notch signaling is specifically required for the differentiation of TAMs (20). In this study, to overcome this RBP-J disruption-mediated developmental arrest of TAMs, we employed conditionally activated NIC transgenic mice. FACS analyses of macrophages in tumors suggested that TAMs existed in a similar number as in the control. This provided us a chance to evaluate the consequence of Notch activation on TAMs phenotypes. Our results indicated that TAMs with forced Notch activation exhibited M1 phenotype and anti-tumor activity. Therefore, in addition to
supporting TAMs differentiation, Notch signaling represses the tumor-promoting activity of TAMs (supplemental Figure S14). However, due to the limitations of the cancer model and gene-modified mice used in this study, more efforts are required to clarify other potential mechanisms underlying the discrepancy between the phenotypes of CD11c-Cre-mediated RBP-J cKO [20] and Lyz2-Cre-mediated NIC\textsuperscript{cA} mice (this study). First, the consequence of NIC overexpression may not be exactly compatible with the phenotype of RBP-J disruption due to the existence of noncanonical Notch signaling that is independent on RBP-J. Second, different Cre transgenic mice were used in the two studies, which might impact differentially on other myeloid cells such as neutrophils in addition to macrophages. Third, LLC cells used in this study might exert different influence on macrophages from that of the MMTV-PyMT mammary cancer cells used in the RBP-J cKO study. Finally and most importantly, it could not be excluded that subcutaneously inoculated LLC tumors might contain different subsets of macrophages from the MMTV-PyMT mammary tumors. How Notch signaling regulates these different subsets of macrophages remains an open question.

Notch signaling modulates the activation of macrophages by targeting a variety of downstream molecules (12-19, 41). Moreover, macrophages, regardless of their embryonic or BM origins, proliferate \textit{in situ} at sites of inflammation, such as cancer (4, 42). This raises the question of how polarized macrophages maintain their phenotypes. miRNAs are a class of non-coding RNAs involved in epigenetic regulation and macrophage activation (43). We identified miR-125a as a novel target
of canonical Notch signaling in macrophages. As a downstream molecule of Notch signaling that regulates macrophage activation, miR-125a has several important properties. First, it enhances M1 polarization and suppresses M2 polarization simultaneously. We have previously shown that disrupting Notch signaling reduces M1 and increases M2 macrophage polarization, even in the presence of M1 inducers (17). We and others have also identified IRF8, CYLD and SOCS3 as downstream targets of Notch signaling in the regulation of macrophage activation (17-19). However, these models consider that M1 polarization is a result of Notch activation and M2 polarization represents a “default” state. In this study, we showed that Notch signaling led to the upregulation of miR-125a, which may actively enhance M1 and suppress M2 polarization through FIH1 and IRF4, respectively (supplemental Figure S14). Second, Foldi et al have shown that the Notch pathway amplifies its own signaling during macrophage activation (44). miR-125a may participate in the self-amplification of Notch signaling strength during macrophage activation by upregulating its own expression through suppressing RYBP, which functions as a transcriptional repressor of the Spaca6A/pri-miR-125a enhancer via interaction with YY1. Last but not least, it has been widely accepted that macrophages proliferate in situ in solid tumors to elicit long-lasting pro-tumor activities (4, 42). Notch-miR-125a signaling may play a role in the epigenetic memory associated with the immune-suppressing and tumor-promoting capacities of TAMs. Further experiments are required to clarify this possibility.
miR-125a expression has been shown to be higher in M2 macrophages and to promote their polarization by targeting KLF13 (26, 27). However, in the current study, we showed that miR-125a was induced in M1 macrophages after LPS+IFN-γ stimulation and that miR-125a overexpression promoted M1 polarization by targeting FIH1 while inhibiting M2 polarization by targeting IRF4. This inconsistency might be a consequence of different experimental conditions employed during the induction of macrophage activation and polarization. In contrast with GM-CSF and M-CSF, which have been previously used to induce M1 and M2 macrophage polarization, respectively (26), we employed LPS+IFN-γ or IL-4 stimulation. Other studies have also shown that miR-125a expression is up-regulated after stimulation with LPS, consistent with our observation and other reports (24). In addition, it should be noted that FIH1 inhibits both Hif-1α and Hif-2α, which play opposite roles in macrophage polarization (34). Therefore, it cannot be formally excluded that under specific conditions, miR-125a might also enhance M2 macrophage activation. A recent report has unveiled other activities of miR-125a (30), also prompting further investigations.

Notch-miR-125a signaling might also be involved in monocyte-to-macrophage differentiation in vitro (Figure 2C; supplemental Figure S1A). Shroeder and Just have reported that Notch signaling promotes myeloid differentiation through RBP-J (45). However, RBP-J KO does not significantly change the number of myeloid colony-forming units in BM (31). Notch signaling has been implicated in various myeloid malignancies (46). Disruption of this signaling pathway leads to
myeloproliferation in mice (47, 48). Additional studies are required to elucidate the mechanism by which Notch signaling regulates myeloid development.

Education of macrophages to elicit anti-tumor activities using miR-125a.

TAMs have been widely recognized as a major tumor-promoting population during tumor initiation, growth, invasion and metastasis (1-3). Macrophage depletion shows tumor-repressive effects in several experimental systems (7, 8). However, as an important cell population in innate immunity, macrophages may also elicit anti-tumor activities (7). It has been speculated that TAMs can be “educated” to perform anti-tumor functions, if their phenotypes are reversible. Indeed, blocking CSF1R signaling in TAMs leads to the conversion of M2-like macrophages to M1-like macrophages (49). Moreover, a recent study has shown that low-dose irradiation of tumors induces massive increases in M1 macrophages in the tumors (9). The results of this study indicated that miR-125a-overexpressing macrophages exhibited strong anti-tumor activities. These macrophages possessed M1 characteristics and enhanced phagocytosis of tumor cells. More importantly, miR-125-overexpressing macrophages exhibited M1-polarized characteristics with increased secretion of TNF-α and IL-12. The immune-microenvironment molded by miR-125a-overexpressing macrophages had higher levels of TNF-α and IL-12 and lower levels of IL-10 and TGF-β, which mediate M1 and M2 polarization, respectively (1, 2, 7). This cytokine milieu would polarize newly recruited macrophages into M1 directly or indirectly, accompanied by enhanced CD8⁺ T-cell infiltration and diminished MDSC recruitment. Moreover, these macrophages proliferated in tumors while retaining their M1 characteristics,
which strengthened their anti-tumor activities. Therefore, our findings may facilitate the development a new therapeutic strategy for tumors based on Notch- or miR-125a-modified macrophages in the future.

Acknowledgments

We thank HL Li and K Rajewsky for mice. The study was performed at the Graduates Innovation Center of the Fourth Military Medical University.

Grant Support

Grants from Ministry of Science and Technology (2015CB553702), National Natural Science Foundation of China (31130019, 31371474, 31570878, 81530018, 31301127, 81170963, 31071291).
References


10. Artavanis-Tsakonas S, Rand MD, and Lake RJ. Notch signaling: cell fate control


Figure legends

Figure 1. Activation of Notch signaling in macrophages repressed tumor growth accompanied by diminished TAM phenotypes. A, NICcA and control (Ctrl) mice were inoculated s.c with $5 \times 10^6$ of LLC cells. Tumors were dissected on day 28 after the inoculation, photographed, and tumor weights were measured. Tumor size was monitored from day 12 after the inoculation. B, CD11b$^+$F4/80$^+$ macrophages were sorted from the tumors, and the expression of the indicated molecules was determined by using qRT-PCR. C, Single cell suspensions were prepared from the tumors, and analyzed by FACS after staining as indicated (supplemental Figure S3C, 3D). MDSCs (CD11b$^+$Ly6G$^+$) and cytotoxic T-cells (CD8$^+$) were quantitatively compared. D, Tumors were sectioned and stained with anti-CD31 followed by counter-staining with Hoechst. Pixels for CD31 were quantitatively compared. Bars, mean ± SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 2. miR-125a was a downstream molecule of Notch signaling in macrophages. A, BMDMs were prepared from RBP-J$^{cKO}$ and Ctrl mice and activated by LPS. miRNA expression was profiled by using microarray hybridization ($n = 3$), and confirmed by using qRT-PCR ($n = 5$). B, BMDMs from RBP-J$^{cKO}$ and Ctrl mice (left), or NICcA and Ctrl mice (right), were stimulated with PBS, LPS+IFN-γ or IL-4. miR-125a expression was determined by using qRT-PCR ($n = 4$). C, Monocytes from normal mice were cultured in the presence of GM-CSF for 4 days. RBP-J mRNA, Hey1 mRNA, and miR-125a were determined on days 0, 1, 2, 3, and 4 ($n = 3$). *, Comparison with day 0; #, comparison with day 1. D, Monocytes from Research.
RBP-J<sup>ckKO</sup> and Ctrl mice were induced to differentiate into BMDMs. miR-125a expression was detected using qRT-PCR (n = 3). E, BMDMs from normal mice were stimulated with PBS, LPS+IFN-γ or IL-4 in the presence of Ctrl, siRNA1, or siRNA2 to Notch1 (supplemental Figure S5A). The expression of miR-125a and miR-99b was determined by qRT-PCR. F, Purified mD1R protein was coated on cultured dishes. BMDMs from normal mice were seeded and cultured in the presence of LPS+IFN-γ. The expression of miR-125a and miR-99b was determined by qRT-PCR. Bars, mean ± SD; *, P < 0.05; ** and ###, P < 0.01; ***, P < 0.001.

**Figure 3. Notch signaling directly regulated the enhancer of pri-miR-125a.**

A, Reporter assay. HeLa cells were transfected with reporters containing different truncated or mutated pri-miR-125a enhancer, together with different amounts (0, 50, or 100 ng) of pEF-BOS-NIC. Relative luciferase activity was determined 48 h after the transfection (n = 6). B, Normal BM monocytes were cultured with GM-CSF and collected on days 0, 1, and 3 for ChIP with IgG, anti-RBP-J or anti-NIC antibody. The precipitated chromatin DNA was amplified by PCR and analyzed on a 2% agarose gel (lower, for day 3) and qPCR (upper) (n = 4). C, pri-miR-125a (left) and miR-99b (right) were determined in differentially stimulated BMDMs derived from RBP-J<sup>ckKO</sup> and Ctrl mice (n = 4). Bars, mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Figure 4. miR-125a regulated macrophage polarization downstream to Notch signaling.** A, BMDMs were transfected with miR-125a mimics or control oligonucleotides and stimulated with PBS, LPS+IFN-γ or IL-4. The expression of iNOS, IL-12, TNF-α and MR was determined by using qRT-PCR (n = 3). B, NO
production was measured in BMDMs in (A). C, BMDMs in (A) were irradiated and co-cultured with CFSE-loaded allogeneic T-cells for 24 h. T-cell proliferation was determined by FACS (n = 6). D, BMDMs from wild-type mice were transfected with miR-125a ASO or control and stimulated with PBS, LPS+IFN-γ or IL-4. The expression of iNOS, IL-12, TNF-α and MR was determined with qRT-PCR (n = 3). E, BMDMs derived from RBP-γKO and control (Ctrl) mice were transfected with miR-125a mimics or control oligonucleotides (Ctrloligo) and treated with PBS, LPS+IFN-γ or IL-4. The expression of iNOS, TNF-α and MR mRNA was determined with qRT-PCR (n = 3). Bars, mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Figure 5.** miR-125a promoted M1 and suppressed M2 polarization by targeting FIH1 and IRF4, respectively. A, Normal BMDMs were transfected with miR-125a mimics or control, and stimulated with PBS, LPS+IFN-γ or IL-4, followed by Western blot 48 h after the transfection. The relative FIH1 and IRF4 protein levels were quantitatively compared (lower; n = 4). B and C, HeLa cells were transfected with miR-125a mimics or control, together with reporters containing wild-type and mutant 3’-UTRs of FIH1 (B) or IRF4 (C). Luciferase activity was determined 24 h after the transfection (n = 4). D and E, Normal BMDMs were transfected with miR-125a ASO or control, together with siFIH1 (D) or siIRF4 (E). Cells were stimulated with PBS (M0), LPS+IFN-γ (M1) or IL-4 (M2), and the expression iNOS, IL-12, TNF-α and MR was determined by qRT-PCR (n = 3). Bars, mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.

**Figure 6.** Macrophages overexpressing miR-125a exhibited strong anti-tumor
activity. A, TAMs were sorted from tumors in Figure 1A, and miR-125a expression was determined by qRT-PCR (n = 5). B, Normal BMDMs were transduced with a lentivirus overexpressing miR-125a and EGFP or EGFP only (Ctrl). Cells (1 × 10^6) were mixed with LLC cells (5 × 10^6) and injected s.c in normal mice. Tumor sizes were monitored on the indicated days (left), and their weights were measured on day 21 after the inoculation (right; n = 5). C, Macrophages (CD11b^+F4/80^+) in tumors in (B) were analyzed by FACS after staining for cytoplasmic iNOS and MR. The numbers of iNOS^+ and MR^+ macrophages in the EGFP^+ and EGFP^− compartments were compared. D, Total RNA was extracted from the tumors described in (B), and the pri-miR-125a, iNOS, TNF-α, IL-12, MR, IL-10, and TGF-β were determined by qRT-PCR (n = 5). E, Tumor-infiltrating cells, splenocytes, and lymph node (LN) cells from the mice described in (B) were analyzed by FACS after staining with anti-CD11b and anti-Ly6G or anti-CD3 and anti-CD8. The numbers of MDSCs (CD11b^+Ly6G^+, left) and CD8^+ T-cells (CD3^+CD8^+, right) were compared (n = 4). F, Normal BMDMs transfected with miR-125a mimics or control (Ctrl) were stimulated with PBS or LPS+IFN-γ. Cells (1 × 10^5) were co-cultured with CFSE-labeled L1210 cells (1 × 10^6) for 2 h and observed under a fluorescence microscope (upper). The numbers of tumor cells engulfed per macrophage were compared (lower; n = 6). Bars, mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.

Figure 7. Self-amplification of miR-125a expression through RYBP/YY1. A, Normal BMDMs transfected with miR-125a mimics or control were stimulated with PBS, LPS+IFN-γ or IL-4. RYBP expression was determined by Western blot 48 h
after the transfection (upper), and the relative RYBP protein levels were quantitatively compared (lower) (n = 3). B, HeLa cells were transfected with miR-125a mimics or control, together with reporters containing wild-type and mutant 3’-UTRs of RYBP. Luciferase activity was determined 24 h after the transfection (n = 6). C, RAW264.7 cells were transfected with pFlag-RYBP or control, plus siYY1 or control, and miR-125a was determined by qRT-PCR (n = 3). D, HeLa cells were transfected with the reporter 1 in Figure 3A, together with siYY1 and pFlag-RYBP. Luciferase activity was determined 48 h after the transfection (n = 3). E, BMDMs transfected with miR-125a mimic or control (Ctrl) were stimulated with PBS, LPS+IFN-γ or IL-4 for 24 h. The expression of pri-miR-125a and miR-99b was determined by qRT-PCR 48 h after the transfection (n = 3). Bars, mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.
Fig 2

A. Heatmap showing the expression levels of various miRNAs in Ctrl and RBP-J^ΔΔKO cells. The colors represent the relative expression levels, with red indicating higher expression and green indicating lower expression.

B. Bar graph showing the relative levels of miR-125a in different conditions: Ctrl, PBS, LPS+IFN-γ, and IL-4. The asterisks indicate statistical significance.

C. Graph showing the relative levels of Hev1 and miR-125a in RBP-J^ΔΔKO cells over different days of GM-CSF stimulation. The asterisks indicate statistical significance.

D. Bar graph showing the relative levels of miR-125a in WT and N1C^ΔΔKO cells in different conditions: Ctrl, PBS, LPS+IFN-γ, and IL-4. The asterisks indicate statistical significance.

E. Bar graph showing the relative levels of miR-125a and miR-99b in different treatments: PBS, LPS+IFN-γ, and IL-4. The asterisks indicate statistical significance.

F. Graph showing the relative levels of miR-125a and miR-99b over different days of GM-CSF stimulation. The asterisks indicate statistical significance.
**Fig 5**

**A**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PBS</th>
<th>LPS + IFN-γ</th>
<th>IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR</td>
<td>Ctrl</td>
<td>miR-125a</td>
<td>Ctrl</td>
</tr>
<tr>
<td>FIH1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

Relative Luc. activity

3'-UTR reporter

miR-125a

Ctrl | FIH1 | mut1 | mut2 |

**C**

Relative Luc. activity

3'-UTR reporter

miR-125a

Ctrl | IRF4 | mut |

**D**

<table>
<thead>
<tr>
<th>Ctrl</th>
<th>ASO</th>
<th>Ctrl + siFIH1</th>
<th>ASO + siFIH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**E**

<table>
<thead>
<tr>
<th>Ctrl</th>
<th>ASO</th>
<th>Ctrl + siIRF4</th>
<th>ASO + siIRF4</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Downloaded from cancerres.aacrjournals.org on January 3, 2021. © 2016 American Association for Cancer Research.
Forced Activation of Notch in Macrophages Represses Tumor Growth by Upregulating miR-125a and Disabling Tumor-Associated Macrophages

Jun-Long Zhao, Fei Huang, Fei He, et al.

Cancer Res Published OnlineFirst January 12, 2016.