Original Paper

MicroRNA Quantitation During Dendritic Cell Endocytosis Using Imaging Flow Cytometry: Key Factors and Requirements

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Key Words

miRNA endocytosis • Flow cytometry • miR-590-5p • Dendritic cells • Argonaute 2 (AGO2) • FRET analysis

Abstract

Background/Aims: MicroRNA (miRNA)-induced suppression of dendritic cells (DCs) has been implicated in many diseases. Therefore, accurate monitoring of miRNA endocytosis by DCs is important for understanding the role of miRNAs in many diseases. Recently, a method for measuring the co-localization of Argonaute 2 (AGO2)-associated miRNAs on laser-scanning confocal microscopy method was proposed to localize the miRNAs. But its definition was limited by the number of observed cells through its accuracy. **Methods:** In this study, a method based on imaging flow cytometry was developed to localize miR-590-5p with fluorescent probes in DCs. miR-590-5p proven to play an important role in tumor immunity. This method enabled the quantification, visualization and localization of the fluorescence intensity in 30,000 individual cells. **Results:** Using this method, the DCs with different endocytotic ability were distinguished. The behaviour of miR-590-5p during endocytosis under the stimulation of tumor antigen in DCs was observed, binding to its cognate target mRNA and degradation in DCs. **Conclusion:** This method based on imaging flow cytometry provide an additional method to study miRNA processing in DCs, which makes it a valuable addition to existing miRNA research techniques

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Introduction

MicroRNAs (miRNAs) are evolutionarily-conserved small non-coding RNA molecules that are involved in the translational regulation or degradation of mRNAs by pairing to the untranslated region (UTR) [1, 2]. Dysregulation of miRNA profiles has been implicated in numerous diseases including cancer, and several therapeutic clinical trials are being conducted to modulate this mechanism [3]. Recent evidence has confirmed that cancer cells secrete certain miRNAs to regulate gene expression in normal cells, such as dendritic cells (DCs), to create the appropriate conditions for the growth and development of cancer cells [4]. Therefore, the availability of technology to accurately monitor how miRNAs enter cells and modulate mRNAs and their translation is important to understanding these mechanisms in many diseases [4-6].

The accurate observation and evaluation of miRNA endocytosis into cells and subsequent regulation of target mRNAs are difficult and typically requires the use of multiple techniques [7-10]. Briefly, quantitative PCR (qPCR) or luciferase reporter genes are the most frequently used methods to monitor levels of miRNAs and their interactions with target mRNAs, but they only indicate the presence of miRNAs and the degradation of target mRNAs [11, 12]. It is still challenging to monitor the behaviour of miRNA in processes such as endocytosis, exocytosis, and RNA interference in real time.

Recently, new technique that combine image flow cytometry with fluorescence resonance energy transfer (FRET) has emerged. One approach measures miRNA with fluorescein (FAM) or cyanine dye 3 (Cy3) fluorophores in tumour cell lines [13], which enables a more accurate observation of miRNA by fluorescence microscopy. The interaction of miRNAs and other molecules can be studied based by the FRET principle. Cytological analysis with labelled miRNA mimics however is limited by the inaccuracy of fluorescence microscopy in detecting miRNA behaviour [14]. This lack of accuracy is due to the low number of detected cells, with approximately 200–400 cells detected per field, though fluorescence microscopy is more accurate and quantitative in single cells [15]. Thus during key cellular events, subcellular information reflecting the cell population of interest is typically not accurate [16].

The ImageStream MkII is a flow cytometry-based platform that simultaneously captures multiple enhanced, separate, but spatially related images from different individual cells at a high acquisition speed of up to 1000 cells/sec [14]. The ability to perform photometric or morphometric analysis of the images of thousands of individual cells thus combines quantitative image analysis with the statistical power of flow cytometry [15]. This technology monitors miRNA behaviour using a fluorescence tag in a high-throughput approach that is amenable to statistical analysis. This feature makes image flow cytometry a potential candidate tool to enumerate cellular images associated with miRNA measurements [17].

DCs are innate immune cells that play key roles in tumour immunology. Recent studies have shown that tumour-specific miRNAs regulate the function of DCs in cancer patients [18-20]. Our previous study showed that miR-590-5p acts as a suppressor in DCs through the transforming growth factor, beta 1 (TGFβ1) pathway [21, 22]. In addition, it has been shown that DCs have endocytotic activity for miRNAs, but its mechanism is not well understood because of the lack of a monitoring method [23]. However, these properties make the DCs a suitable platform for the study of miRNA behaviour, especially endocytosis and RNA interference.

In order to further study the subcellular events of mir-590-5p in tumor-associated immune cells, especially the interaction between dendritic cells and mir-590-5p, a series of techniques based on flow cytometry were developed to detect how mir-590-5p is endocytosed and processed by dendritic cells. This study determines the internalization ratio of miR-590-5p, a suppressor miRNA secreted by tumour cells [24], during endocytosis into mouse bone marrow DCs, and observed that anti-miR-590-5p induced RNA interference through FRET analysis in real time. Moreover, the endocytotic ratio and degradation of mRNA by imaging flow cytometry were validated by qPCR.

Materials and Methods

Approval for experiments on mice was obtained from the Ethics Management Committees of the Second Military Medical University (ID: 81602707). All methods were carried out in accordance with the approved guidelines. All methods of flow cytometry were described according to the MIFlowCyt 1.0 guidelines [39]. All analysis files mentioned are available upon request.

Mouse xenograft and treatment

Thirty 6-week-old nude mice were used in this study. After the mice had been reared under sterile conditions for one week, the hair was removed with a shaving device. HepG2 cell suspensions with 10^7 cells were injected under exposed skin. 21 days after injection a mass of 3mm size appeared at the injection site. The solution of miR-590 and other mimics were injected into the mass (0.05 mg each). The treatment lasted 2 weeks. Intraperitoneal injection of the miR-590-5p-Cy3 into HepG2 tumor-bearing mice and detected Cy3 in peripheral blood mononuclear cells (PBMC) and organs (spleen, iliac lymph nodes, bone marrow and tumor).

Dendritic cell harvesting, culture and stimulation

Six-week-old male C57BL/6J mice (bred at the Experimental Animal Centre, Second Military Medical University) were maintained in a pathogen-free animal facility for one week prior to use.

Mouse bone marrow DCs (BMDCs) were isolated from the femur bone marrow of C57/BL6J mice. After removing red blood cells by lysis, bone marrow cells were cultured in RPMI1640 media containing 10% Fetal Bovine Serum (FBS), 1000 U/ml GM-CSF (R&D Systems, Minneapolis, MN, USA) and 1000 U/ml IL-4 (R&D Systems, Minneapolis, MN, USA) overnight. The next day, non-adherent cells were removed by pipetting gently, and the adherent cells were cultured in the same medium for two more days. These cells were sorted using CD11c magnetic beads (Miltenyi, Bergisch Gladbach, North Rhine-Westphalia, Germany). CD11c+ cells were activated using LPS (Sigma-Aldrich, St. Louis, Missouri, USA) for 24 hours [40].

Blood samples and PBMC isolation

Human peripheral blood samples were collected at Changhai Hospital, Affiliated Hospital - Shanghai Second Military Medical University. Informed consent was obtained prior to the collection of human samples. 10 ml whole blood were collected into coagulant tubes and 10 ml PBS was added and mixed. 10 ml Ficoll was add and centrifugal at 2000rpm for 20 minutes. PBMCs were drawn into another tube for further use.

Tumor antigen stimulation against dendritic cells

10^7 HepG2 cells were collected, washed in PBS and and broken by sonication. Cell lysates were centrifuged at 12000rpm for 30min and supernatant collected. After filtering with a $7\mu\text{m}$ filter, the filtrate was supplemented into culture system of DC cells with LPS.

Co-culture of dendritic cells with HepG2 tumor cells

HepG2 cells were prepared into 10^6 /ml cell suspensions and placed in Millicell standing cell culture ($0.4\mu\text{m}$, 24well CM, PICM01250). At the same time, activated DCs were placed in a 24-wells plate for culture. 24 hours later, half of the supernatant from the each Millicell was discarded and placed into the wells of 24-well plate containing the DCs.

miRNA and small interfering RNA (siRNA)

miRNA mimics, miRNA inhibitors, and other oligonucleotides were purchased from Gene Pharma (Suzhou, China). The sequences of the miRNAs were obtained from the miRbase (<http://microrna.sanger.ac.uk/sequences/>). Primer 3 software was used for primer design (<http://frodo.wi.mit.edu/>). FAM and Cy3 were used to label oligonucleotides at the 5'- end [41] and at the 3'- end [42], respectively. miR-

Table 1. RNA Oligonucleotides

Name	Sequence
miR-mock	UUGUACUACACAAAAGUACUG
miR-590-5p	GAGCUUAUUCUAAAAGUGCAG
Anti-miR-590-5p	CUGCACUUUUUAUGAAUACUC
miR-mock ^{FAM}	FAM-UUGUACUACACAAAAGUACUG
miR-mock ^{FAM-F}	FAM-UUGUACUACACAAAAGUACUG-O-Fluoro
miR-590-5p ^{Cy3}	GAGCUUAUUCUAAAAGUGCAG -Cy3
miR-590-5p ^{Cy3-F}	GAGCUUAUUCUAAAAGUGCAG -Cy3-O-Fluoro
Anti-miR-590-5p ^{FAM}	FAM- CUGCACUUUUUAUGAAUACUC
miR-590-5p ^{Fluor}	FAM- GAGCUUAUUCUAAAAGUGCAG -Cy3

mock-Fam-F and miR-590-5p-Cy3-F were purchased with 2'-O-fluoro-dNTP[43]. The oligonucleotides used are listed in Table 1.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA of DCs was extracted using TRIzol (Life Technologies, New York, USA) reagent according to the manufacturer's protocol. Reverse transcription was performed using Reverse Transcript Kit (QIAGEN, Duesseldorf, Germany). qPCR was performed with miScript SYBR Green qPCR kit (QIAGEN, Duesseldorf, Germany). miR-1228 amplification was performed as was negative control [38]. Beta-actin were amplified as a reference (Fig. 5A). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. All reactions were performed in triplicate. The used primers are listed in Table 2.

Transfection

For transfections of miRNA mimics, positive control, and negative control oligonucleotides into cells, Lipofectamine 2000 (Invitrogen, New York, USA) was used according to the manufacturer's instructions. LPS, ovalbumin and its modification were directly added into the DC culture system, relying on DC endocytosis to enter the cell. Silencing of the target molecules was confirmed by qPCR after transfection at the indicated time points.

Treatments

For the time kinetics of miRNA endocytosis DCs were incubated in RPMI 1640 medium with 5 μ M miR-590-5p-Cy3 for 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 or 6 h. For the time kinetics of miRNA binding, DCs were activated by LPS and incubated in RPMI1640 medium containing 10% FBS with 5 μ M miR-590-5p-cy3 and 5 μ M Anti-miR-590-5p-5p-fam for treatment durations of 6, 7, 8, 9, 10, 12, 15, 18 or 24 h. For the time kinetics of miRNA degradation, DCs were activated by LPS and incubated in RPMI 1640 medium containing 10% FBS with 5 μ M Anti-miR-590-5p and 5 μ M miR-590-5p-fluor for 6, 8, 10, 12, 15, 18, 24, 36 or 48 h. For experiments evaluating the internalization ratios under active conditions, cells were activated with LPS and incubated for 24 hours in the respective media in the presence of 10% FBS. The methods for estimating the internalization ratio are further described below under ImageStream MkII.

Imaging flow cytometry

The ImageStreamX MkII (Merck Millipore, Seattle, WA) one camera system with six lasers was used for the imaging flow cytometry assay. The ImageStreamX MkII system was calibrated with speed beads (Merck Millipore) before the test, and the sample data were acquired under laser excitation. A laser power of 200 mW at 488 nm and 50 mW at 561 nm was used for the endocytosis test, and a laser power of 200 mW at 488 nm only was used for the FRET test. Images (bright field in channel 1, FAM in channel 2 and CY3 in channel 4,) were acquired for each cell; 30,000 individual cells were analysed for each experimental sample, and 10,000 cells were acquired for each control sample. The software INSPIRE (version 6.0.154, Merck Millipore) was used for data collection. Analysis was performed on the compensated image files using algorithms in IDEAS (version 6.1.822.0, Merck Millipore) image analysis software.

For tissue specimens, the method of tissue homogenate is adopted. The tissue was ground into a single cell suspension and digest with collagenase. Products were washed with PBS and suspended. The obtained suspension is filtered with a filter of 400 mesh per inch², prior to application to flow cytometry.

Image Stream Analysis

After data acquisition, single cells were analysed by Area and Aspect Ratio Parameters. From each cell, the bright field, FAM or FITC (505–560 nm emission in Channel 2), and Cy3 (595–642 nm emission in Channel 4) were used for analysis. Internalization ratio analyses were performed using the wizard tools of IDEAS software. The spot count and mean fluorescent intensity of the DCs of interest were determined by employing hierarchical gating schemes and are described in the Results section.

Table 2. Primers (DNA Oligonucleotides)

Name	Sequence
miR-mock-Forward	TTGTACTACACAAAAGTACTG
miR-590-5p-Forward	GAGCTTATTCATAAAAGTGCAG
miR-1228-Forward	TCACACCTGCCTCGCCCCC
miRNA-RT-a	CACTGTCATGCCGTTAGGTAGCGTATCGTTGACAGC TTTT TTTT TTTT TTTT TTTT
miRNA-RT-c	CACTGTCATGCCGTTAGGTAGCGTATCGTTGACAGC TTTT TTTT TTTT TTTT TTTT
miRNA-RT-g	CACTGTCATGCCGTTAGGTAGCGTATCGTTGACAGC TTTT TTTT TTTT TTTT TTTT
R-miRNA-R1	TCATGCCGTTAGGTAGCGTA

The circularity feature measures the degree of the mask's deviation from a circle. Its measurement is based on the average distance of object boundary from its center divided by the variation and therefore the feature value will be high. Vice versa, the more the shape deviates from a circle, the higher the variation and the circularity value will be low.

Statistical Analysis

Data comparing multiple groups were analysed using one-way ANOVA (Microsoft Office Excel 2016, version 16.0.6); if $P < 0.05$, post-hoc testing was performed with Bonferroni correction for multiple comparisons (IBM SPSS Statistics 20.0). The comparisons made with the control groups are detailed in the Results section. The differences were considered statistically significant if the adjusted $P < 0.05$. Where only two groups were compared, the data were analysed using an unpaired two-tailed t test (Microsoft Office Excel 2016, version 16.0.6). Where applicable, the data are expressed as the mean $\pm$ standard error of the mean (SEM) from at least three replicates.

Western Blot

Proteins extracted from cultured cells were dissolved, homogenized, and quantified using BCA Protein Assay Kit (Pierce, Rockford, USA). Protein samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine. Membranes were blocked with 5% non-fat dry milk in PBS, 0.05% Tween-20 and probed with antibodies against ACTB and AGO2 (ab32381 and ab129348, respectively; Abcam, Cambridge, United Kingdom) according to manufacturer's instructions. Blots were developed with ECL reagent (Thermo, Boston, USA), and their images were captured in the FC2 Image Station (Alpha, Bellingham, USA).

Results

The internalization of miRNA as a measure of the target of endocytosis in DCs

DCs from mouse bone marrows were cultured as described in Methods. After sorting using CD11c magnetic beads, DCs were treated with or without lipopolysaccharide (LPS). As expected, DCs cultured in the presence of LPS showed a high level of expression of CD80 and CD86 (Fig. 1A), which are the indicators for the maturation and antigen presentation of DCs [25, 26].

In a further study of the endocytosis of miRNA in DCs, imaging flow cytometry was used to calculate the internalization ratio [27]. The internalization ratio, a parameter used to denote the volume of marked miRNA in a cell was measured by IDEA software and surface marker CD11c was used as a biomarker in DCs cultured with miR-590-5p-Cy3, which is proven to play an important role in the tumor immune process and was expressed and secreted by tumor cells. This was found to be a suppressor of DCs in a previous study [4], either under basal conditions (culture without LPS) or in the presence of LPS (Fig. 1B). The results showed that DCs cultured without LPS had relatively lower internalization ratios, in contrast to the previously published findings for proteins [28, 29]. However, most DCs cultured with LPS had relatively higher internalization ratios (Fig. 1B, left panel) and fluorescence intensity of Cy3 (Fig. 1B, upper and right panel), which indicated an increase in labelled miRNA endocytosis. Compared with basal treated cells, more LPS-treated cells had a rich dendritic structure, including a bigger circularity feature, a larger diameter and cross-sectional area, compared to basal treated cells (Fig. 1C, $n = 60,000$). Circularity feature is a parameter for evaluating the irregular cell contour in image flow cytometry system [30]. However, some LPS-treated DCs with low miR-590-5p-Cy3 fluorescence intensity had a high level of CD11c (Fig. 2, upper and lower panel), which indicates functional differentiation of DCs. Conversely, few DCs with high miR-590-5p-Cy3 fluorescence intensity had a low level of CD11c (Fig. 2, lower panel). These data show that the internalization ratio may be suitable for this type of analysis because it is able to distinguish DCs with different endocytotic ability of miR-590-5p-Cy3 [31].

Fig. 1. LPS influences the endocytosis of miR-590-5p and morphology of DCs. **A.** Ratio of CD80⁺/CD86⁺ mouse DCs by flow cytometry. DCs were cultured in the medium with or without LPS. Compared to DCs cultured in basal media (without LPS, left panel), more cells could be found in top right quadrant, which showed the DCs cultured with LPS (right) exhibited an increased ratio of CD80⁺/CD86⁺. **B.** Representative results of the cellular internalization ratio of Cy3 (Ex: 508-532 nm, Em: 568 nm) in DCs at 3 hours in culture with labelled miR-590-5p in the basal media or LPS treatment media (0.5 μg/ml) analysed by real-time imaging flow cytometry. CD11c, a biomarker of DCs, was labelled with FITC. Left upper: The values of MFI are indicated. MFI: Mean Fluorescent intensity of cells. Left lower: The values of internalization ratio are indicated. Mean ± SEM of three independent experiments. ** P < 0.01, n = 60,000. Right: Image of DCs. Scale bars represent 20 μm. SSC is side scatter, an indicator of a flow cytometry that roughly responds to fine particles in cells. **C.** Representative statistical analysis of the morphological parameters of DCs at 3 hours in culture with labelled miRNAs in basal media or LPS treatment media (0.5 μg/ml) analysed by real-time imaging flow cytometry. Left: Values of the circularity feature are indicated. Middle: Values of the mean diameter are indicated. Right: Values of the mean cross-sectional area are indicated. Mean ± SEM of three independent experiments. ** P < 0.01, n = 60,000.

Fig. 2. miR-590-5p endocytosis in dendritic cells treated by LPS. Excitation: 488nm(50mW) & 561nm (50mW). Upper: Cell image of dendritic cells with high level CD11c and Low Cy3 fluorescence. Lower: Ratio of CD11c+ and miR-590-5p-Cy3+ dendritic cells by image flow cytometry. Show the dendritic cells group with different miRNA-Cy3 endocytic ability

The timing and selectivity of miRNA endocytosis in DCs

To obtain a more accurate measure of endocytosis, the internalization ratios of miR-590-5p-Cy3 and miR-mock-Fam-F were compared. miR-590-5p-Cy3 was synthesized directly, but miR-mock-Fam-F was modified with 2'-O-fluoro (2'-O-methoxyethyl/2'OME), which was a common modification to increase RNA stability [32]. After incubation with miR-590-5p-Cy3 and miR-mock-Fam-F for 3 hours in basally treated DCs, the internalization ratio of Cy3 label in DCs increased significantly, but the internalization ratio of FAM label remained stable (Fig. 3A). The fluorescence intensity of Cy3 increased with the internalization ratio (Fig. 3B). To evaluate the impact of LPS, DCs cultured in the presence of LPS were incubated with miR-590-5p-Cy3 and miR-mock-Fam-F for 3 hours. As a result, LPS treatment increased the fluorescence intensity and internalization ratio of Cy3 in DCs but had little impact on the number of miR-mock-Fam-F spots (Fig. 3C). The results suggests that the phagocytosis of miRNA by DCs is selective.

In addition, in order to verify the effect of miRNA modification, each version of miR-590 was tested in HepG2 cells for the significant inhibitory effect of miR-590 on TGFBR2 [21]. The modifications of miR-590, including 2'-O-fluoro modification or FAM modification, can effectively inhibit TGFBR2, and 2'-O-fluoro modification can even make the miR-590 function for a longer period (Fig. 4), as demonstrated from luciferase reporter assays and qPCR experiments.

To distinguish what causes the low rate of internalization of miR-mock, either the modification of 2'-O-fluoro or the sequence itself, another miR-mock was synthesized without modification by 2'-O-fluoro, named miR-mock-Fam. Another miR-590-5p was synthesized with 2'-O-fluoro-dNTP and named miR-590-5p-Cy3-F. After incubation with miR-590-5p-Cy3-F and miR-mock-Fam, the fluorescence intensity and the internalization rate of FAM increased, whereas Cy3 remained stable (Fig. 3D). These results suggested that miRNA endocytosis by DCs takes approximately 3 hours and that 2'-O-fluoro modification of miRNA inhibits the endocytosis process. As a result, the experiment showed the relationship between the modification of the same miRNA and the endocytosis capacity of the DC, so it can be used as a method to detect the endocytotic ability of cells.

Fig. 3. 2-O-fluoro modification of miRNA interferes with endocytosis into DCs. **A.** Time-dependence of the mean internalization ratios of both FAM and Cy3-labelled miRNAs (miR-mock-Fam-F and miR-590-5p-Cy3, former is 2-O-fluoro modified, latter is non-modified) in DCs stimulated by LPS, analysed by real-time imaging flow cytometry. **B.** Images of DCs cultured with labelled miRNAs (miR-mock-Fam-F and miR-590-5p-Cy3). Scale bars represent 20 μ m. The miRNA label '-F' means 2-O-fluoro modified. **C.** Representative results of the cellular internalization ratios of miR-mock-Fam-F (2-fluoro-modified) (Ex: 488 nm, Em: 516 nm) and miR-590-5p-Cy3 (not 2-fluoro-modified) (Ex: 508-532 nm, Em: 568 nm) in DCs at 3 hours in culture in basal media or LPS treatment media (0.5 μ g/ml) analysed by real-time imaging flow cytometry. Left: Values of MFI are indicated. Middle: The values of the internalization ratios are indicated. Mean $\pm$ SEM of three independent experiments. ** $P < 0.01$, $n = 60,000$. Right: Image of DCs. Scale bars represent 20 μ m. Micro RNA label '-F' means 2-O-fluoro modified. **D.** Representative results of the cellular internalization ratios of FAM labelled on miR-mock and Cy3 labelled on miR-590 with or without 2-O-fluoro-modification, in LPS stimulated DCs at 3 hours in culture by real-time imaging flow cytometry. Left: The values of MFI are indicated. Middle: The values of the internalization ratios are indicated. Mean $\pm$ SEM of three independent experiments. ** $P < 0.01$, $n = 60,000$. ANOVA test was performed between miR-590-5p-f and non-modified miR-590-5p (blue bar) Right: Image of DCs where 2-O-fluoro modification has been swapped between microRNAs. Scale bars represent 20 μ m. The miRNA label '-F' means 2-O-fluoro modified.

Fig. 4. Effect of miRNA modification on miR-590 function. Left : Relative expression of TGFBR2 in HepG2 cells. GAPDH was detected as a reference gene. Control means miR-mock was transfected. No mod means miR-590 without modification was transfected. Right: Relative luciferase content in HepG2 cells. A sequence of TGFBR2 was ligated into pmiRGLO vector and co-transfected with several modification of miR-590.

In Fig. 3C, low rate of internalization of mir-mock is less clear. The manufacturer's synthetic record was searched, and details regarding the synthesis was revealed: commercial miR-mock was 2-o-fluoro modified. A series of experiments were performed to explore whether this modification is caused by the difference lying in the internalization rate. miR-mock was synthesized in 2 copies, one of which was 2-o-fluoro modified while the other was not. When these two miR-mock were added into the culture system of the DCs, respectively, the 2-o-fluoro modified Mir-mock showed a lower internalization rate (Fig. 3D left panel). Then the same procedure was applied to the mir-590-5p, and the results showed that 2-o-fluoro modification also reduced the internalization rate of mir-590-5p. (Fig. 3D Middle panel). This observation implies that 2-o-fluoro modification may interfere with the uptake of miRNA by dendritic cells.

To prove this hypothesis, a batch of miR-mock-fam without 2-o-fluoro modification is synthesized separately, and the original miR-590-5p-cy3 is treated with 2-O-FLUORO-dNTP to make it 2-o-fluoro modified, named miR-590-5p-cy3-F. After incubation with miR-590-5p-cy3-F and miR-mock-fam, the internalization rate of miR-mock-fam increased, whereas internalization rate of miR-590-5p-cy3-F decreased. (Fig. 3D right Panel). This observation, together with result shown in Fig. 3C, suggested that 2-o-fluoro modification can significantly interfere with the absorption of miRNA by dendritic cells. This set of experiment, by simply modifying a miR-mock, can be used to assess the endocytosis capability of dendritic cells.

Differences in miRNA endocytosis between PBMCs

Dendritic cells have shown the ability for miRNA endocytosis, and it has been reported that this endocytotic ability is easily interfered by the secretion of tumor cells [33]. To further assess the endocytosis properties of miRNAs between different immune cells, a series of experiments were performed in dendritic cells. FITC-labeled ovalbumin and miR-590-5p-Cy3 were added into the media of the primary cultured dendritic cells, and dendritic cells were observed to have greater endocytosis of ovalbumin-FITC rather than miR-590-5p-Cy3 (Fig. 5A and 5B). When the dendritic cells are stimulated with LPS and tumor antigens, the results are just the opposite (Fig. 5A and 5B). More interestingly, when dendritic cells were co-cultured with tumor cells, the endocytosis of protein and miRNA both decreased (Fig. 5C). As the expression of CD80 and CD86 in DC cells was significantly decreased (Fig. 5D), this suggested that tumor cells reduced the endocytosis of miRNAs while inhibiting dendritic cells.

Fig. 5. Endocytosis of different cells for miR-590-5p. Ovalbumin-FITC and miR-590-5p-Cy3 were added to DCs at the same time, and then the endocytosis rate was measured by imaging flow cytometry. ** $P < 0.01$, $n = 20,000$. **A.** The fluorescence intensity of DCs which remain inactivated (basal) or stimulated by LPS and tumor antigen. **B.** Upper panel: Imaging flow cytometry showing in the basal states, DCs tend to endocytose proteins rather than miR-590-5p. Low panel: Stimulated by LPS and tumor antigen, DC cells tend to endocytose miR-590-5p. Scale bars represent 20 μm . This image is divided into four columns, showing the photo of the cells in the bright field, FITC, CY3 and Fitc-cy3 channels respectively. The above 2 rows show DC in basal media and 2 lower rows show DC stimulated by LPS. **C.** Co-cultured with the tumor cells, endocytosis of DCs on the protein and miR-590-5p decreased simultaneously. Scale bars represent 20 μm . **D.** Flow cytometry analysis showing more CD80+ or CD86+ cells could be found in basal DCs than DCs co-culture with tumor cells. **E.** Imaging flow cytometry showing endocytosis of miR-590-5p mostly for DC cells (CD11c+). Down-left Panel (legend e) showing the positive rate of miR-590-5p+ in DCs. Down-right Panel (legend f) show the positive rate of CD11c+ in DCs. Upper panel (legend a to d) showing the cell morphology of the miR-590-5p+ DCs. **F.** Imaging flow cytometry from various organs, showing less miR-590-5p+ and CD11c+ could be found in tumor tissues, showing endocytotic DC cells were mainly in the bone marrow. Six independent experiments were done as replicates. The DC cells used in each independent experiment come from the same animal and receive the same pre-treatment. **G.** Immunofluorescence of tumor and bone marrow tissues collected from HepG2 tumor bearing mice. Scale bars represent 20 μm .

Taking into account the important role of miRNAs in tumor immunity [34], a series of experiments were performed in peripheral blood mononuclear cells (PBMCs). When PBMCs from healthy volunteers were isolated and incubated with miR-590-5p-Cy3, a group of cells with significantly higher Cy3 content was found with imaging flow cytometry. Further analysis revealed that most of these cells were CD11c positive (approximately $74.6 \pm 7.7\%$, Fig. 5E). This result confirms that dendritic cells are cells that are the most endocytotic to miRNAs in PBMCs. Potentially more interesting is that less DC cells with high endocytosis was detected in prostate cancer patients, but more highly endocytotic DC cells can be measured in prostatitis patients, which supports the previous results [4]. To further elucidate the endocytotic effect of this miRNA, more experiments were performed on HepG2 xenograft tumor-bearing mice. After miR-590-5p-Cy3 was injected into the tumor-bearing mice, DCs with higher endocytosis were found in the bone marrow, and the spleen and lymph nodes were less (Fig. 5F & Fig. 5G). Six independent experiments were done as replicates. The DC cells used in each independent experiment came from the same animal and received the same pre-treatment. While DC cells were detected around the tumor tissue, almost no endocytotic miR-590-5p-Cy3 was found. This is consistent with the conclusions of our previous study [4] and through these experiments we can demonstrate that miR-590-5p-Cy3 endocytosis is one of the characteristics of DC cell activation. For the analysis of DC cells engulfing miR-590-5p-Cy3, not only can the DC transformation be observed, but also different functional DC cells can be traced.

FRET analysis as a measure of miRNA combination and degradation

To further confirm our assay as an optional method to monitor the miRNA behaviour, FRET analyses were performed in DCs transfected with anti-miR-590-5p-Fam and miR-590-5p-Cy3 under excitation of 200 mW at 488 nm. The degradation of miRNA was measured using the FRET method following our previous research [4]. The 488 nm wavelength is not a suitable wavelength for Cy3 excitation (Fig. 6). In this system, the only method for detecting miR-590-5p-Cy3 fluorescence is to combine it with anti-miR-590-5p-Fam [35]. As expected, under an excitation of 200 mW at 488 nm, few cells were found in the "high intensity" region of the Cy3 channel (Fig. 7A) after transfection with miR-590-5p-Cy3 or anti-miR-590-5p-Fam alone. However, after co-transfection with anti-miR-590-5p-Fam and miR-590-5p-Cy3, increasing the time of miR-590-5p-Fam from 6 to 24 hours led to a visible increase in

Fig. 6. Fluorescence spectrum of different FRET pairs, A: Fluorescence spectrum of Cy3/Cy5B: Fluorescence spectrum of Fam/Tamra, C: Fluorescence spectrum of Fam/Cy3.

Fig. 7. Detection of miRNA combination and degradation by FRET analysis in imaging flow cytometry. A. Mean fluorescence intensity in the FAM channel and Cy3 channel in DCs under excitation at 488 nm at 12 hours after transfection with miR-590-5p-Cy3 or anti-miR-590-5p-Fam. Left: The values of MFI are indicated. Right: Image of DCs. Scale bars represent 10 μ m. B. Time dependence of the relative fluorescence intensity of co-transfected miR-590-5p-Cy3 and anti-miR-590-5p-Fam in DCs under excitation at 488 nm. Left: Image of DCs. Scale bars represent 10 μ m. Right: The values of the relative fluorescence intensity are indicated. Mean $\pm$ SEM of three independent experiments. 30000 cells was measured in each experiment. C. Time dependence of the relative fluorescence intensity of AGO2-APC in DCs co-transfected with miR-590-5p-Cy3 and anti-miR-590-5p-Fam under excitation at 488 nm. Image shows the accumulation of AGO2 in cells transfected with miR-590-5p-Cy3. Scale bars represent 10 μ m. D. Time dependence of the relative fluorescence intensity of co-transfected miR-590-5p-Fluor and anti-miR-590-5p in DCs under excitation at 488 nm. Left: Image of DCs. Scale bars represent 10 μ m. Right: Values of the relative fluorescence intensity are indicated. Mean $\pm$ SEM of three independent experiments.

FRET (Fig. 7B), as measured using the ImageStream MkII. The accumulation of AGO2 (Fig. 7C) was also measured on anti-miR-590-5p-FAM at 24 hours. It has been reported that the AGO2 protein plays key roles in the RNA-induced silencing complex (RISC) and mediates the interaction of siRNA with target RNA [36]. Together, these data not only indicate the specific interaction of miR-590-5p and anti-miR-590-5p but also provide real-time images of RNA complementation. Another study shows that unrelated miRNA (the core region is not complementary) will not increase the FRET signal [37]. The number of FRET-fluorescence spots was not significantly altered by the dose or duration of miR-mock-Fam-F treatment (Fig. 6A and 6B). Subsequent qPCR and luciferase experiments also showed a significant decrease in miR-590-5p function (Fig. 7). These data are consistent with the results with miR-590-5p-Cy3 and anti-miR-590-5p-Fam, and provide a real-time observation of the mechanism of RNA interference through a specific combination in the presence of AGO2 protein, which would lead to the assembly of the RISC [36].

To further observe the process of miRNA degradation after pairing, anti-miR-590-5p and miR-590-5p-Fluor (labelled with FAM at the 5' end and Cy3 at the 3' end on one miRNA mimic) were synthesized. Under stable conditions, miR-590-5p-Fluor emits Cy3 fluorescence under excitation at 488 nm because of the mechanism of FRET. However, after degradation, miR-590-5p-Fluor emits FAM fluorescence because of fluorophore release when anti-miR-590-5p and miR-590-5p-Fluor are co-transfected into DCs. Surprisingly, the amount of FRET-fluorescence was stable after the time point of combination (12 h) rather than disappearing simultaneously (Fig. 7B), which may indicate that the combination with AGO2 was not the only condition of RISC; other elements of RNA interference may delay the process of RNA degradation.

Validating imaging flow cytometry with qPCR experiments and western blotting

To confirm the findings of imaging flow cytometry, qPCR analyses of the levels of miR-mock and miR-590-5p were performed on cell lysates of each (Fig. 8). The results showed that in RPMI1640 media in the presence of miR-590-5p-Cy3, the stimulation of LPS and tumor antigen led to an accumulation of miR-590-5p compared with basal controls (Fig. 8A). The amplification of miR-1228 was performed as the negative control because of its stable expression [38]. This is generally consistent with the findings of imaging flow cytometry, which show that LPS promoted miR-590-5p endocytosis (Fig. 3C). Under activation conditions with miR-590-5p, the qPCR results revealed temporarily increased levels of miR-590-5p compared to the basal controls without LPS (Fig. 8A and 8B), which supports the notion that miRNA endocytotic ability could be enhanced by LPS and the process of RNAi occurs after endocytosis (Fig. 1A and 1B). Additionally, miR-mock contents remained low, which is similar to our imaging flow cytometry data that showed little accumulation of miR-mock-Fam-F by endocytosis (Fig. 3A).

To further confirm the finding of RNAi-induced degradation by anti-miR-590-5p that was observed using ImageStream MkII, active DCs were transfected with anti-miR-590-5p and analysed at nine time points. The qPCR results showed a significant decrease in the relative level of miR-590-5p at 24 hours after transfection (Fig. 8C), which is generally consistent with the results from imaging flow cytometry showing disappearance of FRET fluorescence at the point of origin from miR-590-5p-Fluor (Fig. 7B). In addition, western blot analysis showed an accumulation of AGO2 levels after transfection with miR-590 (Fig. 8D), which confirmed the RNAi occurring in dendritic cells (Fig. 4&Fig. 9). This result supports the conclusion that RNA interference was partially induced by AGO2. Together, these qPCR data suggested that the FRET analysis by imaging flow cytometry is a potential method for detecting this miRNA mechanism.

Fig. 8. Using qPCR experiments and western blot analysis to test the result of imaging flow cytometry. A. Levels of miR-590-5p, miR-1228 and miR-mock in DCs co-transfected with miR-mock-Fam and miR-590-5p-Cy3. miR-1228 served as a negative control. Mean $\pm$ SEM of three independent experiments. B. Time dependence of the relative levels of miR-590-

5p and miR-mock in active DCs (stimulated by LPS and tumor antigen) cultured with labelled miRNAs (miR-mock-Fam-F and miR-590-5p-Cy3), which were analysed by qPCR. Mean $\pm$ SEM of three independent experiments. C. Time dependence of the relative levels of miR-590-5p and miR-mock in DCs (stimulated by LPS and tumor antigen) co-transfected with miR-590-5p, miR-mock and anti-miR-590-5p, which were analysed by qPCR. Mean $\pm$ SEM of three independent experiments. D. Levels of AGO2 in DCs co-transfected with miR-590-5p, miR-mock and anti-miR-590-5p, which were analysed by western blot. β -actin was used as a loading control. All three samples were run under the same experimental conditions. The images of the blots were cropped for clarity, and the full-length blots/gels are presented in Fig.9.

Discussion

The endocytotic ability of DCs has been reported [40], but few studies have described the relationship between miRNA endocytotic ability and stimulation in DCs [23]. In our study, an imaging flow cytometry analysis was performed to evaluate the miRNA endocytotic ability in DCs with or without LPS treatment; the results showed a sharp increase in the miR-590-5p internalization ratio on LPS treatment. These data suggested that the stimulation by LPS increased the internalization ratio of DCs with miRNA endocytotic ability. Previously published findings showed that immature DCs possess a

Fig. 9. Full-length blots of Fig. 8D.

stronger ability for protein endocytosis [44]. Our data show differences in the endocytotic ability of DCs for protein and miRNA. However, not all of the DCs in our study demonstrated an ability for miRNA endocytosis (Fig. 2B). It has been reported that some DCs exerted lower endocytosis function [33, 44], but there is no biomarker to distinguish this group [45, 46]. Our study provided a method for distinguishing DCs with low endocytosis from other mature DC populations without the help of a surface biomarker. We also developed a new approach to observe miRNA endocytosis in real time, where imaging flow cytometry test showed the time-dependent accumulation of miR-590-5p-Cy3, verified by qPCR analysis.

Interestingly, LPS stimulation did not lead to an accumulation of miRNAs with a 2-O-fluoro modification in our imaging flow cytometry assay. However, there was an increase in miR-mock without 2-O-fluoro, which suggested that RNA endocytosis was a process that prefers miRNA without chemical modification. When DCs were cultured with LPS and miR-590-5p, the increase in the miR-590-5p internalization ratios was greater than that observed in cells with 2-O-fluoro miR-590-5p with LPS media and miR-590-5p with basal media, which supports our conclusion that miRNA endocytosis by DCs prefers miRNA without chemical modification and could be increased by LPS. A possible explanation for the lack of accumulation of 2-O-fluoro miR-mock is that the fluorine atom interferes with the antigen recognition process in DCs. It has been reported that the fluorine atom was an inhibitor of NADPH oxidase activity [47].

The results from imaging flow cytometry do not mirror the qPCR data precisely. However, they follow similar trends. The sequence and modification of miR-590-5p for FRET analysis are difficult to design. Typical FRET pairs for RNA such as Cy3-Cy5 [37] were not suitable for our study, as the wavelength of Cy3 emission was too short to excite Cy5 by image flow cytometry (Fig. 6A). Cy3 and Cy5 are problematic for use with miRNAs [37]. Another typical FRET pair FAM-TAMRA has been commonly used in qPCR [48]. However, the emission intensity of this pair is too low to detect (Fig. 4B). However, FAM-Cy3 was an ideal pair for our FRET analysis, as FAM can be attached at the 5' end of miRNA while Cy3 is at the 3' end and the emission wavelength of FAM (516 nm) was in the range of Cy3 excitation (508~532 nm). If the FAM and Cy3 were modified in different RNAs, the FRET pair could be used to detect RNA duplex formation. If the FAM and Cy3 were modified in a single RNA, the FRET pair can be used to detect RNA degradation. Moreover, unlike immunoblotting analysis or microscopy, imaging flow cytometry assay enables the higher-throughput quantitation and visualization of endocytosis of up to 100,000 individual cells.

It has been reported that AGO2 plays a key role in the RNA interference pathway and that other RNA modulators, such as TARBP2, may affect this and other pathways [49, 50]. Thus the use of the AGO2 immunoblot alone was not sufficient in our study to gain a real measure of RNAi processing. However, combining AGO2 flow cytometry with miRNA imaging assays to query the presence of RISC, yielded deeper insight into the RNA interference processes on a larger scale. In this study, imaging flow cytometry assay enabled us to observe and quantify the impact of LPS stimulation and other treatments on RNA endocytosis and interference more accurately in DCs.

Another advantage of our study is that fluorescence-tagged nucleic acid and antibody-stained samples can be used in a single assay. Accordingly, this detail makes it a practical method for nucleic acid localization in clinical studies because of the ease of detection and because sample processing is relatively simple and could be automated (Fig. 10A). However, the limitation of this method is that cells in suspension are required because of the flow cytometry technology. Evaluating endocytosis or RNA interference in primary tissues therefore remains a challenge.

Fig. 10. The schematic summary of this research.

Conclusion

In conclusion, our study demonstrates further application and evidence for our previous method of miRNA analysis using imaging flow cytometry [4]. Moreover, we elucidate key requirements for wider applicability of this method, in particular a key requirement for LPS activation of DCs being critical for miRNA endocytosis, and that fluoro-modified miRNAs exhibit poor uptake. In addition, we suggest that miR-590-5p uptake and detection by our novel method could be an additional marker for DC activation in populations, in addition to CD86 expression.

This method is faster, more robust, more sensitive and simpler than the standard qPCR and immunoblot methods, which can be labour intensive and are technically demanding. Moreover, this method can be helpful in gaining additional insight into the process of RNA interference at two related steps within a large population of cells: RNA binding and RNA degradation. This result could be valuable for investigating miRNA-induced pathway in cells and the effects of miRNA in pathogenesis. Therefore, the miRNA research methods that take advantage of the imaging flow cytometry assay described in this study will be a useful supplement for monitoring cellular miRNA processes.

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Disclosure Statement

No potential conflicts of interest to disclose.

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