RETRACTED ARTICLE: The Notch signal mediates macrophage polarization by regulating miR-125a/miR-99b expression

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**ABSTRACT**

We aimed to explore the mediating role of Notch signal in macrophage polarization. This signal was knocked out from macrophages of Lyz2 cre and RBP-J flox mice. Bone marrow-derived macrophages (BMDMs) were isolated and polarized. The expressions of polarization markers in BMDMs 24 h after transfection were detected by qPCR. After Notch knockout, the expressions of M1 markers decreased but those of M2 markers increased significantly. MiR-125a/miR-99b and Spac6 were highly and lowly expressed upon M1 and M2 polarizations, respectively. The expressions of experimental group were significantly lower than those of control group. Overexpression of miR-125a significantly promoted the expressions of M1 markers, whereas inhibited those of M2 markers. NO release in the culture supernatant of miR-125a overexpression group significantly exceeded that of control group. Transfection with miR-125a inhibitor significantly down-regulated IL-12 expression but up-regulated MR expression in BMDMs. The supernatant secreted by M1 macrophages significantly facilitated BS524 cell apoptosis, with miR-125a inhibitor significantly down-regulated IL-12 expression but up-regulated MR expression in BMDMs. The supernatant secreted by M1 macrophages significantly facilitated BS524 cell apoptosis, with up-regulated MR expression in BMDMs. The supernatant secreted by M1 macrophages significantly facilitated BS524 cell apoptosis, with up-regulated MR expression in BMDMs.

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**Introduction**

Tumours have seriously threatened human health worldwide [1], and are routinely treated by surgery, radiotherapy and chemotherapy. Although tumours can be effectively inhibited, residual tumour cells may cause recurrence, and patients may suffer from drug resistance during repeated treatment [2]. Therefore, researchers have endeavoured to seek targets for improving tumour therapy.

As vital cells in the innate immune system, macrophages are involved in organ development, injury repair, homeostasis maintenance and immune regulation. According to functions and morphologies, they are clarified into classically activated macrophages (M1 phenotype) and alternatively activated macrophages (M2 phenotype) [3]. Tumour-associated macrophages are one of the main immune cell subsets of infiltrating tumours. Tumour cells and macrophages can interact in a variety of ways, and macrophages can facilitate tumour growth by inhibiting immune responses and secreting growth factors. In turn, tumour cells can also enable macrophages to acquire tumour-promoting phenotype by secreting factors or affecting macrophage metabolism [4]. With strong plasticity, macrophages are subjected to functional and state changes depending on the microenvironment [5].

As a highly conserved signalling pathway regulating cell differentiation and development, the Notch pathway widely participates in the onset and progression of various diseases. During the onset and progression of tumours, Notch signal acts on epithelial–mesenchymal transition, angiogenesis, apoptosis and stem cell proliferation [6], and the signal in M1 macrophages is activated stronger than in M2 macrophages [7]. By knocking out the key transcription factor RBP-J of the Notch signalling pathway from mice, the Notch signalling pathway is blocked and macrophages undergo irreversible transformation into M2 phenotype, thereby facilitating tumour proliferation, migration, invasion and immune escape. Recently, Toritsuka et al. reported that RBP-J activated TLR4 via the classical Notch signalling pathway, then up-regulating the JNK pathway [8]. High IRF8 expression was caused through MNK1-e IF4E-IRAK2, thereby inducing the
expressions of MI markers and promoting the inflammatory responses of MI macrophages. Therefore, the Notch signalling pathway is involved in macrophage polarization regulation. Besides, a variety of miRNAs can regulate macrophage polarization [9,10], and changes of the Notch signalling pathway can significantly alter the expressions of miR-125a and miR-99b. Thereby motivated, we herein aimed to clarify the molecular mechanism by which the Notch signalling pathway regulated miR-125a/miR-99b expression to mediate macrophage polarization.

Materials and methods

Experimental animals and cells
RBP-J flox and Lyz2 Cre mice were purchased from Suzhou Industrial Park Ai’er Maite Technology Co., Ltd. (Suzhou, China). Breast cancer BS524 cell line was bought from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China).

Main reagents and antibodies
Eukaryotic expression vectors pCMV-Flag, pCMV-Myc, pGL3-promoter and pGL3-basic, as well as prokaryotic expression vector pET-32b were obtained from Invitrogen (Carlsbad, CA). MiR-125a mimic, miR-125a inhibitor and miR-99b mimic were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). PE anti-mouse F4/80, PE anti-mouse IL-12 and APC anti-mouse IL-6 were purchased from eBioscience (San Diego, CA). APC anti-mouse CD11b and biotin anti-mouse Gr-1 were obtained from BioLegend (San Diego, CA).

Mouse culture and genotype identification
Six-week-old wild type, RBP-J flox and Lyz2 Cre mice were cultured in an SPF animal house. With a light/dark cycle of 12/12 h, all the mice were given free access to food and water. Then the mice aged 8–10 weeks old were separated into different cages and mated.

The detailed steps of genotype identification are shown as follows. 1) Extraction of tail genomic DNA by phenol-chloroform: About 0.5 cm of tail from a 4-week-old mouse was collected, and completely digested with 500 µL of tail buffer (10 mM Tris-HCl, 75 mM NaCl, 25 mM EDTA and 1% SDS) and 5 µL of protease K (20 mg/mL) overnight at 55 °C. Afterwards, the tail tissue was centrifuged at 12,000 rpm for 3 min, and approximately 470 µL of the supernatant was collected, mixed with 470 µL of saturated phenol for 30 min, and centrifuged at 12,000 rpm for 10 min. Then 450 µL of the supernatant was collected, mixed with 300 µL of saturated phenol and a 300 µL mixture of chloroform–isopentanol (24:1) for 30 min, and centrifuged again at 12,000 rpm for 10 min. Subsequently, 400 µL of the supernatant was collected, mixed with a 400 µL mixture of chloroform–isopentanol for 30 min, and centrifuged at 12,000 rpm for 10 min. Then 350 µL of the supernatant was collected, completely mixed with 800 µL of cold absolute ethanol to yield flocculent precipitate, and centrifuged again at 12,000 rpm for 10 min to discard the supernatant, followed by addition of 500 µL of 75% ethanol and centrifugation at 12,000 rpm for 2 min. After the supernatant was discarded, the residue was oven-dried at 50 °C, kept to 1 min until ethanol vaporized. Then 100 µL of sterile deionized water was added to fully dissolve genome at 50 °C for 30 min, which was stored at −20 °C.

2) Genotype identification by PCR: Genotypes were identified with a 30 µL reaction system consisting of 15 µL of 2× Taq Master Mix, 0.3 µL of 100 µM upstream and downstream primers each, 13.4 µL of deionized water and 1 µL of genomic DNA. The primer sequences are listed in Table 1. PCR conditions for Lys2 Cre gene: Pre-denaturation at 94 °C for 5 min (denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min), and extension at 72 °C for 10 min, with 25 cycles of amplification. PCR conditions for RBP-J flox wild type and knockout genotypes: Pre-denaturation at 94 °C for 5 min (denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s), and extension at 72 °C for 10 min, with 35 cycles of amplification. The amplification products were subjected to 1.5% agarose gel electrophoresis, and photographed by a gel imaging system.

Breast cancer cell culture
BS524 cells and cryopreservation solution were added into a centrifuge tube with 9 mL of RPMI 1640 medium containing 10% foetal bovine serum (FBS), centrifuged at 1300 rpm for 3 min to discard the supernatant at negative pressure, resuspended in 8 mL of RPMI 1640 medium and centrifuged again to discard the supernatant. Finally, the cells were resuspended in 8 mL of RPMI 1640 medium and inoculated evenly into a 10 cm culture dish. The medium was refreshed and the cells were passaged according to their state. After digestion, the cells were counted using a cell counting chamber, subpackaged at 5 × 10⁴/tube, centrifuged, resuspended in 100 µL of normal saline, and placed on ice prior to use.
Establishment of tumour-bearing mouse model

Using Lyz2 Cre RBP-J<sup>flox</sup> and Lyz2 Cre RBP-J<sup>+/-flox</sup> as a pair, several pairs of knockout mice and their controls were selected, and each pair was female, from the same source and aged about 6 weeks old, also with similar body weights. One side of the back of a mouse was hair-trimmed to expose the skin which was thereafter injected with 100 μL of tumour cell suspension using a 1 mL syringe. After treatment, the mouse was returned to its cage.

Culture and induction of bone marrow-derived macrophages (BMDMs)

BMDMs were isolated and cultured as follows. The mice aged 8–10 weeks were sacrificed by cervical dislocation and sterilized with 75% ethanol for 10 min. The femur and tibia of hind limbs were carefully removed. The muscle tissues were separated and the ends were cut with scissors to expose the marrow cavity. BMDMs were pipetted by a 5 mL syringe, transferred to a 15 mL centrifuge tube, and centrifuged at 1200 rpm for 5 min at 4°C. The supernatant was discarded, and the cell masses were resuspended with erythrocyte lysis buffer for about 5 min at room temperature and centrifuged at 1200 rpm and 4°C for 5 min to discard the supernatant. Then the cell precipitate was resuspended with α-MEM containing 10% FBS and inoculated evenly into a 10 cm culture dish.

Macrophages were differentiated and induced as follows. The cells in the dish were cultured in a 37°C incubator for 12–18 h and adherent stromal cells, such as fibroblasts were removed through differential adherence, and the suspending cells were collected, centrifuged, resuspended with α-MEM containing 10% FBS, and seeded in a Petri dish or a culture plate at 2 × 10<sup>6</sup>/mL. Meanwhile, 40 ng/mL induction factor GM-CSF was added to differentiate the bone marrow cells into macrophages. The solution was changed by half or totally every three days, and the induction factor was added. The induction was ended 6–8 d later.

To identify BMDMs, the above macrophages were trypsinized and collected into a 15 mL centrifuge tube, blocked with 10 μL of 20% rat serum for 10 min, resuspended with 50 μL of flow cytometry buffer, and incubated with fluorescent antibodies APC anti-mouse CD11b and PE anti-mouse F4/80 for 30 min at 4°C in dark. After staining, the cells were washed twice with flow cytometry buffer, and added 500 μL of this buffer for flow cytometry. Finally, CD11b<sup>+</sup> F4/80<sup>+</sup> double positive cells were counted.

Induction of macrophage polarization

Primary macrophages were induced by LPS at a final concentration of 100 and 20 ng/mL IFN-γ into M1 macrophages, and into M2 macrophages with IL-4 at a final concentration of 20 ng/mL. Polarization type was identified 24 h after induction.

Identification of macrophage polarization

M1 and M2 markers were detected by RT-PCR. In detail, total RNA was extracted from polarized macrophages and reverse-transcribed into cDNA. The mRNA expression levels of molecular markers in differently polarized macrophages were detected with the same procedure. M1 markers: iNOS, IL-12, TNF-a and IL-6; M2 markers: Arg1, MR, IL-10, TGF-β, Ym-1 and Fizz1.

NO release was measured as follows. Cell culture medium was centrifuged, and 50 μL of the supernatant was added into a 96-well plate, followed by addition of 50 μL of solution I of Griess reagent and 50 μL of solution II into each well. The optical density at 540 nm was measured by a microplate reader to determine the NO release amount.

Cell transfection

After induction and differentiation of primary BMDMs, the supernatant was discarded and replaced with serum-free α-MEM, followed by addition of 2.5 μL/mL lipofectamine2000 and 5 nM/mL miRNA mimic/inhibitor. After 4–6 h, the serum-free medium was replaced with complete medium, and 40 ng/mL GM-CSF was added again. After 24 h, cell transfection was continued.

Detection of BS524 cell apoptosis by flow cytometry

After BS524 cells were cultured to the logarithmic growth phase, the medium was removed and replaced with a 1:1 mixture of polarized BMDM culture supernatant and fresh 10% FBS-containing RPMI 1640 medium. After 24 h of incubation, BS524 cells were digested and transferred to a 15 mL centrifuge tube. After washing twice with sterile PBS, the cells were resuspended in 500 μL of binding buffer and added into a clean flow cytometry tube. Subsequently, 5 μL of APC-annexin V was added, and the mixture was incubated at room temperature for 10 min in dark. After centrifugation with PBS, the cells were resuspended in 200 μL of binding buffer and then added 5 μL of PI. Finally, apoptosis was detected by flow cytometry.

Statistical analysis

All data were analysed by GraphPad Prism version 6.0 software (GraphPad Software, La Jolla, CA). The categorical data were expressed as mean ± standard deviation. Inter-group comparisons were conducted by the t test. p < .05 was considered statistically significant.

Results

M1 polarization of macrophages promoted by the Notch signalling pathway

Mouse genotype identification

Transgenic mice were crossed by Lyz2 Cre and RBP-J flox mice. According to Mendel’s law of inheritance, progeny can produce homozygous and heterozygous genotypes. Lyz2 Cre can express Cre enzyme only on one allele, and the Notch signal can be completely blocked by mating between homozygous Lyz2 Cre and RBP-J flox mice. Homozygous RBP-J flox and heterozygous RBP-J<sup>+/-flox</sup> mice were set as control group (Figure 1(A)) and experimental group (Figure 1(B)), respectively. Tails of the obtained mice were cut off, from
which DNA was extracted. Corresponding primers were used for PCR amplification, and their mouse genotype was identified by agarose electrophoresis.

Tumour growth in mice
BS524 cells were inoculated at $5 \times 10^6$/mouse into the lower back. One week later, the tumour length $L$ and width $S$ were measured with a vernier caliper. The tumour volume was calculated according to: $\text{volume} = L \times S^2 \times 0.51$. The above data were measured every day for two weeks, and then the tumour growth trend was observed. In the mice with Notch signal knockout, the tumour growth was significantly faster than that of control mice (Figure 2(A,B)). Meanwhile, the spleen of the experimental group was significantly larger than that of the control group (Figure 2(C)).

Culture, polarization induction and identification of primary macrophages
After primary bone marrow cells were induced and differentiated, the expressions of macrophage-specific molecules were identified by flow cytometry, and the induction efficiency was examined. After 6 d of induction, the proportion of macrophages to adherent cells was over 95% (Figure 3). Different cytokines were added to the macrophages to promote polarization in different directions. BMDM phenotype was observed under light microscope after 24 h, and the expression levels of polarization-related genes were measured. Induction using LPS/IFN-$\gamma$ facilitated the polarization of BMDMs to M1 macrophages with pseudopodia extension. IL-4 induction promoted the polarization of BMDMs to M2 macrophages with pseudopodia contraction and enlarged area (Figure 4).

Promotion of M1 polarization by Notch signal
After GM-CSF-induced differentiation and polarization, the mRNA expression levels of polarization-related genes were detected by qRT-PCR. After Notch knockout, the expressions of M1 markers iNOS and IL-12 decreased significantly, and those of M2 markers, such as MR increased significantly (Figure 5). Therefore, Notch facilitated M1 polarization.
Expression profiling of miRNAs in BMDMs

Cluster analysis of miRNAs
Cluster analysis was performed to plot miRNAs with FC (abs) of >2 and \( p < .05 \). As shown in Figure 6, the red and green miRNAs are up-regulated and down-regulated, respectively, and the black ones are not differentially expressed.

Differential expressions of miRNAs in BMDMs between experimental and control mice
A higher FC (abs) means a larger difference between two samples, and a lower \( p \) values mean that the difference is more reliable. Of the 4440 genes included in the miRNA spectrum, 460 miRNAs had FC (abs) of >2 and \( p < .05 \). They were thereafter sequenced and aligned with the mouse miRNAs in miRbase version 21 by miRDeeP software (Queensland, Australia), and 406 miRNAs with known sequences were screened. Among them, 103 had significant differential expressions between experimental and control groups. Compared with the control group, 48 miRNAs were up-regulated and 53 were down-regulated, of which 10 miRNAs had FC of >3 (Table 2). Thus, miR-125a and miR-99b with large FC values were selected for subsequent experiments.
Promotion of miR-125a/miR-99b expression in macrophages by Notch signal

MiR-125a and miR-99b belong to the same microRNA cluster, which are both located on mouse chromosome 17 and only 600 bp apart (Figure 7(A)). Primary BMDMs of both groups were differently polarized, and blank control was set. MiR-125a/miR-99b and Spaca6 were highly expressed upon M1 polarization, and lowly expressed upon M2 polarization. Meanwhile, the expressions of the experimental group were significantly lower than those of the control group (Figure 7(B)).

Promotion of M1 polarization response by miR-125a

Promotion of M1 polarization by miR-125a

After transfection for 24 h, the expressions of polarization markers in different types of macrophages were detected by real-time qPCR. Overexpression of miR-125a significantly promoted the expressions of M1 markers such as iNOS, IL-12 and TNF-α, whereas inhibited those of M2 markers, such as MR and TGF-β (Figure 8(A)). Additionally, the NO release level in the cell culture supernatant of miR-125a overexpression group significantly exceeded that of control group (Figure 8(B)). After transfection with miR-125a inhibitor, IL-12 expression in BMDMs was significantly down-regulated, but MR expression was up-regulated. In the meantime, the expressions of iNOS and TNF-α slightly decreased (Figure 9).

Enhancement of anti-tumour immune response of macrophages by miR-125a

We then assessed the effects of miR-125a on macrophage functions. After 24 h of co-culture, the supernatant secreted by M1 macrophages significantly facilitated BS524 tumour cell apoptosis, which was further enhanced after miR-125a overexpression (Figure 10(A)). In contrast, the effects on cell proliferation were similar (Figure 10(B)).

Promotion of M1 polarization response by miR-99b

After 24 h of polarization induction, the TNF-α expression of the miR-99b overexpression group increased significantly, whereas that of MR decreased significantly (Figure 11). Accordingly, miR-99b also facilitated the M1 polarization response of BMDMs.

Promotive effects of miR-125a on miR-125a/miR-99b expression cluster

After overexpression of miR-125a in vitro, the expression of miR-125a initial transcript was significantly up-regulated. In addition, the expressions of miR-99b and host gene Spaca6 were also significantly higher than those of the control group (Figure 12). Hence, miR-125a may promote its own transcription and expression through a positive feedback.

Using the 5’-RACE method, we found that the transcription of miR-125a was initiated at about 3200 bp upstream of the mature miRNA sequence, and the miR-125a/miR-99b expression cluster was located at the first intron of initial transcript. The intron contained an RBP-J specific recognition site, indicating that Notch may regulate the expressions of miR-125a/miR-99b and Spaca6 (Figure 13).

Discussion

As tumours develop, a series of changes will occur, such as immune evasion, replication immortalization, loss of energy...
Figure 7. Effects of Notch on miR-125a/miR-99b expression cluster. A: chromosomal locations of miR-125a/miR-99b; B: promotion of miR-125a/miR-99b and Spaca6 expressions by Notch.

Figure 8. Promotion of M1 polarization by miR-125a. A: expressions of M1 markers; B: release of NO.
metabolism, invasion and metastasis, among which the inflammatory microenvironment of tumours is receiving increasing attention [11,12]. How to control the tumour inflammatory microenvironment has gradually become one of the key links in the treatment of tumours and prolonging the survival of patients with cancer. Macrophages, as the main cell subpopulation of the inflammatory response of the body, play an important regulatory role in inflammatory

![Graphs and Diagrams](image-url)

**Figure 9.** Suppression of M1 polarization and promotion of M2 polarization by miR-125a inhibitor.

**Figure 10.** Effects of macrophage secreted supernatant on B524 cells. A: promotion of cell apoptosis after miR-125a overexpression; B: unaffected cell proliferation.
injury and tissue remodelling [13,14]. Macrophages have strong plasticity and can move towards different polarizations under different stimulation conditions: M1 macrophages are stimulated by LPS/IFN-γ, to induce Th1-type immune response, promote local tissue inflammation, and take the anti-tumour immune function [15]; M2 macrophages are stimulated by IL4 or immune complexes, to antagonize inflammatory response, induce Th2-type immune response, promote damage tissue repair, remodelling and angiogenesis, and promote tumourigenesis and development [16]. Therefore, fully understanding the regulation of different polarization modes of macrophages is of great significance for elucidating the molecular mechanism of tumour inflammatory microenvironment and then establishing rapid and effective treatment measures.

With the recent progress in the regulation of the Notch signalling pathway on the development and differentiation of the innate immune system, its influence on the functional plasticity of macrophages has attracted more and more attention [17,18]. Monsalve et al. reported that [19] BMDMs stably expressed ligands Jagged 1, 2 and receptors Notch 1, 2 and 4; when macrophages were induced and stimulated by M1 polarization, the expression levels of Notch1 and Jagged 1 were significantly up-regulated, suggesting that the Notch signalling pathway may be involved in the regulation of BMDM development and function. Subsequent studies have
confirmed that activation of the macrophage TLRs pathway can specifically up-regulate Notch 1 expression, thereby inducing downstream molecular expression and activating the Notch pathway; after inhibition of Notch signalling, release of the inflammatory factor IL-6 is reduced, IL-10 secretion increased, synthesis of iNOS and NO decreased, showing significant characteristics of M2 polarization. Activated DLL4 ligands can promote macrophage inflammatory responses. In this experiment, the blocking of Notch signalling was achieved by conditionally knocking out the key transcription factor RBP-J downstream of Notch signalling in macrophages, and the tumour growth of tumour-bearing mice blocking the Notch signalling pathway was faster than that of the control group. It is revealed that macrophages can affect tumour growth due to the absence of Notch signalling.

Wang et al. found that Notch signalling pathway can promote the polarization of macrophages to M1, but the specific molecular mechanism remains unclear. We postulated that epigenetic modifications may be involved in macrophage polarization regulation. In this study, knockout mouse-derived bone marrow was used to culture macrophages, achieving the goal of blocking Notch signalling. The changes in macrophage phenotype during polarization were observed. It was found that the macrophage morphology after polarization had characteristic changes during the experiment, that is, the M1 phenotype presented a narrow shape, while the M2 phenotype a flat adherence, suggesting that its morphological changes may be related to its implementation of different functions. Real-time quantitative PCR has found that the presence of Notch signalling makes macrophages easy to differentiate into M1 phenotype macrophages, and when blocking Notch signal, macrophages are more likely to differentiate into M2 phenotype.

It has previously been reported that a variety of miRNAs can regulate macrophage polarization. In this study, the chip results show that Notch signal knockout can affect the change of miRNA expression profile of macrophages. In this study, we intended to find out the molecular mechanism by which the Notch signalling pathway regulates macrophage polarization from non-coding RNA.

After verifying and analysing the previous chip results, it was found that Notch signalling can significantly promote the expression of miR-125a/miR-99b cluster. After overexpression of miR-125a and miR-99b, BMDMs significantly promoted M1 polarization and inhibited M2 polarization. Functional experiments also demonstrated the ability of BMDMs to promote apoptosis in tumour cells after miR-125a overexpression. Hematopoietic cells are regulated by many miRNA molecules during differentiation, which has a great influence on the development and maturation of immune cells. While specifically exploring the pro-inflammatory response of miR-125a, we were surprised to find that miR-125a could significantly promote the expression of itself, miR-99b and its host gene Spac6. To unravel the molecular mechanism of regulation, we used RACE to find the initial transcript Pri-miR-125a of this miRNA expression cluster, which contained a binding site of RBP-J at the first intron, where the positive regulation of Notch signalling was likely to be received.

## Conclusions

In summary, the Notch signalling pathway promoted the expression of miR-125a/miR-99b cluster in macrophages. After overexpression of miR-125a, macrophages secreted considerable inflammatory cytokines to kill tumour cells. Overexpression of miR-99b also facilitated inflammatory responses in macrophages. This pathway promoted macrophage M1 polarization by up-regulating miR-125a/miR-99b expression.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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