Downregulation of miR-4772-3p promotes enhanced regulatory T cell capacity in malignant pleural effusion by elevating Helios levels

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Abstract

Background: Malignant pleural effusion (MPE) is a complicated condition of patients with advanced tumors. Further dissecting the microenvironment of infiltrated immune cells and malignant cells are warranted to understand the immune-evasion mechanisms of tumor development and progression.

Methods: The possible involvement of microRNAs (miRNAs) in malignant pleural fluid was investigated using small RNA sequencing. Regulatory T cell (Treg) markers (CD4, CD25, forkhead box P3), and Helios (also known as IKAROS Family Zinc Finger 2 [IKZF2]) were detected using flow cytometry. The expression levels of *IKZF2* and miR-4772-3p were measured using quantitative real-time reverse transcription polymerase chain reaction. The interaction between miR-4772-3p and Helios was determined using dual-luciferase reporter assays. The effects of miR-4772-3p on Helios expression were evaluated using an *in vitro* system. Correlation assays between miR-4772-3p and functional molecules of Tregs were performed.

Results: Compared with non-malignant controls, patients with non-small cell lung cancer had an increased Tregs frequency with Helios expression in the MPE and peripheral blood mononuclear cells. The verified downregulation of miR-4772-3p was inversely related to the Helios$^+$ Tregs frequency and Helios expression in the MPE. Overexpression of miR-4772-3p could inhibit Helios expression in *in vitro* experiments. However, ectopic expression of Helios in induced Tregs reversed the effects induced by miR-4772-3p overexpression. Additionally, miR-4772-3p could regulate Helios expression by directly targeting *IKZF2* mRNA.

Conclusion: Downregulation of miR-4772-3p, by targeting Helios, contributes to enhanced Tregs activities in the MPE microenvironment.

Keywords: Helios; IKAROS family zinc finger 2; Lung cancer; Malignant pleural effusion; MiR-4772-3p; Regulatory T cells

Introduction

Malignant pleural effusion (MPE) is a common event of primary or secondary malignancy of the pleural cavity, affecting over 1 million people worldwide.[1] The presence of MPE in patients is predictive of reduced therapeutic options, a life-shortening condition, and a heavy health-care burden.[2] Lung cancer is the leading cause of MPE, and has the shortest survival rates among all malignancies with MPE.[3] Comprehensive treatment of lung cancer, including molecular targeted anti-cancer drugs, has significantly improved the overall survival of patients; however, because of tumor recurrence, drug resistance, side effects, and limited suitable populations, the clinical efficiency of treatment for advanced progressive lung cancer remains poor.[4-6] Further investigation of the immunological mechanism of the MPE, as a special microenvironment of tumors, to correct immune abnormalities and expand novel immunotherapy targets, is a pressing clinical need.

CD4$^+$ T-helper (Th) cells orchestrate humoral and cellular immunity via subsets, such as Th1, Th2, Th17, Th9, Th22, T follicular helper (Tfh) cells, and regulatory T cells (Tregs).[7,8] The enriched CD4$^+$ T subsets drive tumor progression and/or regression in patients with MPE.[9,10] Tregs are functionally suppressive CD4$^+$ and CD8$^+$ T cells, which are indispensable for restricting aberrant or

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excessive immune activation and maintaining self-tolerance to foreign antigens and self-components.[11] However, Tregs are believed to hinder effective antitumor immunity and are associated with an unfavorable prognosis in both humans and animal models.[12] There is evidence that the aberrant frequency and function of Tregs or an imbalance of Tregs and effector T cells, facilitate the progression of MPE.[13-15] However, the underlying molecular mechanism remains to be clarified.

The forkhead box P3 (FOXP3) transcription factor, a widely recognized marker, is central to the differentiation, development, and function in Tregs.[16] However, accumulating evidence indicates that FOXP3$^+$CD4$^+$ Tregs can lose FOXP3 expression, or be converted into cytokine-producing effector T subsets, such as Th1-like Tregs, Th2-like Tregs, Tfh-like Tregs, and Th17-like Tregs, under some specific environmental conditions.[16-20] Helios (also known as IKAROS family zinc finger 2 [IKZF2]), a upregulatory of transcription factor, stabilizes the phenotype and function of FOXP3$^+$CD4$^+$ and CD8$^+$ Tregs in face of inflammatory responses.[21,22] Co-expressing Helios and FOXP3 transcription factors conferred on Tregs a specific phenotype of highly suppression of the immune system.[23] Recently, it was observed that Helios-expressing FOXP3$^+$ Tregs are of particular relevance in various disorders, including organ transplant reaction, autoimmunity, infectious diseases, and cancer.[23-28] Importantly, selective Helios deletion in CD4$^+$ Tregs induced Tregs with an unstable phenotype and induced the transformation of Tregs into T effector cells in the tumor microenvironment, which enhanced anti-tumor immunity.[29] Therefore, Helios is expected to be a potential modulator to improve Treg-mediated resistance to the antitumor response.

MicroRNAs (miRNAs), as small (19–25 nucleotides) endogenous non-coding RNAs, negatively regulate more than 30% the encoded gene activity by targeting mRNAs.[30-32] Mounting evidence suggests that miRNAs are also involved in the phenotype, balance and functional stability of Tregs. For example, miR-17 modulates the differentiation and function of Tregs by targeting transforming growth factor-beta receptor II and FOXP3 co-regulator EOS (also known as IKAROS family zinc finger 4 [IKZF4]).[33,34] MiR-27 impairs both thymic Treg development and peripheral Treg homeostasis by repressing REL (REL proto-oncogene, nuclear factor-k-gene binding sub-unit) and granzyme B.[35] MiR146a controls Treg-mediated suppression of interferon (IFN)-gamma-independent Th1 response via targeting signal transducer and activator of transcription 1.[36] MiR-10a restricts the acquisition of a Tfh-like Tregs phenotype via targeting transcription repressor B cell lymphoma-6 (BCL-6) and the nuclear receptor co-repressor 2 (NCOR 2).[37] Thus, targeting Helios/IKZF2 using small molecules such as miRNAs or genes to manipulate Treg function in the microenvironment and curb abnormal immunity, might become a promising approach in immunotherapy.[29,38]

In the present study, we used next-generation sequencing to identify differentially expressed miRNAs in mononuclear cells from pleural fluid, and verified the down-regulation of miR-4772-3p expression using quantitative real-time reverse transcription PCR (qRT-PCR) in MPE subsequent to non-small cell lung cancer (NSCLC). We subsequently measured Tregs frequencies and Helios expression in peripheral blood mononuclear cells (PBMCs), in MPE from patients with NSCLC, and in non-malignant pleural effusion (NPE). Correlation assays between miR-4772-3p and functional molecules of Tregs were performed. The interaction between miR-4772-3p and Helios was determined using dual-luciferase reporter assays. Furthermore, the effects of miR-4772-3p on Helios expression were evaluated using an *in vitro* system. We aimed to identify that downregulation of miR-4772-3p, which targets Helios/*IKZF2*, contributes to enhanced Treg activities in the MPE microenvironment.

Methods

Ethical approval

The study was approved by the ethics committee of the Taizhou People's Hospital and informed consent was obtained from all subjects before participation. We strictly obeyed the guidelines of the *Declaration of Helsinki*.

Study subjects

A total of 30 patients (age range, 43–79 years old) were recruited who were newly diagnosed with MPE from NSCLC, as confirmed by the presence of malignant cells in the pleural fluid and/or on pleural biopsy histology. Among the 30 patients with NSCLC, five had squamous cell carcinoma and 25 had adenocarcinoma. Another 30 patients were diagnosed with NPE, including 19 (age range, 20–78 years old) with parapneumonic effusion, and 11 (age range, 24–78 years old) with tuberculous pleural effusion, as evidenced by *Mycobacterium tuberculosis* DNA testing by PCR, growth of *M. tuberculosis* in the pleural fluid, or disappearance of pleural effusion (PE) after anti-tuberculosis chemotherapy. Peripheral blood (PB) samples were also collected from 20 healthy control subjects and 20 patients with MPE before treatment.

The patients were excluded if they underwent any chest trauma within 3 months before admission, or suffered from any invasive procedures directed into the pleural cavity. None of the patients had been treated with any anticancer therapy, anti-tuberculosis treatment, corticosteroids, or other non-steroid anti-inflammatory drugs before sample collection.

Sample collection and processing

PB samples were collected in citrate anticoagulation tubes from patients with MPE and the healthy control subjects. PE was obtained from each patient in heparin-treated tubes, through a standard thoracocentesis technique within 24 h after admission. All obtained PE specimens were immersed in ice and centrifuged at $400 \times g$ for 10 min at 4°C. The cell pellets of PE were re-suspended in phosphate-buffered saline, and the PE mononuclear (PEMC) cells were isolated using Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden)

for subsequent detection. PEMCs from six patients were recruited for small RNA sequencing.

RNA isolation and small RNA sequencing

PEMCs were subjected to total RNA isolation using TRIzol (B511311; Sangon, China) according to the manufacturer's protocol, and the integrity of the purified RNA was determined by electrophoresis through a 1.0% agarose gel. The quality and quantity of the isolated RNA were evaluated using a NanoPhotometer® spectrophotometer (IMPLEN, Westlake Village, CA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Thereafter, six RNA samples of sufficiently high quality were submitted to Sangon Biotech (Shanghai) Co., Ltd., China for subsequent library construction.

A total of 2 μg of RNA from each sample was used as an input material for small RNA library preparations. RNA libraries were constructed using a NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, Ipswich, MA, USA) according to the manufacturer's instructions. Briefly, after the small RNA ends were ligated to the 3′ and 5′ adapters, complementary DNA (cDNA) was synthesized by reverse transcription (M-MuLV Reverse Transcriptase, Sangon). DNA fragments corresponding to 140 to 150 bp were separated using 12% polyacrylamide gel electrophoresis, and then the cDNA library was obtained. Finally, the quality of resulting library was assessed on the Agilent Bioanalyzer 2100 system, and the high-quality libraries were sequenced on an Illumina HiSeq X-ten platform (Illumina, San Diego, CA, USA). Subsequently, the quality of sequence data was evaluated using FastQC (version 0.11.2). Raw reads were filtered and the remaining clean data aligned to the reference genome using HISAT2 (version 2.0). Statistical analyses of the alignment results were performed using RSeQC (version 2.6.1). Significantly and differentially expressed genes between the two groups were analyzed using DESeq2 (version 1.12.4) and demonstrated by heatmaps and volcano plot filtering. MiRNAs were considered to be significantly differentially expressed if the fold change was >2 and the *P*-value was <0.05.

Quantitative real-time reverse transcription PCR

RNA from PEMC samples were subjected to RT and qPCR amplification using a HiScript First Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) and an AceQ qPCR SYBR Green Master Mix (Vazyme). Data collection and analysis were performed on a real-time PCR system (ABI Prism® 7500, Foster City, CA, USA). U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the references for normalization. All PCR reactions were performed in triplicate. The primers were synthesized by Suchow GENEWIZ Co., Ltd. (Suzhou, China) and are detailed below. After the amplification procedure, the RNA level was quantitatively calculated using the formula $-\Delta\Delta Ct$ (ΔCt = Ct target gene − Ct reference; Ct: cycle threshold).

The primer sequences of U6 and hsa-miR-4772-3p were F: 5′ CTCGCTTCGGCAGCACA 3′, R: 5′ AACGCTTCACGAATTTGCGT 3′; F: 5′ AACAAGCCTGCAACTTTGCCT′, R: 5′ CAGTGCAGGGTCCGAGGT′; respectively. Briefly, 1 μL of cDNA, 10 μL of 2× real time PCR master mix (SYBR Green), 2 μL of 10 μmol/L PCR specific primer MIX(F/R) were mixed with water to a total volume of 20 μL. U6 small nuclear RNA was used as internal control for normalization of gene expression. The program for the reactions was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 40 s.

The primer sequences of *IKZF2 (Helios)* and *GAPDH* were F: 5′ ACTGCAGTGCACAAACACAC 3′, R: 5′ GGTGACAATGTCGGGCTCA 3′; F: 5′ GAAGGTCGGAGTCAACGGAT 3′, R: 5′ CCTGGAAGATGGTGATGGG 3′. Briefly, 0.4 μL of ROX reverse dye (50×), 10 μL SYBR premix ex taq, 0.4 μL of 10 μmol/L PCR specific primer F, 0.4 μL of 10 μmol/L PCR specific primer R and 2 μL cDNA, were mixed with water to a total volume of 20 μL. *GAPDH* was used as the internal control. The reaction for *Helios* and *GAPDH* was performed as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 62°C for 34 s.

Target gene prediction for miRNA-4772-3p

Possible miRNA-4772-3p-regulated target genes were predicted using three predicting algorithms (TargetScan, miRWalk, and DIANA micro T). Among the putative targeted genes identified by the intersection of the three databases, we decided to investigate Helios/*IKZF2* because of its impact on the functional stability of Tregs.

$CD4^+CD25^-$ T cell isolation

PBMCs from healthy controls were isolated using Ficoll-Hypaque gradient centrifugation. The magnetic isolation of $CD4^+CD25^-$ T cells was performed according to the manufacturer's instructions. Briefly, $CD4^+$ T cells were directly purified by negative selection on a column (Hu CD4 T Lym Enrichment IMag – DM set, BD, San Jose, CA, USA), and then added with anti-CD25 antibody conjugated to MicroBeads (Anti-Human CD25 Magnetic Particles – DM, BD) within the magnetic field. The unlabeled cells were $CD4^+CD25^-$ T cells, which were subsequently cultured. The purity of the sorted $CD4^+CD25^-$ T cells was > 95% as judged using flow cytometry.

Induced-Treg cell generation and transfection

Purified $CD4^+CD25^-$ T cells (1×10^6) were cultured in Roswell Park Memorial Institute-1640 (Life Technologies, Carlsbad, CA, USA) plus interleukin (IL)-2 (PeproTech, Rocky Hill, NJ, USA; 2 ng/mL), 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and stimulated with plate-bound anti-CD3 monoclonal antibodies (mAbs) (OKT3; 1 μg/mL) and anti-CD28 mAb (CD28.2; 1 μg/mL) in 48-well plates. To induce the generation of Tregs, TGF-1 (PeproTech; 5 ng/mL) was added. These cells were cultured together for 7 days in a 5% CO_2 incubator at 37°C before being employed in transfection assays. The full-length sequence of *IKZF2* was cloned into a recombinant plasmid eukaryotic expression plasmid pcDNA3.1 to form pcDNA3.1-Helios overexpression plasmid by Sango Biotech. Tregs were transfected with miR-4772-3p mimic (5 nmol/L), or inhibitor (50 nmol/L),

and pcDNA3.1-Helios overexpression plasmid, along with negative controls using Lipofectamine-2000 Reagent (Life Technologies) and were harvested 48 h later (transfection efficiency was 50%–60%). The expression levels of *IKZF2* mRNA were detected using qRT-PCR. The protein levels of Helios were measured by flow cytometry.

Construction and transfection of luciferase reporter plasmids

The 3′-untranslated region (UTR) fragment of *IKZF2* was cloned into a pmirGLO Vector by Sango Biotech. Plasmids carrying the mutated sequence in the corresponding position for the seed regions of *IKZF2* were generated. Transfection was carried out using Lipofectamine-2000 Reagent (Life Technologies) according to the manufacturer's protocol. In brief, 5×10^5 HEK293T cells in a 24-cell plate were transfected with pmirGLO Vector containing the wild-type or mutated 3′-UTR luciferase reporter of *IKZF2* and the indicated miRNA plasmid or the control and collected at 24 h of post-transfection for assay. The transfection efficiency was approximately 50%. Luciferase activity was measured using a dual-luciferase reporter assay kit (Promega Corporation, Madison, WI, USA).

Flow cytometry

The expression markers on Treg cells from PBMCs and PEMCs were measured using flow cytometry after surface staining and intra-cellular staining according to the manufacturer's instructions. The antibodies used included those Perp-cy5.5-conjugated anti-CD4, BB515-conjugated anti-CD25, PE-conjugated anti-FOXP3, and Alex647-conjugated anti-Helios from BD Biosciences (San Jose, CA, USA). Briefly, the cells were stained with Fc receptor antibodies, washed twice, re-suspended, and labeled with anti-CD4 and anti-CD25 antibodies. For intra-cellular staining of transcription factor FOXP3 and Helios, cells were fixed and permeabilized. Data analyses were performed on a fluorescence activated cell sorting (FACS) cytometer using CellQuest™ software.

Statistical analysis

Data were expressed as the mean ± standard deviation. Comparisons of the data between two groups were performed using the two-tailed unpaired Student's *t* test, while one-way analysis of variance and Student-Newman-Keuls-q tests were used for multiple comparisons. For non-normally distributed data, we used Man-Whitney tests to examine the differences between two groups. Paired data comparisons were made using the paired Student's *t* test. Spearman rank correlation was used to measure the possible relationship. Analysis was completed using SPSS17.0 statistical software (IBM Corp., Armonk, NY, USA), and $P < 0.05$ was considered to indicate statistical significance.

Results

Helios$^+$ Treg frequency and IKZF2 mRNA expression are increased in patients with MPE

In view of the contribution of immune inhibition in patients with cancer, we investigated the proportion of $CD4^+CD25^+FOXP3^+$ Tregs in PB and PE of patients using flow cytometry. The frequency of Tregs in PBMCs was significantly increased in patients with MPE compared with those in the healthy controls ($4.16\% \pm 0.25\%$ *vs.* $3.44\% \pm 0.29\%$, $P < 0.01$). Helios protein levels were also upregulated in Tregs of patients ($52.51\% \pm 5.80\%$ *vs.* $33.47\% \pm 4.37\%$, $P < 0.01$). Importantly, we further found that the levels of Tregs in MPE were elevated compared with their levels in PBMCs ($4.83\% \pm 0.43\%$ *vs.* $4.16\% \pm 0.25\%$, $P < 0.01$) and NPE ($4.83\% \pm 0.43\%$ *vs.* $3.51\% \pm 0.65\%$, $P < 0.01$). Notably, the MPE had a significantly higher frequency of Tregs expressing Helios in comparison with PBMCs ($2.96\% \pm 0.35\%$ *vs.* $2.18\% \pm 0.24\%$, $P < 0.01$) and NPE ($2.96\% \pm 0.35\%$ *vs.* $1.18\% \pm 0.38\%$, $P < 0.01$). In addition, the relative mRNA expression of *IKZF2* in MPE was also remarkably increased in comparison with that in the NPE ($P < 0.01$) [Figure 1].

MiR-4772-3p is downregulated in PEMCs from patients with MPE

To identify the miRNA expression profile, next generation sequencing-based technology was used to analyze six PEs samples from patients with benign inflammatory diseases ($n = 3$) and patients with NSCLC ($n = 3$). Among 68 differentially expressed miRNAs between NPE and MPE, ten with highly altered expression are shown in Table 1. Of particular interest to our study are downregulated miRNAs that negatively affect the functional molecules in Tregs. Four miRNAs (including two novel miRNAs) were significantly downregulated in MPE compared to NPE [Figure 2A and 2B]. Previous studies showed that alteration of Tregs differentiation and function were related to different miRNAs expression. Thus, we further focused our study on one of the aberrantly expressed miRNA, miRNA-4772-3p, which might be associated with immunosuppressive molecules. To validate the results of previous deep sequencing analysis, the relative expression of miR-4772-3p in PEMCs was measured using qRT-PCR. We found that miR-4772-3p expression was remarkably decreased in MPE compared with that in NPE ($P < 0.01$) [Figure 2C]. To gain insights into the function of miR-4772-3p, we predict its potential targets using three online databases (TargetScan, miRWalk, and DIANA micro T). Among the possible targeted genes predicted by the three algorithms, we decided to select the *IKZF2* gene, encoding Helios, because of its potential modulator role in Treg function. Analysis of the 3′-UTR sequences of *IKZF2* showed one binding site that matched with the sequence of miR-4772-3p.

MiR-4772-3p is inversely correlated with Helios$^+$ Tregs frequency and Helios expression in patients with MPE

Patients with NSCLC had an increased Tregs frequency and Helios protein and mRNA expression in MPE. A significant negative correlation was found between miR-4772-3p and Helios$^+$ Tregs frequency ($r = -0.462$, $P = 0.010$), the Helios protein level ($r = -0.631$, $P < 0.001$), and *IKZF2* mRNA levels ($r = -0.468$, $P = 0.009$) in MPE, but no in the NPE ($r = -0.285$, $P = 0.127$; $r = -0.385$, $P = 0.365$; $r = -0.126$, $P = 0.507$) [Figure 3].

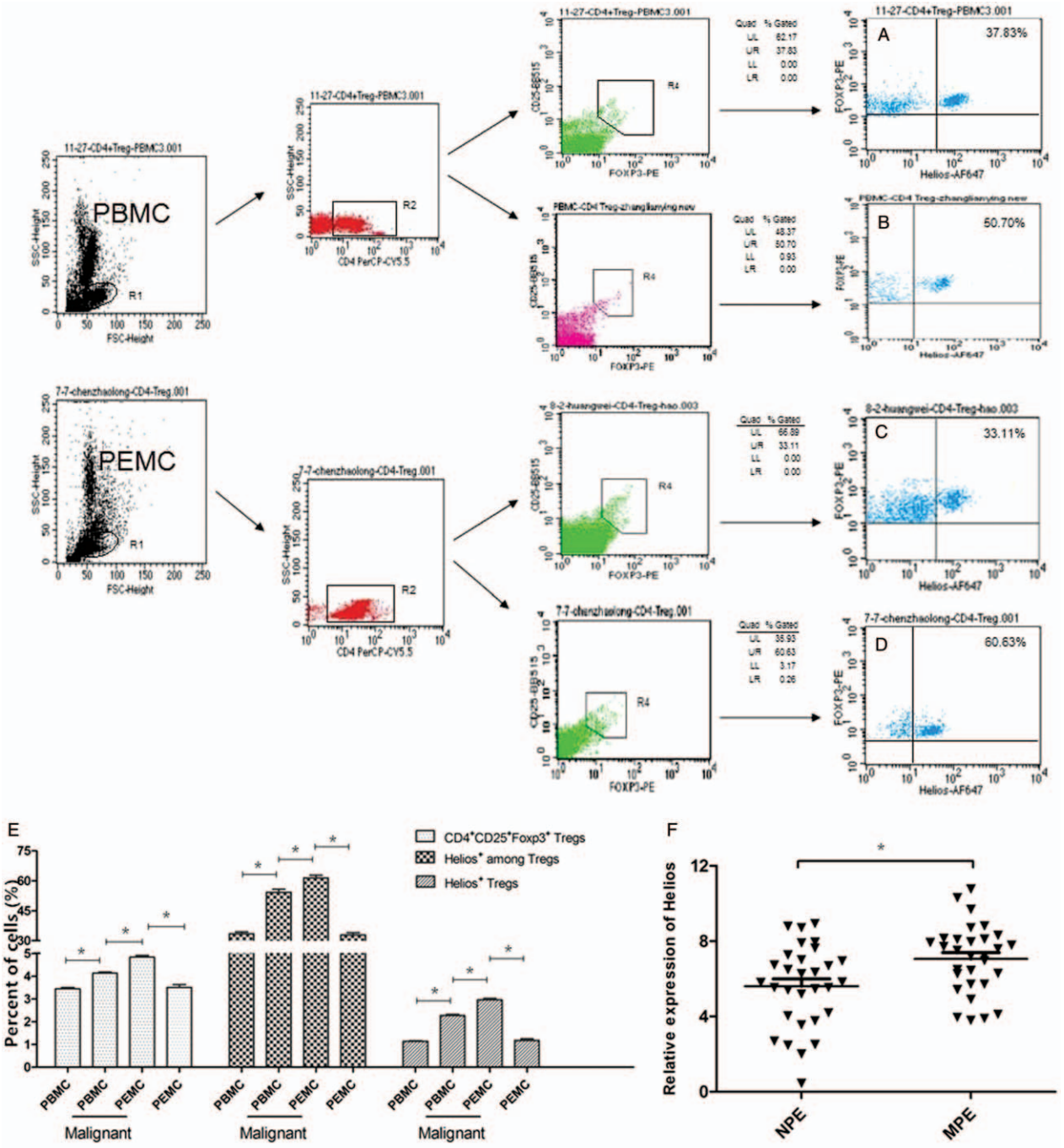

Figure 1: The expression of Treg markers from PBMCs and PEMCs. The $CD4^+$ T cells (R2) from lymphocytes (R1) were identified based on their side- and forward-scatter properties. The percentage of Helios and FOXP3 expression within $CD25^+FOXP3^+$ populations (R4) are shown. Image of Tregs showing Helios expression of $CD4^+CD25^+FOXP3^+$Tregs of the PBMCs in healthy controls ($n = 20$) (A) and patients with NSCLC ($n = 20$) (B). C and D show the Helios expression of $CD4^+CD25^+FOXP3^+$Tregs from the PEMCs of NPE ($n = 30$) and patients with NSCLC ($n = 30$). (E) The cumulative data show that the proportion of $CD4^+CD25^+FOXP3^+$ Tregs and Helios expression in peripheral blood and PE of patients, as analyzed using flow cytometry. (F) The transcript level of Helios in the MPE and NPE was determined by qPCR. $^*P < 0.01$. FOXP3: Forkhead box P3; MPE: Malignant pleural effusion; NPE: Non-malignant pleural effusion; NSCLC: Non-small cell lung cancer; PBMCs: Peripheral blood mononuclear cells; PE: Pleural effusion; PEMCs: PE mononuclear; qPCR: Quantitative polymerase chain reaction; Treg: Regulatory T cell.

MiR-4772-3p regulates the expression of Helios in induced Tregs in vitro

It has been suggested that Helios is associated with Tregs' stable inhibitory capacity. Therefore, we presumed that miR-4772-3p-mediated regulation of Helios might alter the suppressive activity of Tregs. To check this hypothesis, we transfected miR-4772-3p mimic into induced-Tregs for 24 h and then observed the reduced Helios levels in Tregs using flow cytometry [Figure 4]. However, inhibitor of miR-4772-3p produced the completely opposite effect [Figure 4I]. Additionally, transfection of the miR-4772-3p mimic alone caused a significant decrease in *IKZF2* mRNA expression in comparison with the negative control at the

Table 1: List of the top ten up- and downregulated miRNAs in PEMCs from NPE and MPE.

MiRNAs	Folds change	*P*	Regulated
hsa-miR-1269a	9.487468626	0.000252483	Up
hsa-miR-205-5p	8.244451854	0.000175430	Up
hsa-miR-429	8.018726069	0.000094300	Up
hsa-miR-196a-3p	8.000745375	0.002728060	Up
hsa-miR-200a-5p	7.857300366	0.000139828	Up
hsa-miR-615-3p	7.572511095	0.000835971	Up
hsa-miR-493-5p	7.465158693	0.004859225	Up
hsa-miR-18b-5p	4.282359228	0.043863184	Down
hsa-novel-158-mature	4.183866138	0.035719198	Down
hsa-miR-4772-3p	2.680377988	0.044442803	Down

MiRNA: MicroRNAs; PEMCs: Pleural effusion mononuclear cells; NPE: Pleural effusion of non-malignant pleural effusion; MPE: Malignant pleural effusion.

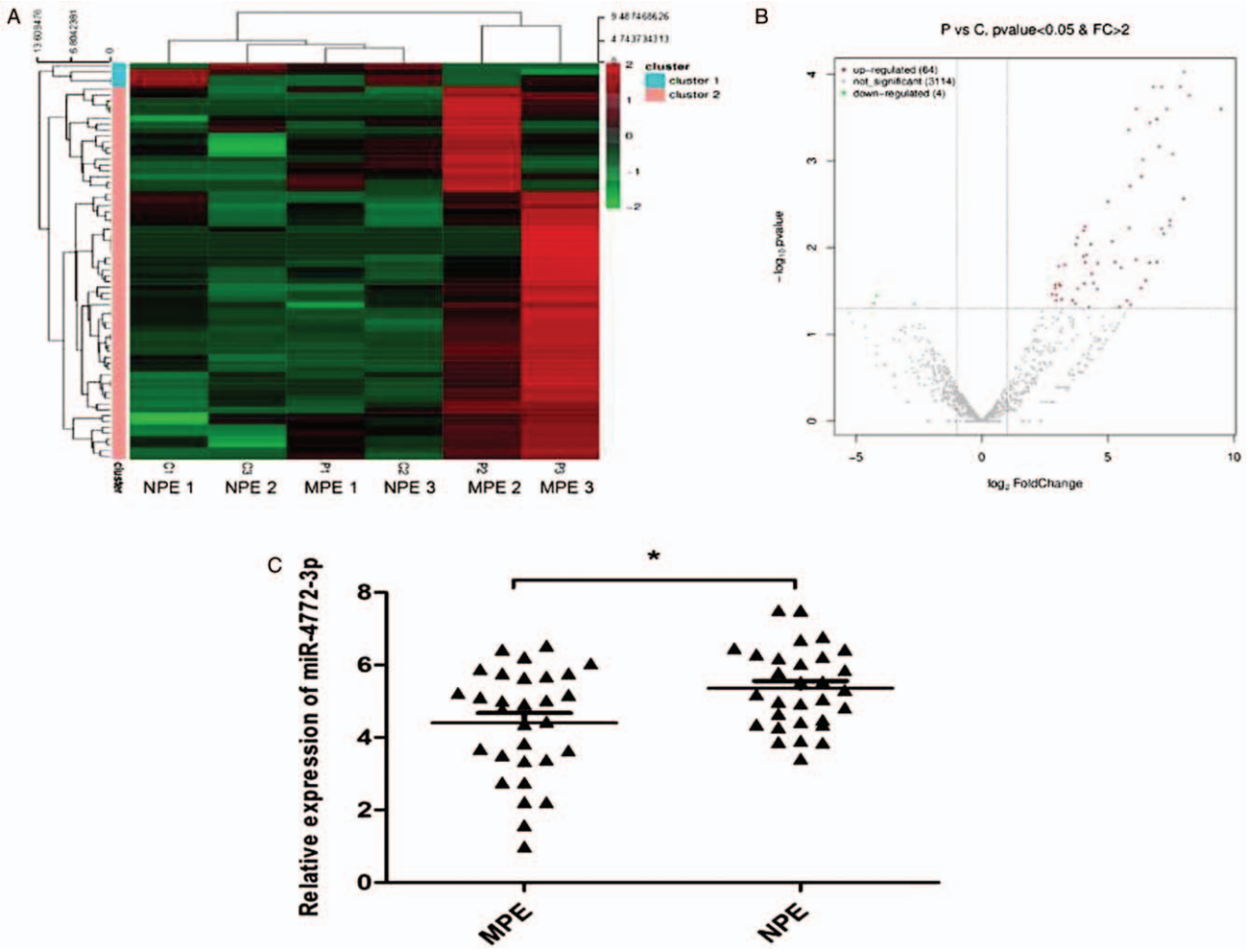

Figure 2: Deregulated miRNAs were identified in PEMCs from patients with benign inflammatory diseases ($n = 3$) and patients with NSCLC ($n = 3$). (A) A representative heatmap of the miRNA expression profile in the MPE (MPE1–MPE3) and NPE (NPE1–NPE3). (B) Differentially expressed miRNAs demonstrated using a volcano plot. (C) The quantification of the candidate miR-4772-3p from the MPE and NPE using RT-PCR. $^{*}P < 0.01$. Green: Downregulation; miRNA: MicroRNAs; MPE: Malignant pleural effusion; NPE: Pleural effusion of non-malignant pleural effusion; NSCLC: Non-small cell lung cancer; Red: Upregulation.

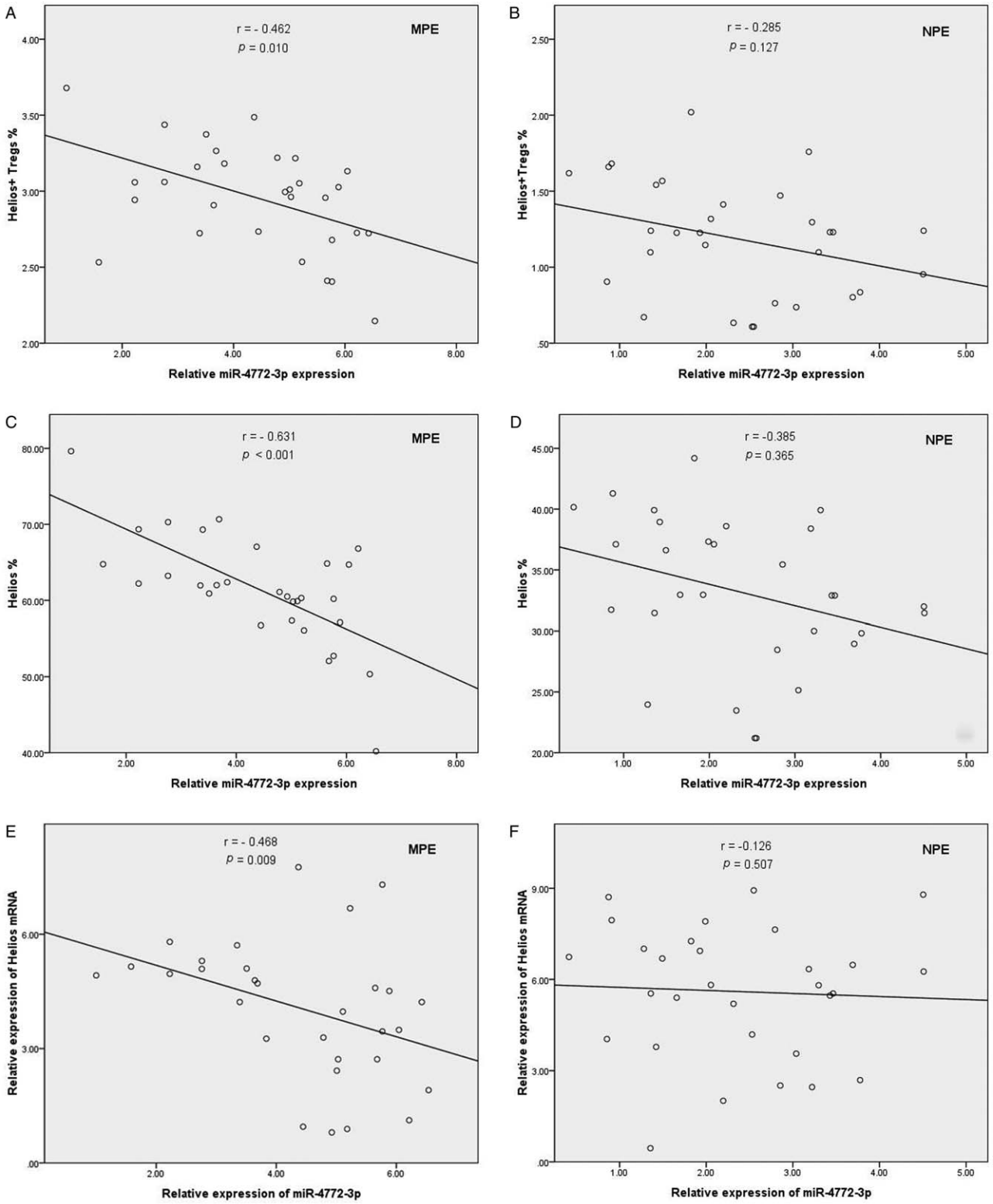

Figure 3: Scatter diagram of the correlation between miR4772-3p and Tregs frequency, Helios protein levels, and mRNA expression in MPE and NPE. Helios$^+$ Tregs %: (A) ($r = -0.462$, $P < 0.05$) in MPE; (B) ($r = -0.285$, $P = 0.127$) in NPE. Helios %: (C) ($r = -0.631$, $P < 0.001$) in MPE; (D) ($r = -0.385$, $P = 0.365$) in NPE. Helios mRNA expression: (E) ($r = -0.468$, $P < 0.01$) in the MPE; (F) ($r = -0.126$, $P = 0.507$) in the NPE. MPE: Malignant pleural effusion; NPE: Non-malignant pleural effusion; Treg: Regulatory T cell.

same time point ($P < 0.001$) [Figure 4K]. By contrast, the miR-4772-3p inhibitor increased Helios mRNA in induced Tregs ($P < 0.05$). We further found that ectopic expression of Helios by plasmid transfection in Tregs compensated for the effect of miR-4772-3p ($P < 0.01$) [Figure 4J and 4K].

IKZF2 is a direct target gene of miR-4772-3p

IKZF2 (Helios) was predicted as the putative target gene of the miR-4772-3p by all three databases (TargetScan, miRWalk, and DIANA-micro T). To confirm *IKZF2* as the

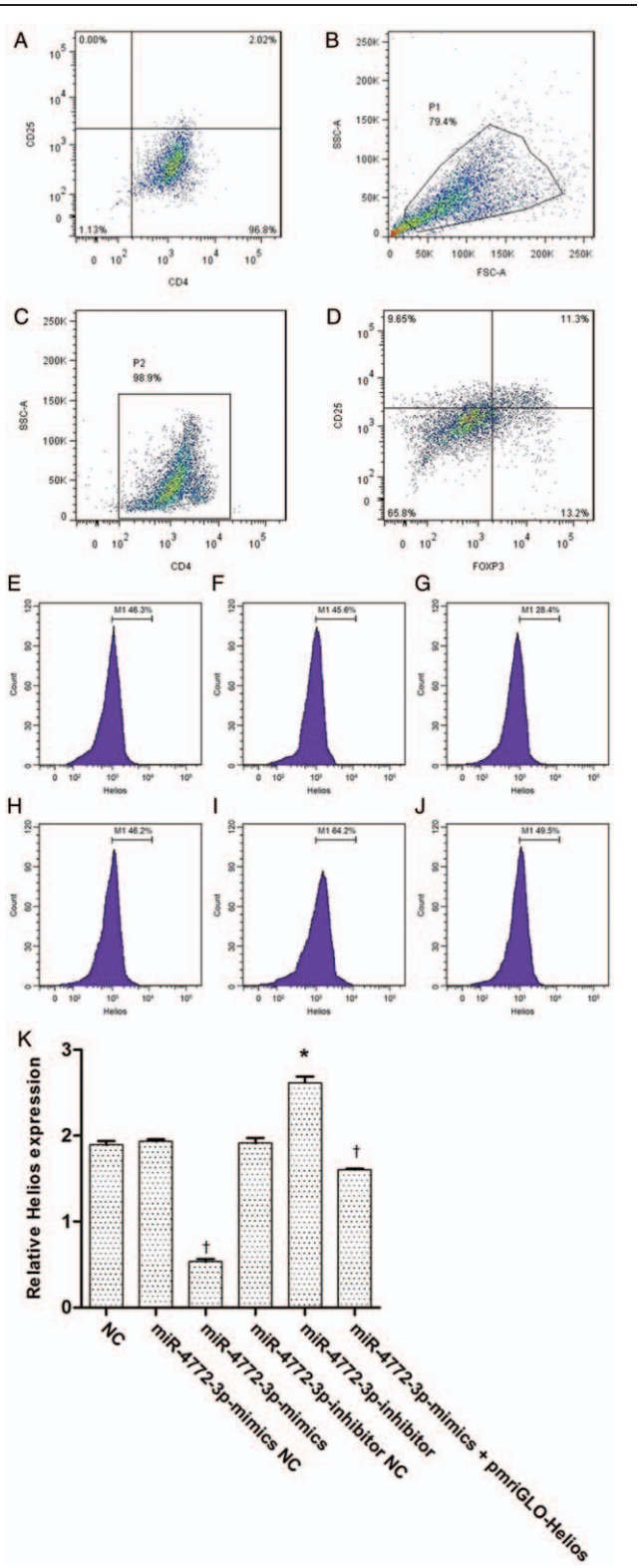

Figure 4: Regulation of Helios expression in Tregs by miR-4772-3p *in vitro*. Freshly isolated $CD4^+CD25^-$ T cells from PBMCs of healthy controls were determined using flow cytometry. The purity of $CD4^+CD25^-$ T cells was >95% (A). $CD4^+CD25^+FOXP3^+$ Tregs were generated after culturing in the cytokines (B–D). The frequency of induced Tregs within $CD4^+$ T cells was over 11%. Induced Tregs were transiently transfected with miR-4772-3p mimic, inhibitor, pmriGLO-Helios, and their negative controls, and 24 h later the fluorescence intensity was measured using flow cytometry (E: NC, 46.3%; F: Mimics NC, 45.6%; G: Mimics, 28.4%; H: Inhibitor NC, 46.2%; I: Inhibitor, 64.2%; J: Mimics + pmriGLO-Helios, 49.5%). (K) The relative level of Helios/*IKZF2* mRNA expression in miR-4772-3p mimics, inhibitor, pmriGLO-Helios and their negative controls transcribed in iTregs. $^*P < 0.05$, $^+P < 0.01$. IKZF2: IKAROS family zinc finger 2; PBMCs: Peripheral blood mononuclear cells; Treg: Regulatory T cell.

direct target gene of the miR-4772-3p, we cloned the 3′-UTR of *IKZF2* into a dual-luciferase UTR reporter plasmid and co-transfected with the miR-4772-3p mimic, inhibitor, or negative control into 293 T cells. As shown in Figure 5, the firefly luciferase activity of the reporter that contained wild-type 3′-UTR of *IKZF2* was significantly inhibited by miR-4772-3p mimic ($P < 0.01$); however, the inhibition was rescued when the target site in the 3′-UTR of *IKZF2* was mutated. This indicated that miR-4772-3p directly acts on the *IKZF2* 3′-UTR.

Discussion

In the present study, we demonstrated that significantly accumulated $CD4^+CD25^+FOXP3^+$ Tregs in the MPE contained a high level of Helios compared with that in the NPE and the PB of NSCLC patients. We selected miR-4772-3p using high-through sequencing, as one of the downregulated miRNA that was associated with the elevated expression of Helios in the microenvironment of MPE. Mechanistically, downregulated expression of miR-4772-3p, which increased Helios levels in Tregs, contributed to a specific immunosuppressive state in PEs from patients with NSCLC.

A previous clinical study found that the proportion of $CD4^+CD25^+$T cells and $CD4^+CD25^+FOXP3^+$ Treg cells was significantly increased in the MPE.[13-15] The expression of functionally related genes such as cytotoxic T-lymphocyte associated protein 4 (*CTLA-4*), *CD28*, *FOXP3*, and glucocorticoid-induced TNFR-related protein *(GITR)* in $CD4^+CD25^+$ T cells were higher than those in $CD4^+CD25^-$ T cells from the MPE of lung adenocarcinoma.[14] Moreover, a high proportion of Treg cells, compared with effector T cells, was associated with worse overall survival in patients with MPE.[9,39,40] In line with a previous study,[14] we found that increased levels of $CD4^+CD25^+FOXP3^+$Treg cells with high Helios expression also supported enhanced Treg cells regulatory potential in the microenvironment of the MPE.

Current understanding suggest that Helios is an activated marker and potential modulator in the suppressive capacity of Tregs.[41-44] It not only directly binds to at least two sites on the *FOXP3* promoter,[21] and augments transactivation of *FOXP3*, but also has been implicated in Treg development and stability by silencing the IL-2 gene promoter.[43,45] In a Helios reporter mouse model, $Helios^+$ Tregs were shown to exhibit superior immunosuppressive characteristics compared with $Helios^-$ Tregs.[43] In human Tregs, siRNA-mediated Helios knockdown attenuated the expression of *FOXP3* and impaired the suppressive function.[21] Additionally, the $FOXP3^+Helios^-$ T cell subset comprised a larger proportion of non-suppressive T cells and secreted significant levels of effector cytokines, such as IFN-γ, IL-2, and IL-17.[46,47] Importantly, the transcription factor Helios ensures the maintenance of Treg lineage phenotype and functional stability under pressure from intense inflammatory responses.[22,29]

Emerging clinical evidence also suggests that aberrant Helios expression participates in the progression of a wide variety of cancers. In the PB from childhood with pediatric

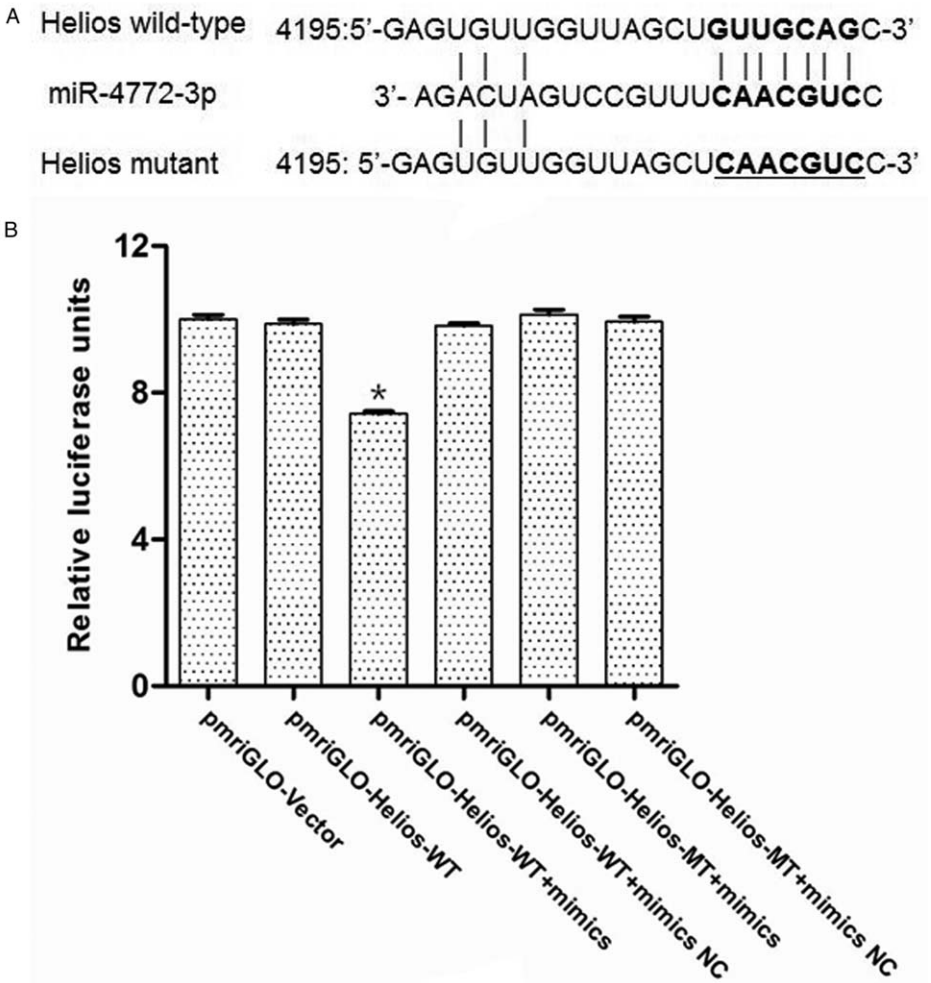

Figure 5: The *IKZF2* (Helios) gene is a direct target of miR-4772-3p. Bioinformatics website predicated the possible binding site at the 3′-UTR of Helios (A). 3′-UTR luciferase reporter assay with vectors harboring WT, mutated *IKZF2* 3′-UTR, or no 3′-UTR (control) co-transfected with miR-4772-3p mimic or non-targeting miRNA. Twenty-four hours later, luciferase activity was measured (B). $^{*}P < 0.01$. IKZF2: IKAROS family zinc finger 2; miRNA: MicroRNAs; UTR: Untranslated region; WT: Wild-type.

precursor B-cell acute lymphoblastic leukemia, Helios^{+}FOXP3^{+}CD4^{+}Tregs, an enhanced subset of total CD4^{+} T cells, promoted angiogenesis by activating the vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 pathway, which affected on the growth of leukemia cells by upregulating the expression of anti-apoptotic protein BCL-2 (BCL2 apoptosis regulator).[48] Preferential accumulation of a FOXP3^{+}Helios^{+} Treg subset in the breast cancer microenvironment presented diverse immunosuppressive characteristics with the co-expression CTLA-4 and programmed cell death 1 (PD-1).[28] These Tregs and diverse immunosuppresive molecules create an immune-subversive environment for breast tumor cells and might contributed to unfavorable patient prognosis.[28] In addition, compared with non-tumoral mucosa and PB from patients with colorectal cancer (CRC), significantly accumulated Helios^{high} Tregs in tumors had a highly demethylated Treg-specific demethylated region of the *FOXP3* locus and high levels of TNF receptor superfamily member 4 (OX40) and ectonucleoside triphosphate diphosphohydrolase 1 (CD39) expression.[49] These Tregs with multiple activities not only suppressed anti-tumor immunity mediated by effector T cells, but also released IL-17 that was derived from the Th17-like Tregs polarization, or switched into T follicular regulatory cells which might impair the Tfh response, thus, contributing to CRC establishment and progression.[49] In addition, Helios-deficient intra-tumoral Tregs displayed higher levels of GITR/PD-1 and affinity for tumor-associated self-antigens; however, intra-splenic or peripheral lymphoid Tregs did not.[38] These genetic and phenotypic changes of Helios-deficient Tregs within the environment of tumors might provide the basis for manipulating Helios as a potential target to improve anti-tumor immune responses.[29,38]

Previous studies have revealed that miRNAs affect the development, biological function, and phenotypic stability of Treg cells. Therefore, the differential expression of miRNAs in the PE from NSCLC and inflammatory conditions was assessed using next-generation sequencing technology. The results showed that miR-4772-3p, as a downregulated miRNA, might be associated with the immune dysfunction in the PE microenvironment. Thus, *IKZF2* (Helios) was further predicted as a potential target gene of miR-4772-3p by TargetScan, miRWalk, and DIANA micro T. Our data demonstrated that, at both the mRNA and protein levels, the miR-4772-3p mimics reduced the expression of Helios in iTreg cells.

Overexpression of *IKZF2* in iTreg cells reversed the promoting effects induced by transfection of miR-4772-3p. The endogenous Helios protein level was also elevated after transfection with miR-4772-3p inhibitors in iTreg cells. This may be associated with the lower expression of endogenous miR-4772-3p in iTreg cells. Importantly, miR-4772-3p could regulate Helios expression by directly targeting *IKZF2* mRNA in a firefly luciferase gene assay. In addition, there was a significant negative correlation between the miR-4772-3p level and Helios$^+$ Tregs in PEMCs from patients with NSCLC. These results indicated that *IKZF2* (Helios), as a potential target gene downregulated by miR-4772-3p, influences the suppressive function of Treg cells in the MPE microenvironment from NSCLC.

In conclusion, preferential accumulation of CD4$^+$CD25$^+$FOXP3$^+$ Tregs with relatively upregulated expression of Helios predominantly occurs in MPE from NSCLC and might have a negative effect on patient prognosis. Furthermore, miR-4772-3p expression was downregulated in PEMCs and might be closely associated with highly immunosuppressive conditions in the tumor-infiltrating microenvironment in patients with NSCLC. MiR-4772-3p, by targeting *IKZF2* (Helios), might represent a mechanism that actively promotes tumor evasion from the host immunological surveillance and represents a potential therapeutic molecular target for MPE.

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Conflicts of interest

None.

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