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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

MiR-21 Participates in the PD-1/PD-L1 Pathway-Mediated Imbalance of Th17/Treg Cells in Patients After Gastric Cancer Resection

Xiaoyu Zheng, MD[1], Linlin Dong, MD[1], Kun Wang, MD[1], Huichao Zou, MD[1], Su Zhao, MD[3], Yu Wang, MD[1], and Guonian Wang, MD, PhD[1,2]

[1]Department of Anaesthesiology, Harbin Medical University Cancer Hospital, Harbin, China; [2]Pain Research Institute of Heilongjiang Academy of Medical Sciences, Harbin, China; [3]Department of Thoracic Surgery, Harbin Medical University Cancer Hospital, Harbin, China

ABSTRACT

Background. The programmed cell death-1/programmed cell death-ligand 1 (PD-1/PD-L1) pathway has been shown to be involved in trauma-induced immunosuppression and to influence $CD4^+$ T cell differentiation. MicroRNA (miR)-21 is a critical player in immune responses. However, it remains largely unknown whether miR-21 is regulated by PD-1 and influences $CD4^+$ T-cell lineage choice after gastric cancer resection.

Methods. In the present study, we analyzed the percentages of T-helper (Th)-17/regulatory T (Treg) cells and PD-1/PD-L1 expression on peripheral blood mononuclear cells (PBMCs) during the perioperative period. We also detected the secretion of interleukin (IL)-17 and transforming growth factor (TGF)-β1 using enzyme-linked immunosorbent assays (ELISAs). Furthermore, PBMCs isolated from patients were transfected with or without adenovirus-short hairpin-PD-1 (Ad-sh-PD1), pre-miR-21 or adenovirus–green fluorescent protein (Ad-GFP), and the percentages of Th17/Treg cells and related transcription factors were measured.

Results. In patients who underwent gastric cancer resection, the number of Th17 cells decreased, whereas the number of Treg cells increased, accompanied by an increased expression of PD-1/PD-L1. In addition, the expression of RORγt and IL-17 decreased, whereas the expression of Foxp3 and TGF-β1 increased. In vitro, silencing PD-1 via Ad-sh-PD1 promoted the expression of miR-21 and increased the percentage of Th17 cells, but decreased the percentage of Treg cells. The overexpression of miR-21 increased the percentage of Th17 cells but decreased the percentage of Treg cells.

Conclusions. Our study demonstrated that gastric cancer resection altered the balance of Th17/Treg cells and increased PD-1/PD-L1 expression. In the in vitro experiments, the transfection of Ad-sh-PD1 ameliorated Th17/Treg cell imbalance partially by increasing the expression of miR-21.

Gastric cancer is the second most common cause of cancer mortality globally, and surgical removal remains a mainstay in its cure.[1] Although surgical excision of tumors can save or extend a patient's life, it has long been acknowledged that the surgical insult itself may precipitate or accelerate tumor recurrence.[2] Abdominal surgery with blood loss causes a depression of cell-mediated immune responses, resulting in increased susceptibility to infectious complications with increased mortality.[3,4] The postoperative immunosuppression is induced by multiple factors, including inflammatory cytokines,[5] the neuroendocrine response (i.e. the release of catecholamines,[6] prostanoids[7,8] and cortisol[9]), low blood pressure/low blood volume,[10,11] ischemia–reperfusion injury,[11] and use of opioids.[12] Surgical trauma not only suppresses the innate immune response but also inhibits the adaptive immune responses, including cellular immune responses that are essential for the clearance of residual tumor cells. Consequently, surgical trauma might promote the metastasis and recurrence of tumors.[13] The imbalance of $CD4^+$ T-cell differentiation plays a major role in adaptive immune dysfunction.[14] Thus,

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G. Wang, MD, PhD
e-mail: guonianwang@163.com; wangguonian609cn@aliyun.com

exploring the regulatory signaling mechanism underlying the imbalance of $CD4^+$ T-cell differentiation after gastric cancer resection is fundamentally important and can aid the development of promising treatment strategies to prevent the immunosuppression that occurs during the postoperative period and eliminate residual tumor cells.

After interacting with their cognate antigen, naive $CD4^+$ T cells proliferate and polarize toward different $CD4^+$ lineages depending on the cytokine microenvironment.[15] $CD4^+$ lineages mainly include T-helper (Th)-1 cells, Th2 cells, Th17 cells, and regulatory T (Treg) cells.[16,17] Multiple reports have indicated that surgery and trauma cause selective suppression of Th1 function and a shift toward a Th2 cytokine pattern with cell-mediated immune suppression.[3,18] Recently, both experimental and clinical studies have observed an imbalance of Th17/Treg cells in multitrauma patients and animals.[17,19] Excessive Treg immunity is harmful for patients who have undergone surgery. Therefore, maintaining the balance of Th17/Treg cells is essential, particularly for cancer patients who have undergone surgery.

The generation of Th17 or Treg cells from naive precursors is heavily influenced by the activation of the T-cell receptor (TCR) and co-stimulatory molecules. Programmed cell death-1 (PD-1) is a novel negative co-stimulatory molecule that is expressed on activated $CD4^+$ and $CD8^+$ T cells and binds two known ligands, programmed cell death ligand (PD-L) 1 and PD-L2, which have been found on antigen-presenting cells (APCs).[20] Previous studies have shown that the abnormal expression of PD-1/PD-L1 can induce an imbalance of $CD4^+$ T-cell differentiation.[21,22] Recent studies have shown that expression of PD-1 increases after surgical trauma.[23,24]

Thus, effectively controlling the expression of PD-1/PD-L1 negative molecules may be one strategy for treating immunosuppression after gastric cancer resection. Therefore, we investigated how gastric cancer resection influences the balance of Th17/Treg cells, and the role of the PD-1/PD-L1 pathway in this process.

PATIENTS AND METHODS

Patients

The study procedures were approved by the Ethics Committee of Harbin Medical University Cancer Hospital. Written informed consent was obtained from participating patients (or their relatives) and healthy volunteers upon enrollment. We recruited patients with gastric cancer who were about to undergo gastrectomy, but excluded patients who received anticancer treatment (such as chemotherapy and radiotherapy) prior to surgery or patients with autoimmune diseases. The control group consisted of 16 healthy subjects. The characteristics of the patients and healthy controls are presented in Table 1.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque density gradient centrifugation as described by Boyum et al.[25] The cells were then suspended in RPMI-1640 with 10% fetal calf serum and antibiotics.

Flow Cytometry Analysis

PBMCs were re-suspended at a density of 1×10^6 cells/100 μL in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin. To determine the percentage of Treg cells, the PBMCs were incubated with FITC-labeled anti-CD4, APC-cy7-labeled anti-CD25, and PE-labeled anti-Foxp3 antibodies for 30 min at room temperature. To determine the percentage of Th17 cells, the cells were stimulated with a cell stimulation kit (eBioscience, USA) for 4 h in the presence of 10 μg/mL brefeldin A, before being incubated with FITC-labeled anti-CD4 and PE-labeled interleukin (IL)-17 antibodies. The cells were incubated with APC-labeled anti-PD-1 and FITC-labeled anti-CD4 for 30 min at room temperature to detect the expression of PD-1 on $CD4^+$ T-cell surfaces. The expression of PD-L1 on PBMCs was also labeled with a PE-labeled anti-PD-L1 antibody.

Isolation, Stimulation and Transfection of $CD4^+$ T Lymphocytes

PBMCs were isolated from patients on day 3 after gastric cancer resection ($n = 16$), as described above. $CD4^+$ T lymphocytes were isolated from PBMCs using a magnetic cell separation (MACS) magnetic column. The isolated $CD4^+$ T lymphocytes were treated with αCD3/CD28-precoated antibodies (Invitrogen Life Technologies) in RPMI-1640 supplemented with 10% calf bovine serum, 1% penicillin–streptomycin, and 100 U/mL IL-2 at a concentration of 2×10^5 cells/mL, and cultured for 48 h in a 24-well plate at 37 °C in 5% CO_2. Subsequently, the $CD4^+$ T lymphocytes were transfected with adenovirus-short hairpin-PD1 (Ad-sh-PD1) or pre-miR-21 adenovirus (Hanbio Technology Ltd, China) for 48 h.

Western Blot

Protein was extracted with protein lysis buffer. The protein concentration was determined using a BCA protein assay kit (Thermo Scientific, USA). Proteins were

TABLE 1 Baseline and surgical characteristics

Characteristics	Control (n = 16)	Patients (n = 16)	p-value
Age, years [mean (SD)]	57 ± 6.6	57 ± 8.7	0.838
Height, cm [mean (SD)]	168 ± 6.1	168 ± 7.0	0.900
Weight, kg [mean (SD)]	65.7 ± 7.46	67.0 ± 8.3	0.643
Male sex	7 (43.75)	5 (31.25)	0.716
ASA			
I	3 (18.75)	3 (18.75)	0.828
II	11 (68.75)	12 (75.00)	
III	2 (12.50)	1 (6.25)	
Cancer stage			
I	–	5 (31.25)	–
II	–	4 (25.00)	
III	–	7 (43.75)	
Degree of differentiation			
1	–	8 (50.00)	–
2	–	4 (25.00)	
3	–	2 (12.50)	
4	–	2 (12.50)	

Data are expressed as n (%) unless otherwise specified

ASA American Society of Anesthesiologists, *SD* standard deviation

Cancer stages: Stage I: T1, N0, M0/T2, N0, M0/T1, N1, M0; Stage II: T3, N0, M0/T4a, N1, M0/T3, N1, M0/T2, N2, M0/T1, N3, M0; Stage III: T2, N3, M0/T3, N2, M0/T3, N3, M0/T4a, N2, M0/T4a, N3, M0/any T4b, any N, M0; Stage IV: any T, any N, M1

Degrees of differentiation: 1 = poorly differentiated; 2 = moderately differentiated; 3 = well-differentiated; 4 = other/unknown differentiated

separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with Tris-buffered saline (TBS)/5% skim milk. Subsequently, the membrane was incubated at 4 °C overnight with anti-PD-1 (1:1000), anti-PD-L1 (1:1000) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10,000) antibodies (Abclonal, China). The membrane was then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody and detected with an enhanced chemiluminescence (ECL) Western blotting analysis kit.

Real Time-Polymerase Chain Reaction

Total RNA was purified using an RNA isolation kit (Invitrogen Life Technologies). Complementary DNA (cDNA) was reverse transcribed using random hexamer primers and RNase H-reverse transcriptase (Thermo Fisher). The primers were designed and synthesized by Invitrogen™, and the primer sequences were as follows:

- RORγt: forward primer 5′-AATCTCATCCTCGGAAA AGTG-3′ and reverse primer 5′-TCTCAAAGCAG GAGCAATGGA-3′
- Foxp3: forward primer 5′-TGGAGGAACTCTGGGAA TGTG-3′ and reverse primer 5′-GGTGCTGGAGAA GGAGAAGCT-3′
- GAPDH: forward primer 5′-CATGTTCGTCATGGG TGTGAA-3′ and reverse primer 5′-GGCATGGACT GTGGTCATGAG-3′.

Enzyme-Linked Immunosorbent Assay

Blood samples were collected and immediately centrifuged at 4000 g for 10 min at 4 °C. The plasma was immediately frozen and used to detect the levels of IL-17 and transforming growth factor (TGF)-β1. Serum levels of IL-17 and TGF-β1 were measured using enzyme-linked immunosorbent assay (ELISA) kits (Abclonal) according to the manufacturers' instructions. All samples were measured at least in triplicate.

Statistical Analysis

Statistical analyses were carried out using SPSS 13.0 software (serial 5,031,432, Stats Data Mining Co., China). Data were presented as the mean ± standard deviation (SD). Differences between the two groups were examined

with unpaired Student's *t*-tests, and one-way analysis of variance (ANOVA) was used to compare multiple groups. The clinical characteristics of the healthy controls and patients were analyzed using Pearson's Chi square tests. A *p*-value < 0.05 was considered statistically significant.

RESULTS

The Balance of T-helper (Th)-17/Regulatory T (Treg) Cells was Disturbed in Patients Who Underwent Gastric Cancer Resection

As shown in Fig. 1, the percentage of Th17 cells ($0.35 \pm 0.11\%$) decreased (Fig. 1a, b; $p < 0.01$), whereas the percentage of Treg cells ($18.3 \pm 4.32\%$) increased in patients on day 3 compared with their respective preoperative levels (3.21 ± 0.75 and $10.02 \pm 2.88\%$, respectively) (Fig. 1c, d; $p < 0.01$). Moreover, we observed that the plasma level of IL-17 was lower on day 3 (225 ± 52 pg/mL) than on day 0 (270 ± 47 pg/mL) (Fig. 1e; $p = 0.02$), and the plasma level of TGF-β1 was higher on day 3 (1035 ± 180 pg/mL) than on day 0 (870 ± 148 pg/mL) (Fig. 1f; $p = 0.008$).

Gastric Cancer Resection Upregulated PD-1/PD-L1 Expression and Downregulated MiR-21 Expression in Gastric Cancer Patients

We analyzed $CD4^+$ PD-1/PD-L1 expression on PBMCs isolated from patients with gastric cancer prior to surgery (day 0) and 3 days after surgery (day 3). $CD4^+$ PD-1 expression on $CD4^+$ T cells was higher on day 3 (Fig. 2a, b; $p < 0.05$) than on day 0. The expression of PD-L1 was also higher on day 3 (Fig. 2c, d; $p < 0.01$) than on day 0. We also detected PD-1 and PD-L1 protein expression levels using Western blot. The expression levels of both PD-1 and PD-L1 were elevated on day 3 compared with day 0 (Fig. 2e). In addition, the expression of miR-21 decreased in patients after gastric cancer resection (Fig. 2f; $p < 0.01$).

Transfection of Ad-sh-PD1 Changed the Expression of MiR-21 and Altered the Percentages of Th17 and Treg Cells In Vitro

We first observed that the miR-21 expression was significantly increased in the Ad-sh-PD1 group, thus indicating the role of PD-1 as a negative regulator of miR-21 (Fig. 3f; $p < 0.05$). We then explored the role of PD-1 in Th17 and Treg cell differentiation. Compared with $CD4^+$ T cells transfected with adenovirus–green fluorescent protein (Ad-GFP), the percentage of Th17 cells was higher (Fig. 3a, b; $p < 0.01$) and the percentage of Treg cells was lower in $CD4^+$ T cells transfected with Ad-sh-PD1 (Fig. 3c, d; $p < 0.01$). RORγt expression was increased in the Ad-sh-PD1 group compared with that in the Ad-GFP group (Fig. 3g; $p < 0.01$), whereas Foxp3 expression was decreased (Fig. 3h; $p < 0.01$).

Overexpression of MiR-21 Altered the Percentages of Th17 and Treg Cells, and the Expression of RORγt and Foxp3 In Vitro

To explore the role of miR-21 in Th17 and Treg cell differentiation, miR-21 was overexpressed by pre-miR-21. We observed a higher percentage of Th17 cells (Fig. 4a, b; $p < 0.01$) but a lower percentage of Treg cells in $CD4^+$ T cells transfected with pre-miR-21 than in the pre-miRNA control group (Fig. 4c, d; $p < 0.05$); these changes were accompanied by higher expression levels of RORγt (Fig. 4e; $p < 0.05$) and lower expression levels of Foxp3 (Fig. 4f; $p < 0.01$).

DISCUSSION

Our present study demonstrated, for the first time, that gastric cancer resection alters the balance of Th17/Treg cells. Moreover, the PD-1/PD-L1 pathway may contribute to this imbalance of Th17/Treg cells, in part due to modulation of miR-21. Our main detailed findings are as follows: First, there was a Th17/Treg cell imbalance in patients who underwent gastric cancer resection. Second, the percentage of $CD4^+$ $PD\text{-}1^+$ T cells and PD-L1-positive cells increased in patients on day 3 following gastric cancer resection. Meanwhile, the expression of miR-21 decreased. Third, the in vitro studies showed that PD-1 inhibition or miR-21 overexpression reduced the percentage of Treg cells but enhanced the percentage of Th17 cells.

Several studies have shown that severe thoracic trauma, thoracotomy and post-traumatic sepsis perturbed the balance of Th17 and Treg cells.[26–28] Whether surgical trauma induced by gastric cancer resection impacts the Th17/Treg balance is unclear. In this study, our results demonstrated that following gastric cancer resection, the proportion of Th17 cells decreased, whereas the proportion of Treg cells increased. Meanwhile, the plasma level of IL-17 decreased and the plasma level of TGF-β1 increased. All these results suggest that gastric cancer resection induced a shift in the Th17/Treg balance toward Treg cells.

The mechanisms governing the imbalance of Th17/Treg cells resulting in immunosuppression after gastric cancer resection remain incompletely understood. The cytokine environment is one of the major determinants of the Th17/Treg balance. TGF-β1 is a signature cytokine secreted by Treg cells and may provide a powerful positive feedback

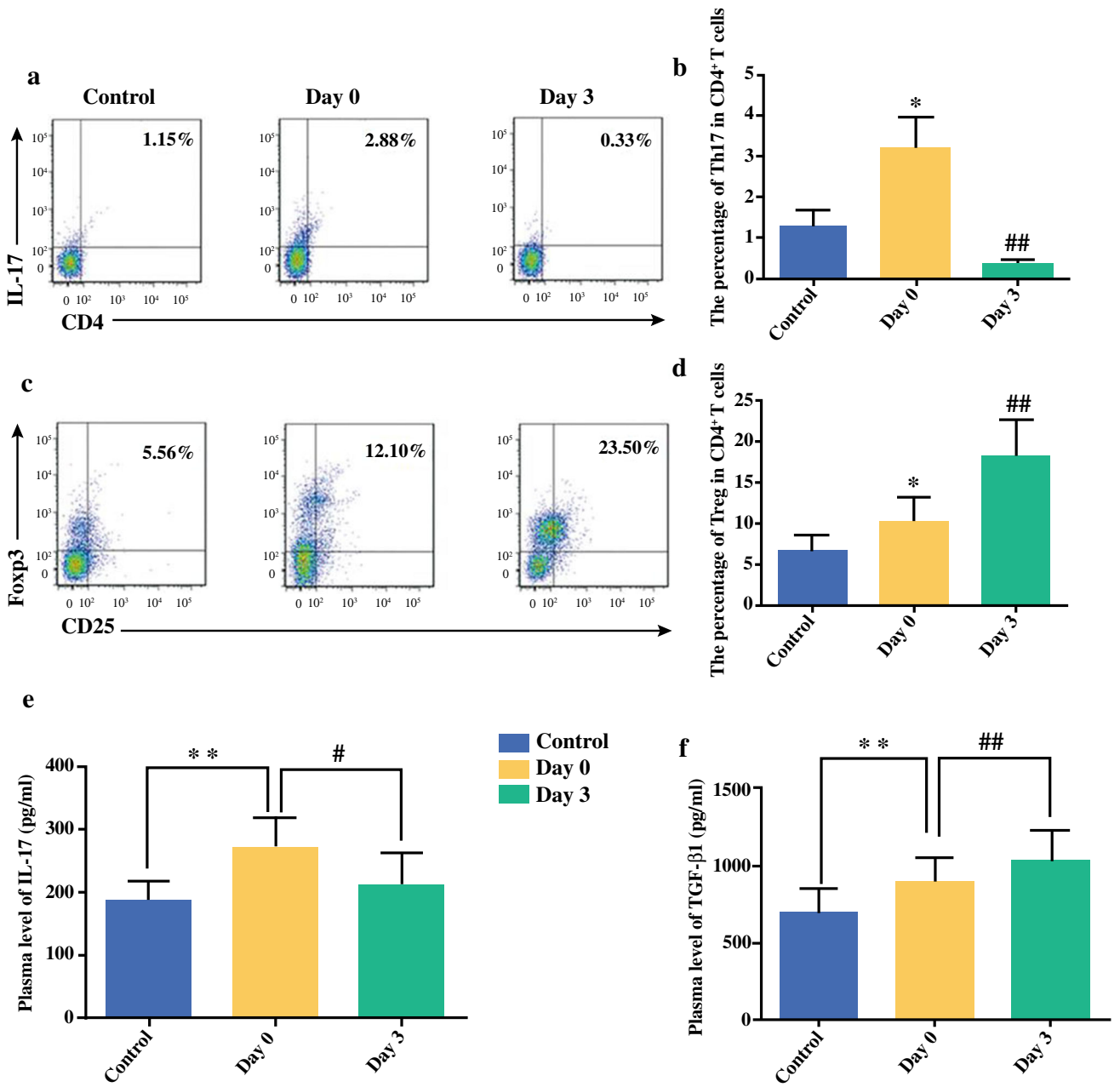

FIG. 1 Gastric cancer resection influenced the imbalance of Th17 and Treg cells. **a-d** Flow cytometry was used to analyze the percentages of Th17 and Treg cells in $CD4^+$ T cells. **a, c** Representative diagram of the percentages of Th17 and Treg cells in $CD4^+$ T cells from healthy controls and patients prior to surgery (day 0) or on day 3. **b, d** Percentages of Th17 and Treg cells in $CD4^+$ T cells. **e** Plasma levels of IL-17 in the three groups. **f** Plasma levels of TGF-β1 in the three groups. Data are expressed as the mean ± SD ($n = 16$). * $p < 0.05$; ** $p < 0.01$ compared with the control group; # $p < 0.05$ compared with day 0; ## $p < 0.01$ compared with day 0. *IL* interleukin, *Th17* T-helper 17, *Treg* regulatory T, *TGF* transforming growth factor, *SD* standard deviation

loop for the induction of Treg cells.[15] In our study, we found that gastric cancer resection promoted the production of TGF-β1, which may be one reason for the gastric cancer resection-induced Th17/Treg cell imbalance. Moreover, severe trauma altered the ability of conventional dendritic cells (cDCs) to secrete proinflammatory cytokines, thereby impairing their ability to effectively prime Th1 and Th17 T-cell responses.[29]

Co-stimulatory molecules such as PD-1 are also critical factors in controlling the differentiation of Th17 and Treg cells.[30] Arai et al.[31] found that PD-1 expression on $CD4^+$

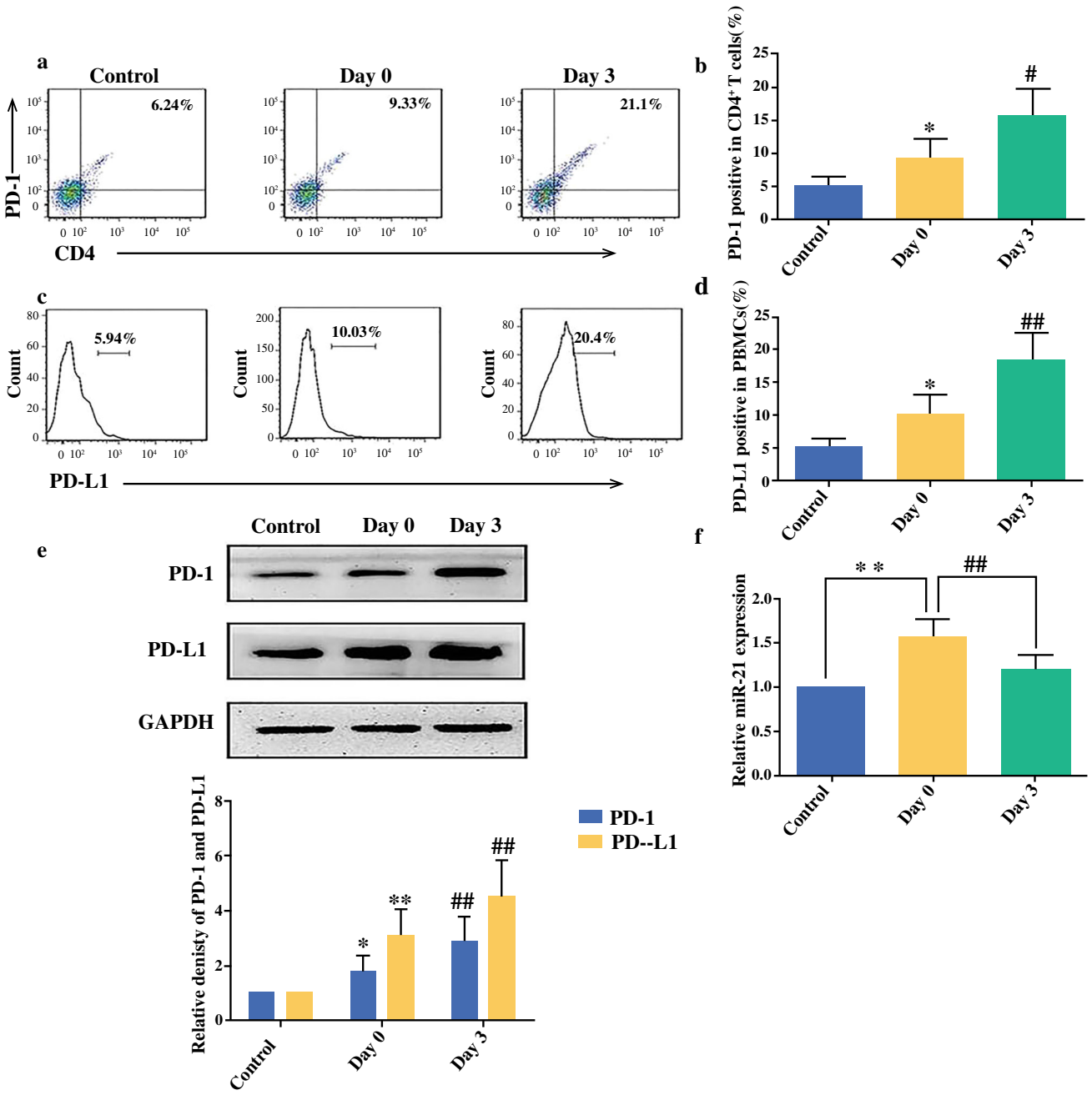

FIG. 2 Expression of PD-1 on the surface of $CD4^+$ T cells, expression of PD-L1 on PBMCs, and expression of miR-21 after gastric cancer resection. **a, c** PBMCs were isolated prior to surgery (day 0) and after surgery (day 3). Flow cytometry was used to analyze the expression of PD-1 on the surface of $CD4^+$ T cells and the expression of PD-L1 on PBMCs. A typical scatter diagram of PD-1/PD-L1 expression prior to surgery (day 0) and 3 days after surgery (day 3) is shown. **b, d** Percentages of PD-1-positive cells in $CD4^+$ T cells and PD-L1-positive cells in PBMCs. **e** The protein expression levels of PD-1 and PD-L1. The results showed that the levels of PD-1 and PD-L1 were higher in the day 0 group than in the control group, and in the day 3 group than in the day 0 group. **f** The expression of miR-21 was determined by quantitative real-time PCR. Data are presented as the mean ± SD ($n = 16$). * $p < 0.05$; ** $p < 0.01$ compared with the control group; $^{\#}$ $p < 0.05$ compared with day 0; $^{\#\#}$ $p < 0.01$ compared with day 0. *PBMCs* peripheral blood mononuclear cells, *PD-1* programmed cell death-1, *PD-L1* programmed cell death-ligand 1, *PCR* polymerase chain reaction, *SD* standard deviation

and $CD8^+$ T cells increased markedly after surgery for colorectal cancer and was associated with postoperative dysfunction of cell-mediated immunity. To evaluate whether the expression of the PD-1/PD-L1 pathway was upregulated after surgical trauma induced by gastric cancer resection, we first measured the expression of PD-1 and

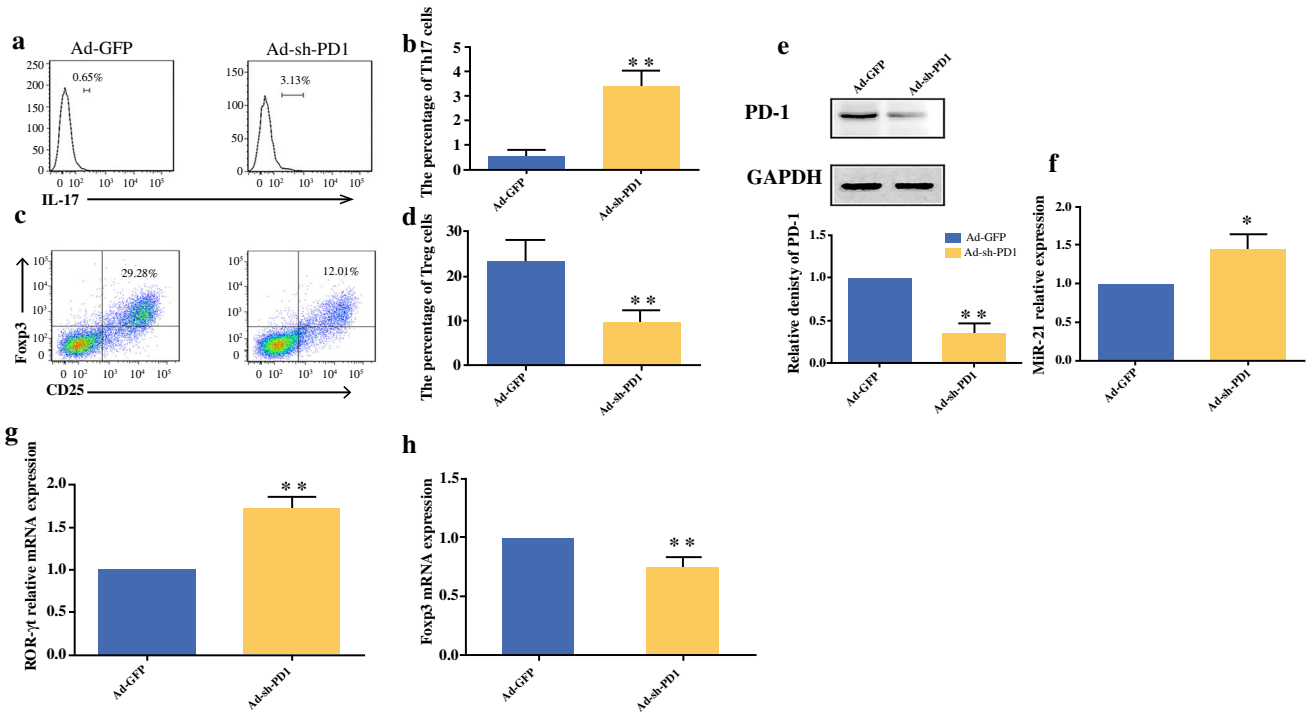

FIG. 3 Transfection of Ad-sh-PD1 changed the expression of miR-21 and regulated the differentiation of Th17 and Treg cells. **a, b** Transfection of Ad-sh-PD-1 increased the percentage of Th17 cells. **c, d** Transfection of Ad-sh-PD-1 decreased the percentage of Treg cells. **e** The expression of PD-1 protein in PBMCs transfected with Ad-GFP or Ad-sh-PD1 was detected by Western blot. **f** The expression of miR-21 was determined by quantitative real-time PCR. **g** Expression of RORγt in the Ad-GFP and Ad-sh-PD1 groups. The results showed that transfection of Ad-sh-PD-1 promoted the expression of the Th17-specific transcriptional factor RORγt. **h** Expression of Foxp3 in the Ad-GFP and Ad-sh-PD1 groups. The results showed that transfection of Ad-sh-PD-1 decreased the expression of the Treg-specific transcriptional factor Foxp3. The data are representative of three independent experiments and represent the mean ± SD. * $p < 0.05$, ** $p < 0.01$. *Ad-sh-PD1* adenovirus-short hairpin-PD-1, *Ad-GFP* adenovirus–green fluorescent protein, *PBMCs* peripheral blood mononuclear cells, *PD-1* programmed cell death-1, *PCR* polymerase chain reaction, *SD* standard deviation, *Th17* T-helper-17, *Treg* regulatory T, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *IL* interleukin

PD-L1 on PBMCs. The results showed that the percentages of $CD4^+$ $PD\text{-}1^+$ T cells and PD-L1-positive cells increased in patients on day 3 following gastric cancer resection, which demonstrated that gastric cancer resection led to increased expression of the PD-1/PD-L1 pathway. The significantly upregulated expression of PD-1/PD-L1 prompted us to further investigate whether the PD-1/PD-L1 pathway participated in the imbalance of Th17/Treg cells in patients after gastric cancer resection. We transfected Ad-sh-PD-1 into $CD4^+$ T cells to inhibit the expression of PD-1, and detected the percentages of Th17 and Treg cells. The results showed that the percentage of Th17 cells increased and the percentage of Treg decreased after transfection with Ad-sh-PD-1. The network of transcription factors is critically important for determining $CD4^+$ T-cell differentiation and function. RORγt, a transcription factor specific to Th17 cells, induces the transcription of the IL-17 gene in naive helper T cells. Foxp3, a specific transcription factor in Treg cells, is required for the secretion of TGF-β1 by Treg cells and for the suppression of effector T-cell function.[32] Thus, we also measured the expression of RORγt and Foxp3. Our data indicated that the expression of RORγt increased and the expression of Foxp3 decreased after transfection with Ad-sh-PD-1. Most importantly, our results showed that inhibition of PD-1 restored the imbalance of Th17/Treg cells in $CD4^+$ T cells from patients who underwent gastric cancer resection.

Emerging evidence indicates that microRNAs (miRNAs) play an important role in the regulation of the immune response.[33] MiR-21 is upregulated in several types of cancer. Specifically, miR-21 mediates tumor growth and promotes proliferation, and the observation of miR-21 overexpression in various human cancers suggests that miR-21 may act as an oncogene.[34] Iliopoulos et al.[35] reported that miRNA analysis of antigen-specific $CD4^+$ T cells revealed a significant upregulation of miR-21 in $PD\text{-}1^{-/-}$ T cells compared with wild-type controls. In our present study, we found that PD-1 expression increased, while the expression of miR-21 decreased. To explore the relationship between PD-1 and miR-21, we inhibited PD-1 expression in vitro via Ad-sh-PD1. We found that the inhibition of PD-1 increased miR-21 expression and

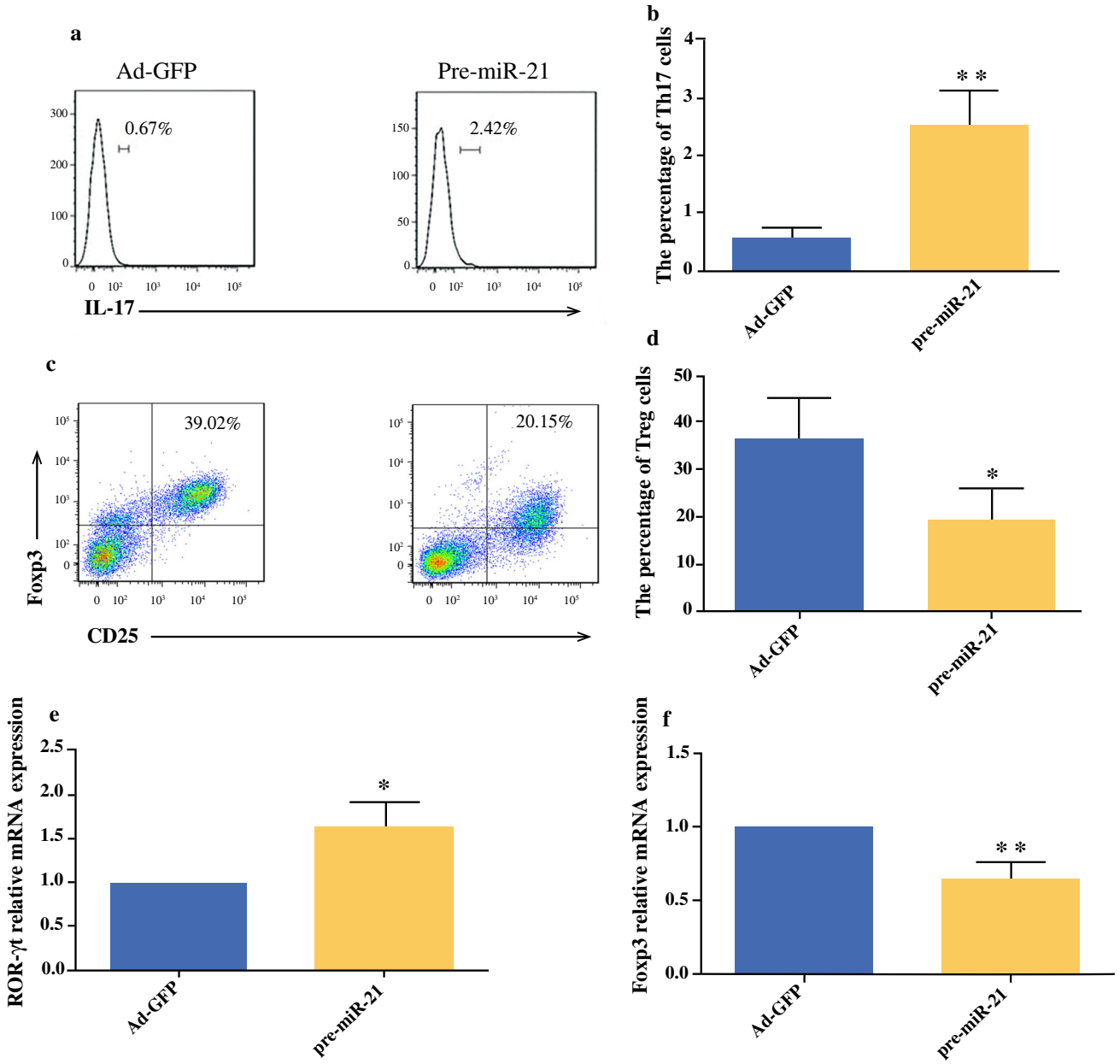

FIG. 4 Overexpression of miR-21 regulated the differentiation of Th17 and Treg cells. **a, b** Transfection of pre-miR-21 increased the frequency of Th17 cells. **c, d** Transfection of pre-miR-21 decreased the frequency of Treg cells. **e** Expression of RORγt in the Ad-GFP and pre-miR-21 groups. The results showed that transfection with pre-miR-21 promoted the expression of the Th17-specific transcriptional factor RORγt. **f** Expression of Foxp3 in the Ad-GFP and pre-miR-21 groups. The results showed that transfection of pre-miR-21 decreased the expression of the Treg-specific transcriptional factor Foxp3. The data are representative of three independent experiments and represent the mean ± SD. $*p < 0.05$, $**p < 0.01$. *Ad-GFP* adenovirus–green fluorescent protein, *SD* standard deviation, *Th17* T-helper-17, *Treg* regulatory T, *IL* interleukin

decreased the percentage of Treg cells, but increased the percentage of Th17 cells, indicating that PD-1 is a negative regulator of miR-21 and may influence the Th17/Treg balance in patients following gastric cancer resection. Thus, we speculated that PD-1 may impact the differentiation of Th17/Treg cells partially by regulating miR-21 expression in $CD4^+$ T cells. To further explore the direct link between miR-21 and Th17/Treg cell differentiation, we overexpressed miR-21 in $CD4^+$ T cells in vitro and found that the percentage of Th17 cells increased and the percentage of Treg cells decreased, thus demonstrating that miR-21 played an important role in the differentiation of Th17 and Treg cells. Thus, our results characterize a previously unknown miRNA pathway that

regulates Th17/Treg differentiation, and identify miR-21 as a potential therapeutic target in the amelioration of complications due to gastric cancer resection.

There were several limitations in our study. First, one potential limitation of this study was that the PD-1/PD-L1 pathway was not blocked by a specific antibody against PD-1 or PD-L1 in vitro. We only used Ad-sh-PD1 to knock down PD-1 and determine the effect of the PD-1/PD-L1 pathway on the imbalance of Th17/Treg cells. Second, the role of the target gene of miR-21 in the differentiation of Th17/Treg cells was not explored. Previous studies have shown that miR-21 targets mIL-12p35 and PDCD4 mRNA post-transcriptionally to regulate Th1 and Treg differentiation.[36] Whether mIL-12p35 or PDCD4 participate in miR-21-related Th17/Treg imbalance in gastric cancer resection will be further evaluated in a future study. Third, an anti-PD-1 antibody was not administered to patients to investigate the effects of the PD-1/PD-L1 pathway on the Th17/Treg cell imbalance in vivo in the present study. Moreover, we did not examine the differential expression of microRNAs using an miRNA array in patients after gastric cancer resection. Other miRNAs may be related to the surgical trauma induced by gastric cancer resection. In the present study, we described a phenomenon specific to patients after gastric cancer resection. However, the in vitro manipulation in PBMCs from control patients and patients without gastric cancer undergoing abdominal surgery, or patients with gastric cancer not undergoing abdominal surgery, will be performed in the future. A further study is needed to validate our conclusions.

CONCLUSIONS

Our results showed that gastric cancer resection altered the balance of Th17/Treg cells, accompanied by increased PD-1/PD-L1 expression and decreased miR-21 expression. In addition, in the in vitro experiments, a blockade of the PD-1/PD-L1 pathway by transfection with Ad-sh-PD1 alleviated this imbalance and increased the expression of miR-21. The present study highlights the important role of the PD-1/PD-L1 pathway in the pathogenesis of gastric cancer resection-related Th17/Treg cell imbalance partially via miR-21, which may provide a novel therapeutic intervention strategy for the postoperative immune dysfunction of clinical patients.

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