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$CD4^+$ $CD25^+$ $GARP^+$ regulatory T cells display a compromised suppressive function in patients with dilated cardiomyopathy

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Summary

Dilated cardiomyopathy (DCM) is a lethal inflammatory heart disease and closely connected with dysfunction of the immune system. Glycoprotein A repetitions predominant (GARP) expressed on activated $CD4^+$ T cells with suppressive activity has been established. This study aimed to investigate the frequency and function of circulating $CD4^+$ $CD25^+$ $GARP^+$ regulatory T (Treg) cells in DCM. Forty-five DCM patients and 46 controls were enrolled in this study. There was a significant increase in peripheral T helper type 1 (Th1) and Th17 number and their related cytokines [interferon-γ (IFN-γ), interleukin (IL-17)], and an obvious decrease in Treg number, transforming growth factor-β_1 (TGF-β_1) levels and the expression of forkhead box P3 (FOXP3) and GARP in patients with DCM compared with controls. In addition, the suppressive function of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells was impaired in DCM patients upon T-cell receptor stimulation detected using CFSE dye. Lower level of TGF-β_1 and higher levels of IFN-γ and IL-17 detected using ELISA were found in supernatants of the cultured $CD4^+$ $CD25^+$ $GARP^+$ Treg cells in DCM patients compared with controls. Together, our results indicate that $CD4^+$ $CD25^+$ $GARP^+$ Treg cells are defective in DCM patients and GARP seems to be a better molecular definition of the regulatory phenotype. Therefore, it might be an attractive stategy to pay more attention to GARP in DCM patients.

Keywords: dilated cardiomyopathy; glycoprotein A repetitions predominant; immune system; regulatory T cells.

Introduction

Dilated cardiomyopathy (DCM) is a lethal inflammatory heart disease characterized by the presence of myocardium disorder with dilatation of the ventricles, but not coronary artery disease, hypertension or valvular abnormalities.[1–3] It is generally accepted that DCM caused by viral myocarditis is associated with hepatitis C virus, Coxsackievirus B, adenoviruses and parvovirus B19.[4,5] Previous studies have also reported the underlying pathogenic mechanisms of viral myocarditis, which can eventually lead to cardiac dysfunction.[6,7] Although no clear mechanism of the transition from viral myocarditis to DCM has been established, there is no doubt that the regulation of immune system plays a vital role in the pathogenesis of DCM.[8–10]

$CD4^+$ regulatory T (nTreg) cells are essential for maintaining immune tolerance by inhibiting the hyperactivation of effector T cells and have an indispensable role in preventing the progression of several inflammatory diseases.[8,11] Forkhead box P3 (FOXP3), a master transcriptional regulator of nTreg cells, is specifically expressed in naturally arising $CD4^+$ Treg cells and can be used to define the classical Treg phenotype.[12–14] However, FOXP3 as an intracellular molecular marker has hampered viable cell sorting for function assay, so discriminating Treg cells from activated T cells based on other member markers was an imminent strategy. Recently, glycoprotein A repetitions predominant (GARP), which is highly expressed on the surface of stimulated Treg clones but not on T helper (Th) clones, was identified as a new specific marker of activated human Treg cells.[15–17] It has been proved that GARP serves as a receptor for latent transforming growth factor-β (TGF-β), and can bind directly to latency-associated peptide on the surface of activated human Treg cells.[18] More notably, $CD25^+$ T cells sorted for high GARP expression can dissect the duality of

Treg/T helper type 17 (Th17) cell differentiation and displayed more potent suppressive activity compared with $CD25^+$ $GARP^-$ cells.[19,20] Our group's previous research found that the number and suppressive function of circulating $CD4^+$ $CD25^+$ $GARP^+$ Treg cells were impaired in patients with acute coronary syndrome, which can result in excess effector T-cell proliferation.[21–23] We also reported that atorvastatin could improve the inflammatory response by up-regulating the expression of GARP on regulatory T cells.[24]

However, available data concerning the impact of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells in patients with DCM has not yet been defined. Therefore, we tried to investigate whether $CD4^+$ $CD25^+$ $GARP^+$ Treg cells influence the pathological process of patients with DCM. Here we compared the circulating frequency of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells in patients with DCM and control groups and explored the potential mechanisms by which the special Treg cells regulate adaptive immunity.

Materials and methods

Study subjects

Based on the exclusion and inclusion criteria as presented below, 45 patients with DCM (mean age ± SD = 48·6 ± 12·5 years, 10 female, 35 male) without detectable aetiology between June 2015 and February 2016 in the Union Hospital of Huazhong University of Science and Technology (Wuhan, China) were enrolled in this study. Diagnosis of DCM was based on clinical criteria and other diagnostic procedures according to the guidelines of the World Health Organization. Inclusion criteria for DCM were the presence of left ventricular dilatation, with left ventricular end-diastolic dimension ≥ 5·5 cm and left ventricular ejection fraction < 40%, assessed by echocardiography. Moreover, all of them were treated with diuretics, spironolactone and angiotensin-converting enzyme inhibitor according to guidelines. Patients were excluded if any of the following were present: other autoimmune diseases, valvular disease (except relative mitral and/or tricuspid regurgitation), tumour, severe vascular lesions, serious infection recently, endocrine disease, clinically serious hepatic or renal disease, receiving immunosuppressive agents, and lack of informed consent from the patient. Normal controls were those who have no heart organic diseases, no other serious diseases (mean age ± SD = 45·6 ± 9·5 years, 15 female, 30 male). All patients and normal controls provided written informed consent before the study. Ethical approval for this study was granted by the ethics committee of Tongji Medical College of Huazhong University of Science and Technology and this investigation conformed to the principles outlined in the Declaration of Helsinki.

Blood samples preparation

Peripheral blood samples were obtained from all the patients by sterile venepuncture within 24 hr after admission. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and then used for cell culture, flow cytometric analysis, Western blot and real-time PCR. Serum was collected after centrifugation, aliquoted, and frozen at −80° for determination of cytokine levels.

Flow cytometric analysis of Th1, Th2, Th17 and Treg

Analysis of Treg cells. The PBMCs were washed twice with PBS and then resuspended at a density of 2×10^6 cells/ml in complete culture medium (RPMI-1640 suspended with 10% heat-inactivated fetal calf serum; Gibco BRL, Grand Island, NY).The cell suspension was seeded in 24-well culture plates. For GARP induction, cells were stimulated with soluble anti-CD3 and anti-CD28 antibodies (2 μg/ml each; eBioscience, San Diego, CA) for 24 hr. The incubator was set to 37° under a 5% CO_2 environment. For Treg analysis, PBMCs (stimulation with CD3/CD28 for 24 hr or no stimulation) were stained with anti-human CD4-FITC (R&D Systems, Minneapolis, MN), anti-human CD25-APC (R&D Systems), and anti-human GARP (PE) (eBioscience) for 30 min at 4°. Anti-mouse IgG1-PE (eBioscience) isotype controls were used to enable normalization and confirm antibody specificity. After surface staining, the cells were washed with PBS, fixed and permeabilized with fix/permeabilization reagent at 4° for 30 min, and washed twice in 1 × permeabilization buffer and incubated with an anti-human Foxp3-PEcy5 (eBioscience) at 4° for 30 min.

Analysis of Th1, Th2 and Th17. For Th cell analysis, PBMCs were seeded at a density of 2×10^6 cells/well in RPMI-1640 medium with 10% heat-inactivated fetal calf serum (Gibco BRL) and stimulated with PMA (50 ng/ml; Sigma, St Louis, MO) plus ionomycin (1 μM, Sigma) and monensin (500 ng/ml, eBioscience) for 4 hr at 37° under 5% CO_2. After stimulation, the cells were washed with PBS and then incubated with anti-human CD4-FITC (R&D Systems) antibody at 4° for 30 min, washed twice in 1 × permeabilization-buffer and stained with anti-human interferon-γ (IFN-γ) PE-cy7 (eBioscience), anti-human interleukin-17 (IL-17) -PE (R&D Systems) or anti-human IL-4-PE (R&D Systems) antibody at 4° for 30 min. Anti-mouse IgG1-PE and anti-rat IgG2-PEcy7 (eBioscience) isotype controls were used to enable normalization and confirm antibody specificity. The catalogue numbers of all antibodies used in this study are listed in Table S4.

Flow cytometric acquisition was performed using a FACSCalibur (BD Immunocytometry Systems, San Jose,

CA), and all analyses were performed using FLOWJO software (FlowJo, LLC, Ashland OR97520).

Proliferation and functional suppression assays

After 24 hr of stimulation with anti-human CD3 and anti-human CD28 antibodies, PBMCs from controls ($n = 6$) and patients with DCM ($n = 6$) were stained with anti-human CD4-Peridinin chlorophyll protein-Cy5.5 (eBioscience), anti-human CD25-APC (R&D Systems), and anti-human GARP-PE (eBioscience) for 30 min at 4°. After the surface staining, the responder T cells (Tresp cells; $CD4^+$ $CD25^-$ $GARP^-$ T cells) and $CD4^+$ $CD25^+$ $GARP^+$ Treg cells were obtained by FACS sorting using a FACs Aria (BD Biosciences, Franklin Lakes, NJ). The purity of $CD4^+$ $CD25^-$ $GARP^-$ T cells was ≥ 92%, and the purity of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells was ≥ 90%. $CD4^+$ $CD25^-$ $GARP^-$ T cells were resuspended in PBS (2×10^6/ml) were labelled with 2 μM carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Carlsbad, CA) for 10 min for 37° under a 5% CO_2 environment while rotating once at 5 min according to the manufacturer's protocol.[25] Then cells were cultured as following set-up: (i) 5×10^4 CFSE-labelled T cells of each group cultured alone without stimulation; (ii) fixed number (5×10^4) of CFSE-labelled T cells of each group were cultured alone or co-cultured with autologous Treg cells at different ratios (Treg : Tresp ratios: 1 : 1, 1 : 2, 1 : 4, 1 : 8) with stimulation of anti-CD3 and anti-CD28; (iii) for crossover experiment between control group and DCM group, 5×10^4 Tresp cells from control group were co-cultured with Treg cells from the DCM group, and 5×10^4 Tresp cells from the DCM group were co-cultured with Treg cells from the control group (Treg : Tresp ratios: 1 : 1, 1 : 2, 1 : 4, 1 : 8) with stimulation of anti-CD3 and anti-CD28. All the T cells were incubated in complete culture medium (RPMI-1640 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, and with 10% heat-inactivated fetal calf serum, Gibco BRL) with a final volume of 200 μl/well in 96-well plates with or without plate-bound anti-CD3 (10 μg/ml; eBioscience) and soluble anti-CD28 (5 μg/ml; eBioscience) for 4 days. Proliferation of Tresp cells were analysed by flow cytometry based on CFSE dilution of gated $CD4^+$ T cells. The difference in the percentages of $CFSE^{low}$ cells in each group and the ratio of these percentages were calculated.

Real-time PCR

Total RNA was extracted with TRIzol extraction (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and converted into cDNA using PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan). The 10-μl PCR mixture contained 1 μg of total cDNA and 5 pmol each of the primers which were purchased from Applied Biosystems (Foster City, CA). All reactions were performed with SYBR Premix Ex TaqII (TaKaRa) and incubated in an Applied Biosystems 7500 Real-Time PCR system (BIO-RAD, Singapore) in a 96-well plate according to the manufacturer's protocol.

Primer sequences used in this study are listed in the Supplementary material (Table S2). For each sample, the mRNA expression level was normalized to that of GAPDH.

Western blot analysis

Total protein was isolated from cultured PBMCs and quantified using a BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL). The following primary antibodies were used: anti-rat/human GAPDH (1 : 1000 dilution; Immunoway, Plano, TX) and anti-human GARP (0·3 μg/ml, R&D Systems). Protein samples were resolved on an 10% SDS–PAGE gel and then immunoblotted to PVDF membranes. The blots were blocked with 5% non-fat milk for 2 hr at room temperature, then probed with the specific primary antibody and incubated at 4° overnight. The membranes were washed and further incubated with secondary horseradish peroxidase-conjugated antibody (1 : 5000 dilution; Cell Signaling Technology, Danvers, MA) for 2 hr at room temperature. Finally, the Western blot bands were washed and developed with super ECL reagent (Thermo Scientific, Waltham, MA), and then they were semi-quantitatively analysed using densitometric methods in each group, with GAPDH as the internal control.

Cytokines detection

Levels of TGF-β_1, IL-10, IFN-γ and IL-17 in patient plasma and cell culture supernatant were measured by an ELISA according to the manufacturer's instructions (both from R&D Systems). The intra-assay and inter-assay variation coefficients for all ELISA were < 10%. All samples were measured in duplicate.

Statistical analysis

Values were expressed as mean ± SD in the text and figures. The Student's *t*-test was used to detect differences between two groups and one-way analysis of variance for multiple comparisons, followed by a post hoc Student–Newmann–Keuls test when necessary. A probable value of $P < 0{\cdot}05$ was considered to be statistically significant.

Results

Basic clinical characteristics

Basic clinical characteristics of the study population were summarized in Table 1. Age and gender showed no

significant differences between the two groups, but the left ventricular ejection fraction and left ventricular end-diastolic dimension were markedly lower in the DCM group compared with the control group. Detailed information of the patients selected to participate in the suppressive function assessment of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells are listed in the Supplementary material (Table S1).

The circulating $CD4^+$ $CD25^+$ $GARP^+$ and $CD4^+$ $CD25^+$ $FOX3^+$ Treg cell frequencies are decreased in patients with DCM

To determine the number of $GARP^+$ and $FOX3^+$ Treg cells, PBMCs were obtained from patients with DCM and age-matched controls, followed by flow cytometry analysis. We found that the frequency of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from PBMCs without stimulation was significantly lower in the DCM group (0·56 ± 0·3%) compared with the control group (1·17 ± 0·15%, $P < 0·01$); meanwhile, the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells was also lower in the DCM group compared with the control group (Fig. 1a–e). Additionally, as shown in Fig. 1(f), the mRNA expression of GARP and FOXP3 was consistent with the results of flow cytometry, showing lower in DCM patients compared with the control groups (both $P < 0·01$).

Table 1. Clinical characteristics of the study population

Characteristics	DCM group (n = 45)	Control group (n = 47)
Age (years)	48·6 ± 12·5	45·6 ± 9·5
Sex (male/female)	30/15	30/17
NYHA (II/III/IV)	II/III	–
LVEF (%)	27·8 ± 8·1#	64·4 ± 4·6
LVEDd (cm)	7·3 ± 1#	4·7 ± 0·3.
NT-proBNP (pg/ml)	3665·5 ± 2574·5#	137·1 ± 84·2
Hypertension (n, %)	8 (17·8%)*	5 (10·6%)
Diabetes (n, %)	6 (13%)*	3 (6. 7%)
Medication (%)		
ACEI/ARBs	40 (88·9%)#	6 (12·7%)
Digitalis	12 (26·7%)#	0 (0)
β-blocker	32 (71·1%)*	10 (21·3%)
Diuretics	42 (93·3%)#	0 (0)

Data are presented as the mean ± SD, percentages or numbers. '–' indicates that the result is negative.

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; DCM, dilated cardiomyopathy; LVEF, left ventricular ejection fraction; LVEDd, left ventricular end-diastolic dimension; NYHA, New York Heart Association. '–' indicates that the result is negative.

*$P < 0·05$ versus control.

#$P < 0·01$ versus control.

Levels of TGF-β_1 and IL-10 in serum and PBMCs in two groups

We tested levels of TGF-β_1 and IL-10 in serum using ELISA in patients with DCM and the control groups. As shown in Fig. 2(a), the level of TGF-β_1 was decreased in patients with DCM compared with the control groups ($P < 0·01$), showing a positive correlation with the frequency of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells. Contrastingly, IL-10 level was increased significantly in the DCM group compared with the control group in Fig. 2(b) ($P < 0·05$). In addition, as shown in Fig. 2(c,d), the mRNA expression of TGF-β_1 in PBMCs showed lower and IL-10 expression showed higher in patients with DCM compared with controls.

The frequencies of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells and $CD4^+$ $CD25^+$ $GARP^+$ $FOXP3^+$ Treg cells after *in vitro* T-cell receptor stimulation are decreased in patients with DCM

To further investigate the expression of GARP, we next measured the frequency of activated Treg cells after 24 hr of T-cell receptor stimulation. There was no significant difference in the percentage of $CD4^+$ T cells in PBMCs with stimulation between patients with DCM and normal controls ($P > 0·05$), whereas the frequency of $CD4^+$ $CD25^+$ T cells in patients with DCM (12·5 ± 5·2%)was statistically reduced compared with normal controls (18·5 ± 4·6%, $P < 0·01$) (see Supplementary material, Table S3). These results are in line with previous research showing the frequency of $CD4^+$ $CD25^+$ Treg cells present a downward trend in patients with DCM.[26,27] In addition, the frequency of $CD4^+$ $GARP^+$ cells was decreased in patients with DCM compared with controls, and the frequency of $CD4^+$ $FOXP3^+$ cells was also lower in patients with DCM than that in the control group (Fig. 3a,c). Simultaneously, we analysed the $CD25^+$ Treg cells in stimulated PBMCs. The frequencies of $CD4^+$ $CD25^+$ $GARP^+$ and $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells were both markedly lower in patients with DCM compared with the control groups (Fig. 3b,d). Meanwhile, we found that the frequency of $CD4^+$ $CD25^+$ $GARP^+$ $FOXP3^+$ Treg cells was also lower in patients with DCM than that of controls (Fig. 3b,d), though the percentage of $CD4^+$ $CD25^+$ $GARP^+$ $FOXP3^+$ cells among the $CD4^+$ $CD25^+$ $GARP^+$ fraction showed no significant difference between patients with DCM and control groups, as shown in Fig. 3(e).

In the present study, we also examined both mRNA and protein expression levels of GARP on total PBMCs. As shown in Fig. 3(f,g), both expressions of GARP mRNA and GARP protein were remarkably reduced in patients with DCM compared with control groups ($P < 0·01$ and $P < 0·01$).

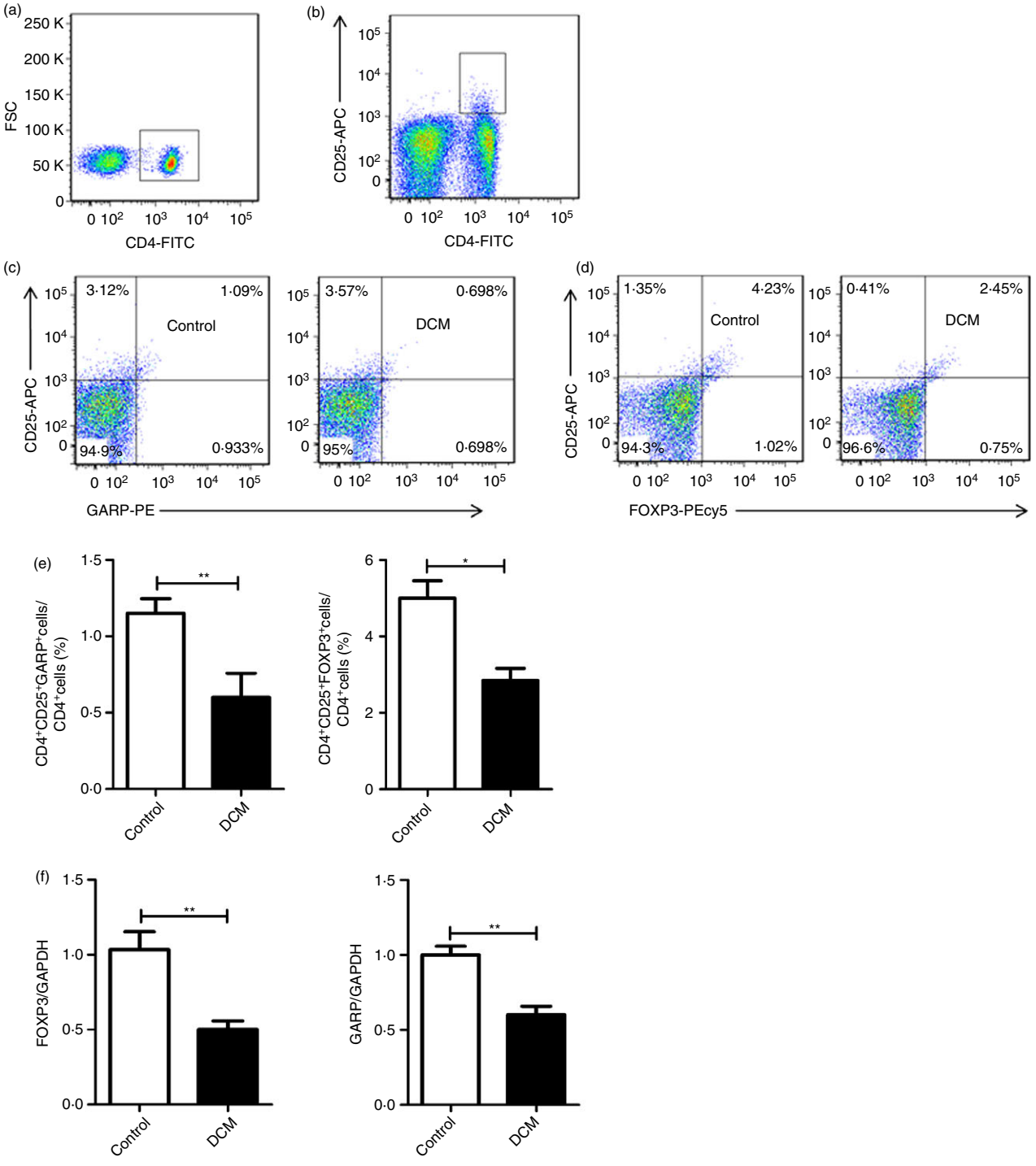

Figure 1. Identification and sorting of $CD4^+$ $CD25^+$ $GARP^+$ and $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T (Treg) cells in human peripheral blood mononuclear cells (PBMCs). The expression of glycoprotein A repetitions predominant (GARP) and forkhead box P3 (FOXP3) from human PBMCs were isolated and analysed by FACS and real-time PCR [dilated cardiomyopathy (DCM): $n = 22$; control: $n = 24$]. (a) Representative FSC/SSC dot plot shows the gated $CD4^+$ T cells. (b) Representative dot plot shows the gated $CD4^+$ $CD25^+$ T cells. (c, d) Representative FACS plots of $CD4^+$ $CD25^+$ $GARP^+$ and $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells in human peripheral blood. $CD4^+$ T cells were first gated on the side-scatter (FSC)/CD4 plots, and then the expression of GARP and FOXP3 was analysed in the gated $CD4^+$ T cells. (e) Comparison of the $CD4^+$ $CD25^+$ $GARP^+$ and $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells based on FACS analyses between the patients with DCM and the control groups. (f) RT-PCR analysis to determine GARP and FOXP3 expression in human PBMCs. $^*P < 0{\cdot}05$ versus control; $^{**}P < 0{\cdot}01$ versus control. [Colour figure can be viewed at wileyonlinelibrary.com]

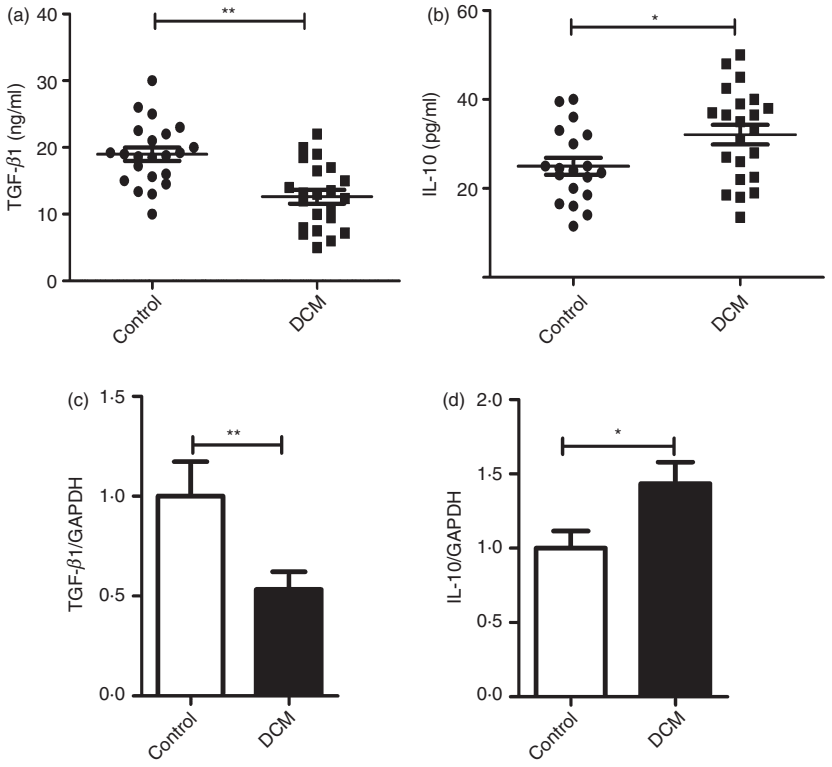

Figure 2. Transforming growth factor-β_1 (TGF-β_1) and interleukin-10 (IL-10) secretion in patients with dilated cardiomyopathy (DCM) and control groups. The expression levels of TGF-β_1 and IL-10 were analysed by ELISA and real-time PCR. (DCM: $n = 26$; control: $n = 28$). (a, b) Levels of TGF-β_1 and IL-10 were reduced in DCM patient serum analysed by ELISA compared with that in control patients (c, d) Levels of TGF-β_1 and IL-10 from human peripheral blood mononuclear cells (PBMCs) in two groups analysed by RT-PCR. *$P < 0{\cdot}05$ versus control; **$P < 0{\cdot}01$ versus control.

Circulating Th1 and Th17 frequencies were increased in patients with DCM

As shown in Fig. 4(b), the frequency of Th1 ($CD4^+$ IFN-$\gamma^+/CD4^+$ T cells) was markedly higher in patients with DCM compared with the control groups. Similarly, we observed that the frequency of Th17 ($CD4^+$ IL-$17^+/CD4^+$ T cells) was also higher in patients with DCM ($4{\cdot}12 \pm 1{\cdot}01\%$) compared with the control groups in Fig. 4(c). But, the frequency of Th2 ($CD4^+$ IL-$4^+/CD4^+$ T cells) showed no significant difference between DCM patients and control groups, as shown in Fig. 4(d). In addition, Th1 and Th17 mRNA expression levels from PBMCs were remarkably higher in patients with DCM compared with normal controls (both $P < 0{\cdot}001$), though the expression of IL-4 showed no obvious difference in the two groups (Fig. 4e).

$CD4^+$ $CD25^+$ $GARP^+$ Treg cells from patients with DCM presented a compromised suppressive function on Tresp cell proliferation

To determine the suppressive effect of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells on the proliferation of Tresp cells, we used a CFSE-based assay to quantify T-cell proliferation in co-cultures of Treg and Tresp cells in different ratios (1 : 1, 1 : 2, 1 : 4 and 1 : 8) stimulated by anti-CD3/28. The basic clinical characteristics of patients were summarized in the Supplementary material (Table S1). We first isolated $CD4^+$ $CD25^+$ $GARP^+$ Treg cells and $CD4^+$ $CD25^-$ $GARP^-$ Tresp cells by FACS (Fig. 5a). Then, Treg and Tresp cells were co-cultured at different ratios and crossover experiments were carried out in the patients with DCM and control groups. $CD4^+$ $CD25^+$ $GARP^+$ Treg cell and Tresp cells cultured alone without stimulation of CD3/28 did not proliferate and showed no significant difference in the patients with DCM and control groups, as seen in Fig. 5(b). $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from the patients with DCM and the control groups were both able to suppress the proliferation of Tresp cells to different extents. But $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from the patients with DCM showed an impaired suppressive function compared with the control groups (Fig. 5c). In addition, the suppressive potential of Treg cells had a close correlation with the ratio of Tresp : Treg cells, as increased cell ratio resulted in a decreased percentage of suppression (Fig. 5d). In conclusion, the experimental data indicated that $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from patients with DCM presented a compromised suppressive function.

We next assessed the levels of IFN-γ and IL-17 in the co-cultured T cells. As seen in Fig. 6(a,b), $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from patients with DCM exhibited a reduced capacity to suppress IFN-γ and IL-17 secretion compared with normal controls. We also measured the TGF-β_1 level in the supernatants of cultured $CD4^+$ $CD25^+$ $GARP^+$ Treg cells in two groups. As shown in Fig. 6(c), TGF-β_1 level was reduced in the DCM group compared with the normal group.

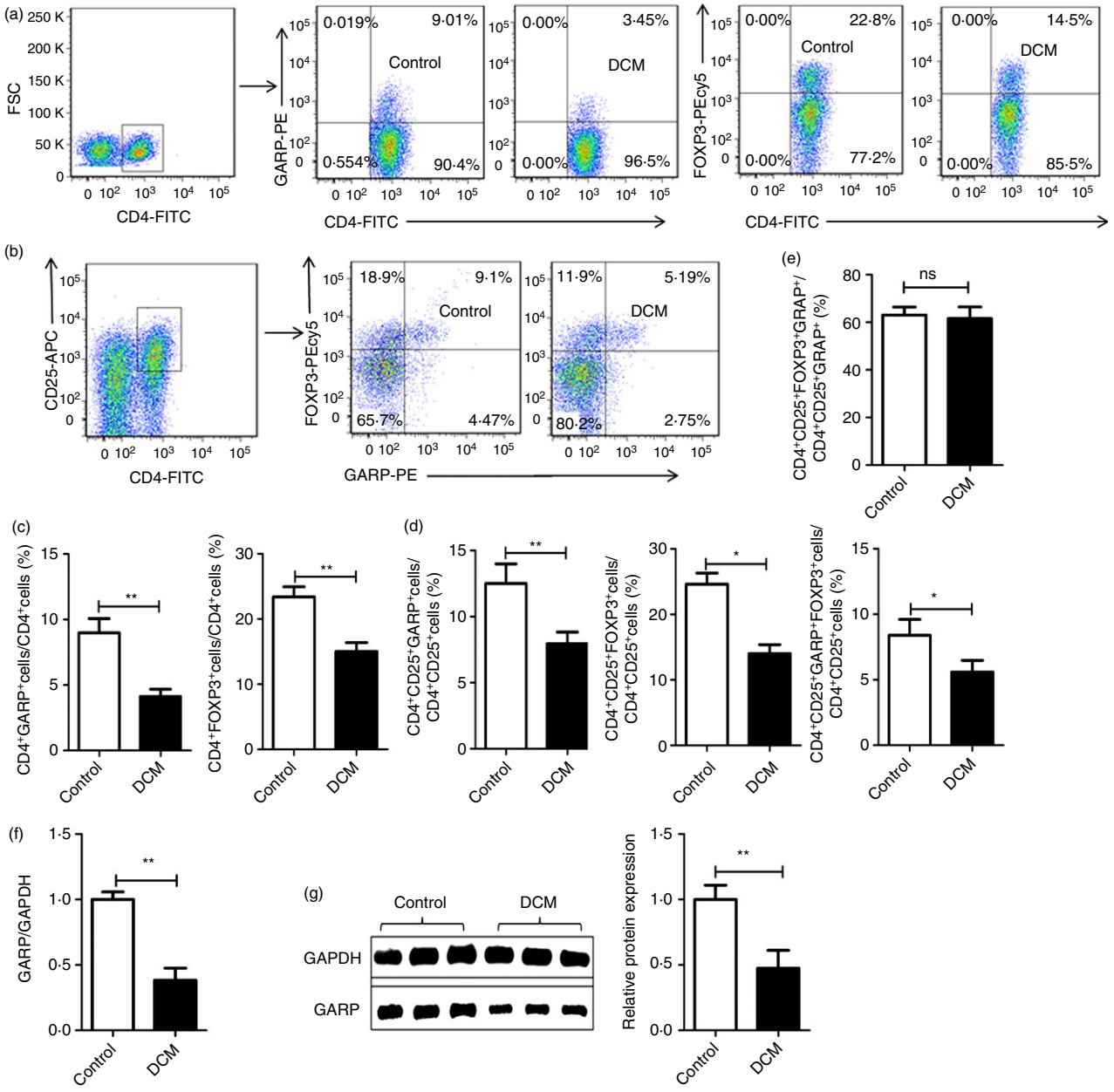

Figure 3. The expression of glycoprotein A repetitions predominant (GARP) and forkhead box P3 (FOXP3) in CD3/28-stimulated peripheral blood mononuclear cells (PBMCs). PBMCs were freshly isolated from patients with dilated cardiomyopathy (DCM; $n = 28$) and controls ($n = 26$), and stimulated with CD3/CD28 for 24 hr, then the cells were stained with anti-human CD4-FITC, anti-human CD25-APC, anti-human GARP-PE and analysed the data by FACS or directly used to analyse the mRNA and protein expression by RT-PCR and Western blot. (a) Representative FACS plots of $CD4^+$ $GARP^+$ and $CD4^+$ $FOXP3^+$ regulatory T (Treg) cells in human PBMCs. $CD4^+$ T cells were first gated on the side-scatter (FSC)/CD4 plots, and then the expression of GARP and FOXP3 was analysed in the gated $CD4^+$ T cells. (b) Representative FACS images of single patients from each group show the frequencies of $GARP^+$, $FOXP3^+$ and $FOXP3^+$ $GARP^+$ Treg cells gated on expanded $CD4^+$ $CD25^+$ T cells. (c–e) Comparison of the $CD4^+$ $CD25^+$ $GARP^+$, $CD4^+$ $CD25^+$ $FOXP3^+$ and $CD4^+$ $CD25^+$ $FOXP3^+$ $GARP^+$ Treg cells between the patients with DCM and control groups. (f) Relative mRNA expression of GARP in stimulated PBMCs of patients with DCM and controls. (g) Relative protein expression of GARP in stimulated PBMCs of patients with DCM and controls. $^*P < 0{\cdot}05$ versus control; $^{**}P < 0{\cdot}01$ versus control. [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

It is evident that the autoimmune mechanism acts as a key role in the development and pathogenesis of DCM characterized by infiltration of mononuclear cells, ventricular chamber enlargement and autoantibodies against the myocardium.[28–30] Accumulating evidence has proved that naturally occurring $CD4^+$ Treg cells are indispensable for

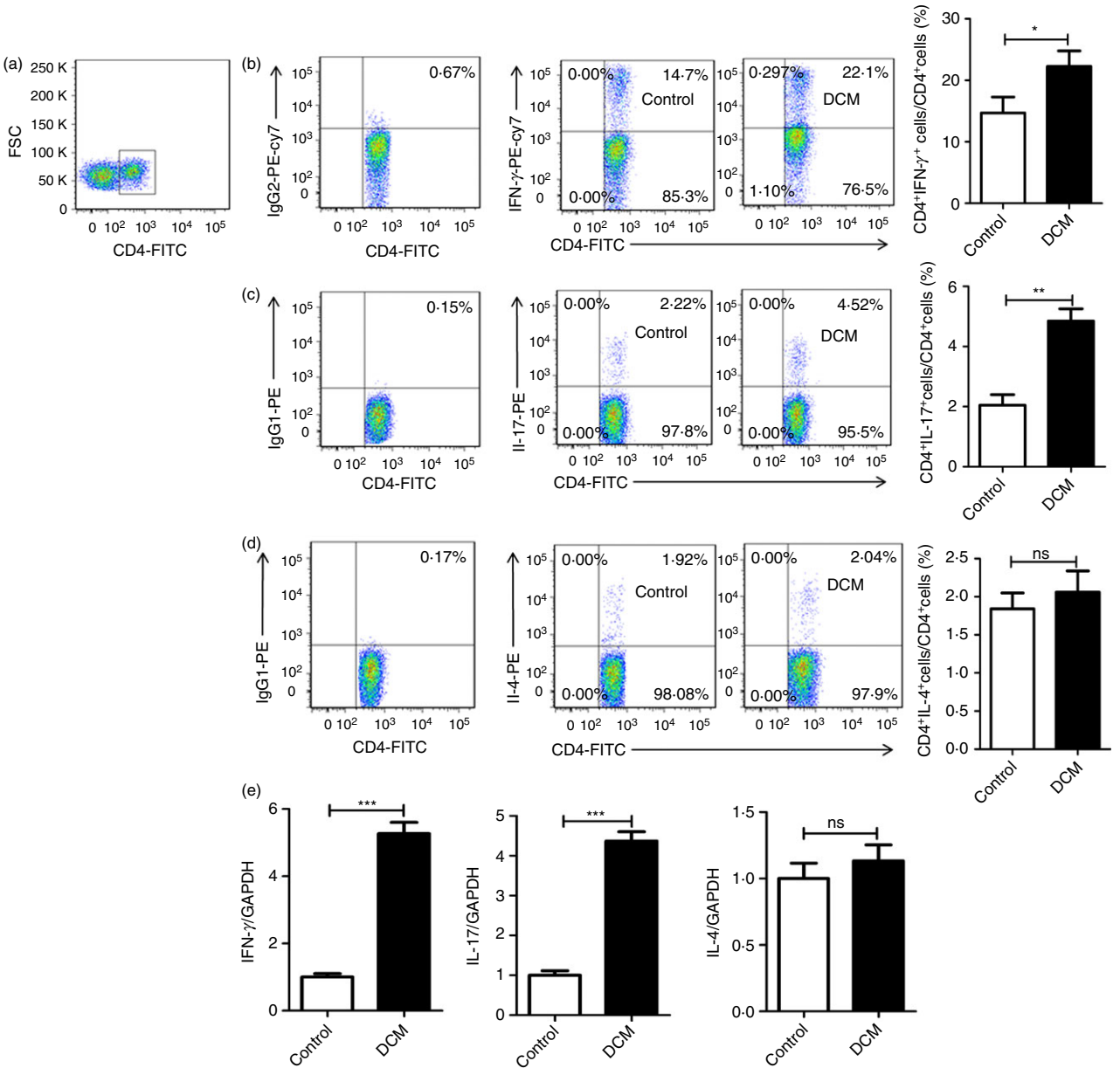

Figure 4. Circulating frequencies and mRNA expression of T helper type 1 (Th1), Th2 and Th17 in human peripheral blood mononuclear cells (PBMCs). PBMCs from patients with dilated cardiomyopathy (DCM; $n = 18$) and control groups ($n = 20$) were stimulated with PMA, ionomycin and monensin for 4 hr, and then stained with labelled antibodies as described in the Materials and methods. (a) Representative dot plot shows the gated CD4 T cells on the FSC/SSC. (b–d) Representative FACS pictures from a single patient in each group and the percentages of interferon-γ (IFN-γ) (b), IL-17 (c) and interleukin-4 (IL-4) (d) were comparable between the patients with DCM and control groups. (e) Relative mRNA expression of IFN-γ, IL-17 and IL-4 in stimulated PBMCs of patients with DCM and controls. *$P < 0{\cdot}05$ versus control; **$P < 0{\cdot}01$ versus control; ***$P < 0001$ versus control. [Colour figure can be viewed at wileyonlinelibrary.com]

maintaining the homeostasis of immune and immunological self-tolerance by suppressing the pathological autoreactive immune response.[31,32] However, specific *in vivo* targeting of activated Treg cells was precluded due to the lack of appropriate surface markers. Recently, GARP was newly identified as a special Treg cell surface molecule that mediates suppressive signals.[33] Therefore, we tried to study whether this new surface molecule expressed on activated Treg cells has a special role in cardiovascular disease. In previous experiments, we elucidated the impaired $CD4^+$ $CD25^+$ $GARP^+$ Treg cells in patients with acute coronary syndrome and atherosclerosis.[21–24] In the present study, our results indicated that the frequencies of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells in patients with DCM are reduced and their suppressive function on Tresp cell proliferation is compromised.

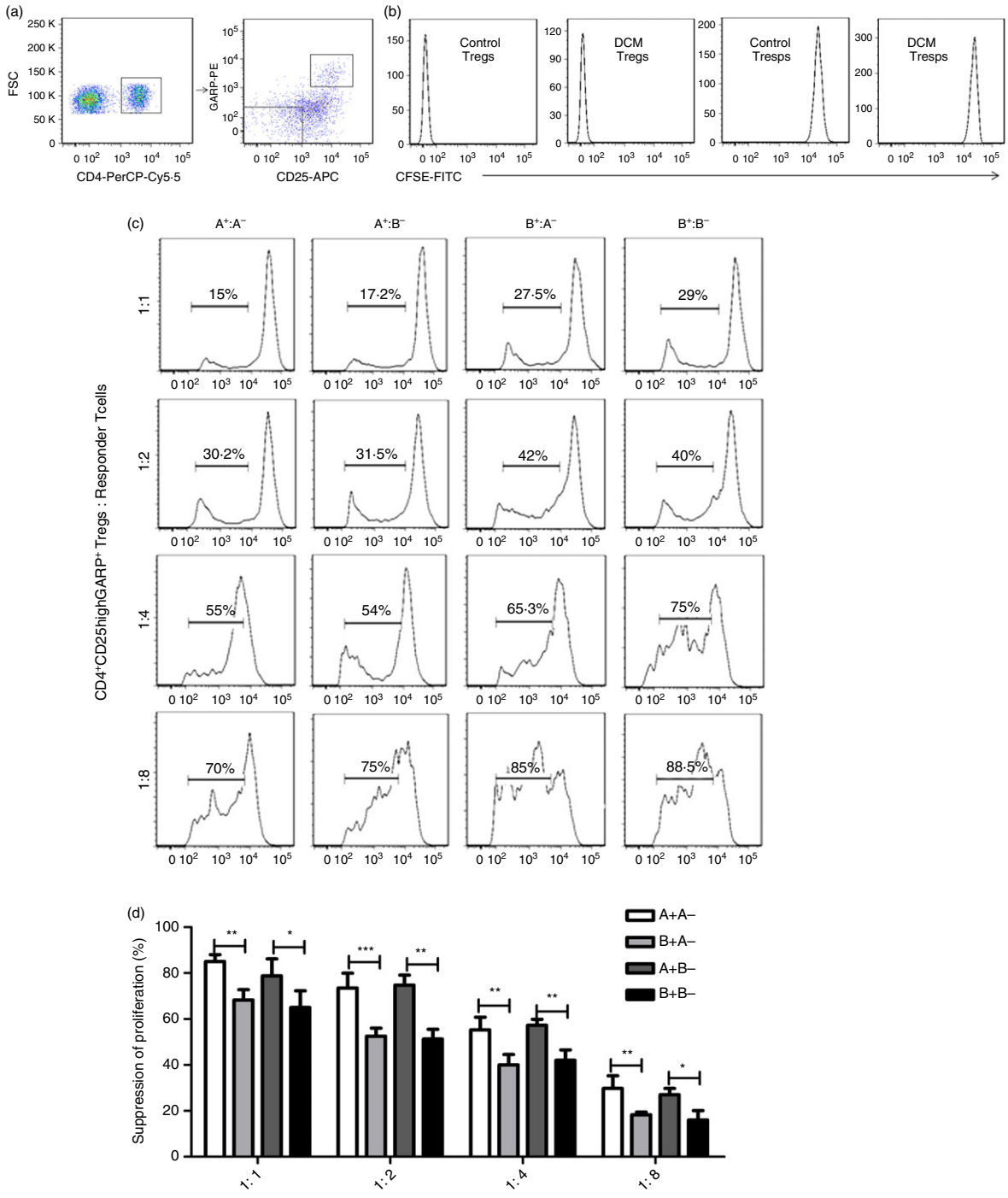

Figure 5. The suppressive function of $CD4^+$ $CD25^+$ $GARP^+$ regulatory T (Treg) cells from patients with dilated cardiomyopathy (DCM) was impaired. $CD4^+$ $CD25^+$ $GARP^+$ Treg cells and $CD4^+$ $CD25^-$ $GARP^-$ responder T (Tresp) cells from patients with DCM ($n = 6$) and controls ($n = 6$) were purified by FACS sorting, and then CFSE-labelled Tresp cells were co-cultured with Treg cells with plate-bound anti-CD3 and soluble anti-CD28 for 96 hr, alone or at different ratios for suppression and proliferation assay. (A+: $GARP^+$ Treg cells purified from normal controls; A−: $GARP^-$ Tresp cells purified from normal controls; B+: $GARP^+$ Treg cells purified from DCM patients; B−: $GARP^-$ Tresp cells purified from DCM patients.). (a) Induction and sorting of $GARP^+$ T cells. Peripheral blood mononuclear cells (PBMCs) were stimulated through T-cell receptor for 24 hr and stained for CD4, CD25 and glycoprotein A repetitions predominant (GARP). (b) Representative FACS analyses of Tresp cells from each group cultured alone without the stimulation of CD3/28. (c) Representative FACS analyses of crossover experiments (Tresp cells co-cultured with Treg cells at different ratios). Cells were then analysed by flow cytometry based on CFSE dilution. (d) Reduced suppressive function of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from patients with DCM, as indicated by suppression assay. $^*P < 0{\cdot}05$ versus control; $^{**}P < 0{\cdot}01$ versus control; $^{***}P < 0{\cdot}001$ versus control. [Colour figure can be viewed at wileyonlinelibrary.com]

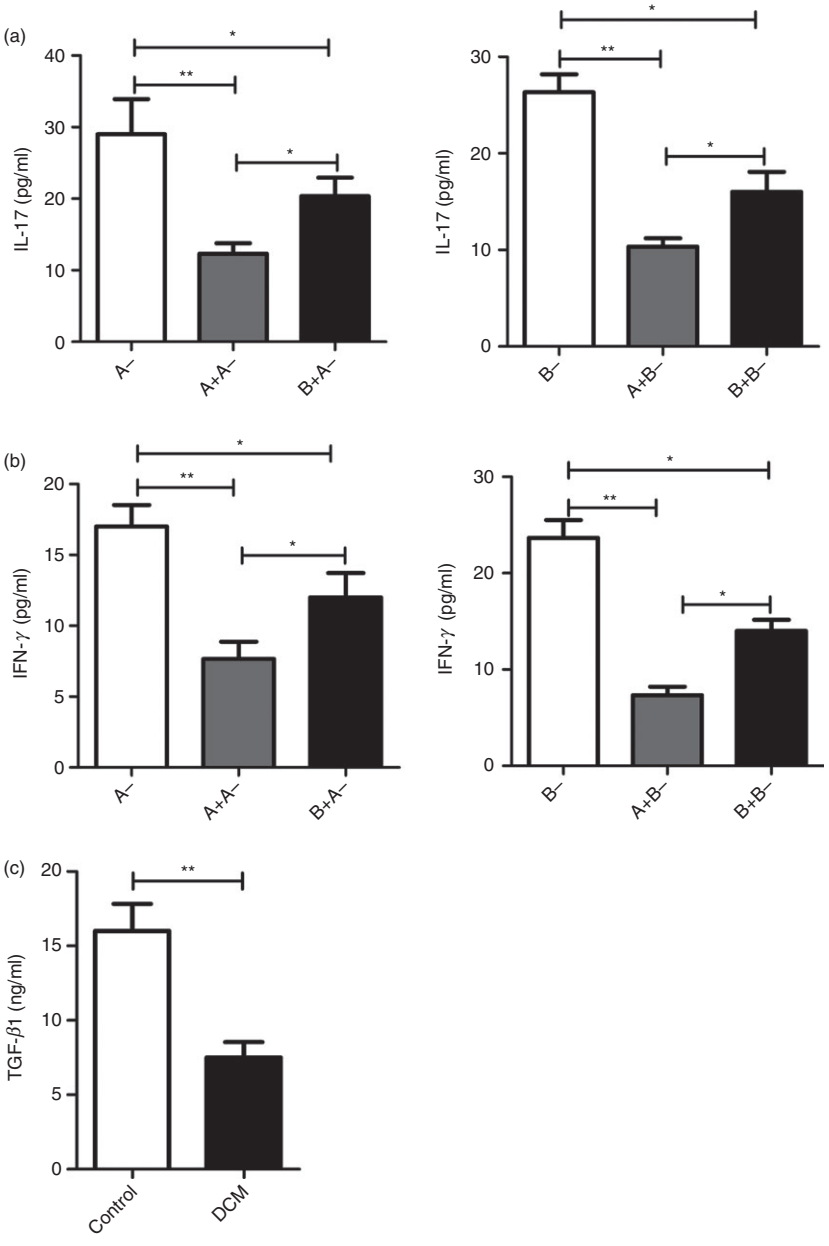

Figure 6. $CD4^+$ $CD25^+$ $GARP^+$ regulatory T (Treg) cells from patients with dilated cardiomyopathy (DCM) showed defective function to regulate the secretion of inflammatory factors. $CD4^+$ $CD25^+$ $GARP^+$ Treg cells and $CD4^+$ $CD25^-$ $GARP^-$ responder T (Tresp) cells from patients with DCM ($n = 6$) and controls ($n = 6$) were co-cultured at a 1 : 1 ratio for inflammation-associated cytokines assay. Four days later, supernatants from $CD4^+$ $CD25^+$ $GARP^+$ Treg cells cultured alone and co-cultured cells were assayed for transforming growth factor-β_1 (TGF-β_1), interferon-γ (IFN-γ) and interleukin-17 (IL-17). (A+: $GARP^+$ Treg cells purified from normal controls; A–: $GARP^-$ Tresp cells purified from normal controls; B+: $GARP^+$ Treg cells purified from patients with DCM; B–: $GARP^-$ Tresp cells purified from patients with DCM). $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from patients with DCM failed to suppress the IFN-γ (a) and IL-17 (b) secretion, compared with normal controls. (c) levels of TGF-β_1 in the supernatants of the cultured $CD4^+$ $CD25^+$ $GARP^+$ Treg cells. $^*P < 0{\cdot}05$ versus control; $^{**}P < 0{\cdot}01$ versus control; $^{***}P < 0{\cdot}001$ versus control.

Undoubtedly, the balance of different immune cells and their secretion of inflammatory cytokines play an important part in inflammatory disease. In humans, growing evidence has demonstrated that Treg cells are compromised with a quantitative or a qualitative abnormality in patients with autoimmune diseases, including DCM.[26,27,34] In this study, our data showed that the frequency of $CD4^+$ $CD25^+$ Treg cells and the expression of FOXP3 were markedly lower in patients with DCM compared with control groups with or without T-cell recepetor stimulation, which conformed to the findings of previous studies showing that the percentage of suppressor/cytotoxic T cells was lower in patients with DCM compared with controls.[26,35] In contrast, the frequencies of Th17 and Th1 were higher in patients with DCM compared with control groups, though the frequency of Th2 was comparable in these two groups. Beyond that, one of the Treg-related cytokines (TGF-β_1) was reduced in DCM, showing a positive correlation with Treg cells. The result suggested that TGF-β_1 may play a protective role in the onset of DCM partly through acting as one of the effective factors of Treg cells. However, the level of IL-10 was increased in the serum of patients with DCM, perhaps in an effort to drive a protective reaction for terminating inflammation and heart injury.[9] In conclusion, all of the data reflected disturbed Th17/Treg and Th1/Th2 cytokine profiles during the development and progression of DCM. So identifying

the precise effect and mechanism of autoimmune disorders in the development of DCM is particularly important.

Although FOXP3 is a key regulator of nTreg cells and can be used to define the classical Treg phenotype, many activated (non-regulatory) human T cells also express FOXP3, which would limit its use as a bona fide marker for human nTreg cells.[12,36] Other surface markers, which are not only expressed in nTreg cells but also up-regulated in activated effector cells, appear to be important for the identification of the Treg phenotype. It has been reported that GARP, which is highly expressed on the surface of stimulated Treg clones but not on Th clones, is more reliable in characterizing activated Treg cells than FOXP3 in diseases with chronic immune activation, such as HIV.[19] In the current study, we found that the frequency of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells showed a significant decline in patients with DCM, and the decreased amplitude was more obvious than that of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells, though the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells was also decreased in patients with DCM. Meanwhile, the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ $GARP^+$ Treg cells was also lower in patients with DCM with T-cell receptor stimulation compared with the control groups. Therefore, our data were not only consistent with previous studies,[18,19,26] but also indicated that GARP seems to be a more sensitive marker of Treg cell activation than FOXP3 in patients with DCM.

Suppressor T lymphocyte function in patients with DCM has been studied by some researchers with discrepant results, which may be caused by many uncontrollable factors.[37,38] No matter what the findings, defining the suppressor cells for assessing cell-subset function in patients with DCM seems to be a valid solution to explore the pathogenesis of the disease. In this study, we found that human $CD4^+$ $CD25^+$ $GARP^+$ Treg cells could suppress the proliferation of Tresp cells and have an effect on secretion of pro-inflammatory cytokines (IFN-γ and IL-17) in the co-culture system. These data demonstrated that human $CD4^+$ $CD25^+$ $GARP^+$ Treg cells and $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells could share the same mechanism to control Tresp cells in some ways. Previous studies have shown that down-regulation of GARP mediated by small interfering RNA in Treg cells could have substantial negative influences on the expression and suppressive function of FOXP3.[18] Furthermore, it has been reported that GARP is an effector molecule downstream from FOXP3 that can directly display inhibitory signalling once it is expressed and blocking GARP signals can amplify immune responses.[15] However, a limitation of the study was that we have no way to eliminate the influence of FOXP3 on $CD4^+$ $CD25^+$ $GARP^+$ Treg cells because of its intracellular expression. Nevertheless, we analysed the expression ratio of FOXP3 in $CD4^+$ $CD25^+$ $GARP^+$ Treg cells. We found that a sizeable portion of GARP-expressing Treg cells (about 80%) can express FOXP3, which indicated that FOXP3 appears to be required for GARP expression and both genes may be co-regulated during human thymic Treg cell development. This thought-provoking result made us deduce that the inhibitory function of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells may partly depend on the expression of FOXP3 in patients with DCM. On the other hand, GARP expression on $CD4^+$ Treg cells normally correlated with FOXP3 expression and can be better used to isolate and purify Treg populations in certain pathological conditions for its cell surface expression.

The crossover experiments showed that the suppressive function of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from patients with DCM on the proliferation of Tresp cells was compromised, indicating that the lymphocyte response disturbance of patients with DCM could be attributed to the incompetent $CD4^+$ $CD25^+$ $GARP^+$ Treg cells rather than the impaired sensitivity of Tresp cells. This result seemed to have a potential conflict with the previous study, which reported that the resistance of $CD4^+$ $CD25^-$ T cells to suppression was the major cause of autoimmune disorder in patients with DCM.[26] The discrepancy may be explained by the differences in the cultivation of the cell concentration, the purity of sorted T cells, and many uncontrollable factors. Nevertheless, our data confirmed that $CD4^+$ $CD25^+$ $GARP^+$ Treg cells with damaged function played an important role in patients with DCM. In addition, these data were in concert with the previous studies by Wang *et al.* and Probst-Kepper *et al.*,[19,39] GARP expression was shown to correlate with the suppressive activity of Treg cells and this suppressive activity was impaired in the absence of GARP.

Moreover, it has been demonstrated that soluble GARP under inflammatory conditions could not only promote Th17 differentiation, but also cooperate with TGF-β_1 to induce Treg cell differentiation.[40] Active TGF-β_1 is a pleiotropic cytokine that could regulate FOXP3 expression and influence the function of Treg cells by inducing SMAD2/3 phosphorylation, which acts as a positive role in the initial stages of inflammation.[41] Additionally, Veronika Nindl *et al.* have demonstrated that IFN-γ was a major effector cytokine driving the initial inflammatory process and that the cooperation of IFN-γ and IL-17A was essential for the development of the progressive disease.[5] In this study, our data indicated that $CD4^+$ $CD25^+$ $GARP^+$ Treg cells could secrete TGF-$\beta_1$and inhibit the secretion of IFN-γ and IL-17 and, more notably, this capacity of secretion anti-inflammatory cytokines and inhibition of pro-inflammatory cytokines was impaired in patients with DCM. For the reason of the reduced capacities of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells, the resistance of inflammatory cytokine secretion was weakened in patients with DCM, which led to the development of pathological process and aggravation of cardiac function.

In summary, our study is the first to show that the frequency of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from patients with DCM is lower than that from normal controls. Moreover, $CD4^+$ $CD25^+$ $GARP^+$ Treg cells from patients with DCM showed a compromised suppressive function on the proliferation of Tresp cells. As the previous study, our results demonstrate that it is a practical measure to open the possibility to use soluble GARP as a potent immunomodulator of inflammatory diseases for its crucial role in modulation of peripheral tolerance and T effector cell function,[40] and the increase and enhancement of the number and function of $CD4^+$ $CD25^+$ $GARP^+$ Treg cells may be a feasible therapeutic approach for the treatment of patients with DCM.

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Disclosures

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical characteristics of the patients.

Table S2. Primer sequences for RT-PCR.

Table S3. $CD4^+$ T-cell subset within peripheral blood mononuclear cells from patients with dilated cardiomyopathy and normal controls and the percentages of CD25 expression on gated $CD4^+$ T cells.

Table S4. Catalogue numbers of all antibodies.