Original Article

OPEN

Circulating senescent angiogenic T cells are linked with endothelial dysfunction and systemic inflammation in hypertension

Gaoxing Zhang[b,*], Yuanya Liu[a,b,c,d,*], Yumin Qiu[a,c,d,*], Jianning Zhang[a,c,d], Jiapan Sun[a,c,d], Zhe Zhou[a,c,d], Zhichao Wang[a,c,d], Peishan Zeng[a,c,d], Jun Tao[a,c,d], and Jiang He[a,c,d]

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Objective: Angiogenic T cells (T_{ang} cells), a recently discovered T-cell subset, have been reported involved in the repair of endothelial injury. The purpose of this study was to explore the correlation of immunologic senescence and pro-inflammatory capacity of T_{ang} cells with endothelial dysfunction in hypertensive patients.

Methods: Immunological characteristics of T_{ang} cells ($CD3^+CD31^+CXCR4^+$) from hypertensive patients with or without endothelial dysfunction were elucidated by surface immunophenotyping and intracellular cytokine staining. Endothelial function was measured by flow-mediated dilation (FMD).

Results: The frequency of $CD28^{null}$ subset in $CD4^+$ T_{ang} cells was notably elevated in hypertensive patients with endothelial dysfunction, which was negatively associated with FMD. The high frequency of $CD28^{null}CD4^+$ T_{ang} cells was an independent risk factor of endothelial dysfunction with good diagnostic performance in ROC curve analysis. Immunophenotyping revealed that this specific subset of T_{ang} cells exhibited senescent profile and has low hTERT expression. $CD28^{null}CD4^+$ T_{ang} cells produced high levels of inflammatory cytokines, IL-6, IFN-γ and TNF-α, and significantly correlated with the systemic inflammation in hypertensive patients with endothelial dysfunction.

Conclusion: Collectively, our findings demonstrate for the first time that $CD28^{null}$ subset in $CD4^+$ T_{ang} cells with senescent and pro-inflammatory phenotype is dependently correlated with impaired FMD and systemic inflammation, which might contribute to the immunopathologic mechanism of endothelial dysfunction. Identification of a pathogenic $CD4^+$ T_{ang}-cell subset lacking CD28 may offer opportunities for the evaluation and management of endothelial dysfunction in hypertension.

Keywords: angiogenic T cells, endothelial dysfunction, hypertension, immunosenescence, inflammation

Abbreviations: CI, confidence interval; CRP, C-reactive protein; ED, endothelial dysfunction; eGFR, estimated glomerular filtration rate; ESC/ESH, European Society of Cardiology/European Society of Hypertension; FMD, flow-mediated dilation; HDL, high-density lipoprotein; hTERT, human telomerase reverse transcriptase; IFN-γ, interferon gamma; LDL, low-density lipoprotein; MFI, mean fluorescent intensity; ROC, receiver-operating characteristic curve; SLE, systemic lupus erythematosus; T_{ang}, angiogenic T cells; TNF-α, tumor necrosis factor-alpha

INTRODUCTION

Hypertension is a major pathophysiologic contributor to cardiovascular diseases [1]. Dysfunction of vascular endothelium is the hallmark of hypertension, which is characterized as impaired endothelium-dependent vasorelaxation with a pro-inflammatory state and prothrombic properties [2]. Accumulated evidence has widely identified endothelial dysfunction as the first step of the atherosclerotic process and as a fundamental mechanism in the pathophysiology of hypertensive target organ damage and cardiovascular diseases [3].

Chronic inflammation has been considered as a critical trigger of hypertension and contributed to an alteration of endothelial structure and function [4,5]. Aging of the immune system, defined as immunosenescence [6], contributes to the development of chronic inflammation and has been thought to underlie vascular endothelial injury [7,8]. Altered T-cell subsets and dysregulated cytokine profile are marked features of immunosenescence [9]. However, any relationship

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[a]Department of Hypertension and Vascular Disease, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, [b]Department of Cardiovascular Disease, Jiangmen Central Hospital of Sun Yat-sen University, Jiangmen, Guangdong, [c]National-Guangdong Joint Engineering Laboratory for Diagnosis and Treatment of Vascular Disease, The First Affiliated Hospital of Sun Yat-sen University and [d]Key Laboratory on Assisted Circulation of Ministry of Health, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

Correspondence to Jiang He, Department of Hypertension and Vascular Disease, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China 510080; National-Guangdong Joint Engineering Laboratory for Diagnosis and Treatment of Vascular Disease, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China 510080 and Key Laboratory on Assisted Circulation of Ministry of Health, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China 510080. Tel: +86 13480265018; fax: +86 2087335700; e-mail: hejiang7@mail.sysu.edu.cn.

*Gaoxing Zhang, Yuanya Liu, and Yumin Qiu contributed equally to this article.

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between the immunosenescence of T cells and impaired endothelial function in hypertension remains to be elucidated.

Senescent T cells exhibit characteristic phenotype, such as lack expression of CD28 and abnormal secretory phenotype [10]. Previous studies found that $CD28^{null}CD8^{+}$ T cells increased in patients with hypertension, suggesting a role for senescent T-cell-driven inflammation in hypertension [11]. Recent studies suggest that a specific T-cell subset, termed angiogenic T cells (T_{ang} cells), involve in endothelial repair and promote formation of new blood vessels. T_{ang} cells are characterized by the co-expression of CD3, CD31, and the receptor for the CXC chemokine stromal cell-derived factor-1 (CXCR4) [12,13]. Altered circulating T_{ang} cell frequencies and senescent subsets have been reported to be associated with cardiovascular risk factors in rheumatoid arthritis [13], systemic lupus erythematosus (SLE) [14] and diabetes [15]. The role of senescent T_{ang} cells in hypertension and endothelial dysfunction has yet to be determined.

On the basis of the uncertainties mentioned above, in the present study, we compare the frequencies of T_{ang} cell subsets in hypertensive patients with or without endothelial dysfunction confirmed by flow-mediated dilation (FMD) and investigate the relationship between senescent T_{ang} cells and FMD. We also explore the pro-inflammatory functional phenotype and senescent profile of $CD28^{null}$ T_{ang} and the association between senescent T_{ang} cells and elevated serum pro-inflammatory cytokines in hypertensive patients with endothelial dysfunction. In summary, this study suggests the linkage between senescent T_{ang} cells and endothelial dysfunction and systemic inflammation in hypertension.

METHODS

Study population

The present study included 80 consecutively enrolled patients diagnosed with essential hypertension at the Department of Hypertension and Vascular Disease, the First Affiliated Hospital of Sun Yat-sen University from June 2019 to August 2019. The inclusion criteria for the essential hypertensive patients were as follows: aged 40–65 years; office blood pressure raised with SBP at least 140 mmHg and/or DBP at least 90 mmHg; without any antihypertension medication, herbal supplements, antidepressants or other traditional Chinese medication therapy history. The exclusion criteria were as follows: secondary hypertension; (ii) systemic diseases, such as diabetes, HIV/AIDS, liver disease, chronic renal failure, tuberculosis, and autoimmune diseases; diabetes or prediabetes mellitus; medical history of cardiovascular disease: acute myocardial infarct, stable angina, unstable angina, heart failure, atrial fibrillation, atrioventricular blockade, peripheral vascular disease or cerebrovascular accident. BP measurements were performed according to *2018 ESC/ESH Guidelines for the management of arterial hypertension* [16]. All of the participants underwent three BP measurements in two different visits, after 30 min of rest, and the measurements were spaced by 5–10 min intervals, on both the left and right arms, in the sitting and lying positions. Patients underwent a physical examination and laboratory examination at the time of initial enrollment. The baseline characteristics of the study population are summarized in Table 1. Informed consent was provided by all participants, and the study protocol was approved by the Ethics Committees of the First Affiliated Hospital of Sun Yat-sen University.

TABLE 1. Demographic characteristics and clinical parameters of study population

	HT without ED ($n=32$)	HT with ED ($n=48$)	*P* value
Age (years)	54.1 ± 7.6	52.9 ± 4.1	0.650
Sex (% male)	22 (68.8)	30 (62.5)	0.566
BMI (kg/m^2)	23.4 ± 1.8	23.5 ± 2.3	0.787
Heart rate (beat/min)	65.5 ± 9.0	64.5 ± 10.1	0.661
Plasma glucose (mmol/l)	5.42 ± 0.37	5.47 ± 0.44	0.587
Triglyceride (mmol/l)	1.26 ± 0.52	1.27 ± 0.37	0.964
Creatinine (μmol/l)	80.4 ± 19.8	79.5 ± 20.3	0.436
eGFR (ml/min per 1.73 m^2)	90.4 ± 17.5	89.6 ± 18.2	0.615
Total cholesterol (mmol/l)	5.11 ± 0.45	5.24 ± 0.58	0.265
LDL cholesterol (mmol/l)	2.75 ± 0.35	2.71 ± 0.40	0.622
HDL cholesterol (mmol/l)	1.44 ± 0.46	1.52 ± 0.29	0.399
Current smokers (%)	14 (43.8)	23 (47.9)	0.148
Hypertension (%)	100*	100*	–
SBP (mmHg)	161.3 ± 8.1	160.9 ± 9.8	0.087
DBP (mmHg)	100.3 ± 4.6	101.4 ± 5.7	0.353
Flow-mediated Dilation (%)	7.8 ± 0.6	5.8 ± 0.7	<0.001

ED, endothelial dysfunction; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HT, hypertension; LDL, low-density lipoprotein.
*$P < 0.05$ is considered significant.

Measurement of flow-mediated dilation

A high-resolution ultrasonography equipment specialized to measure FMD (UNEXEF18G, UNEX Co, Nagoya, Japan) was used to evaluate FMD according to the guidelines of the American College of Cardiology [17], as previously described in our previous study [18].

FMD was performed in a temperature-controlled room (22 °C) with participants in a resting supine state between 0800 and 1000 h, after at least an 8-h fast. Diameter of brachial artery was automatically imaged by a high-resolution linear artery transducer coupled to computer-assisted analysis software. A blood pressure cuff was placed around the forearm. The brachial artery was visualized longitudinally 5–10 cm above the antecubital crease. Pulsed Doppler flow was evaluated at baseline and during peak hyperemic flow. The baseline longitudinal image of the artery was acquired for 30 s, and then the cuff was inflated 50 mmHg greater than systolic pressure for 5 min. The longitudinal image of the artery was recorded continuously until 1 min after releasing occlusion. FMD was automatically expressed as the percentage change in peak diameter from the baseline diameter. Percentage of FMD [(peak diameter − base-baseline diameter)/baseline diameter] was used for analysis. Endothelial dysfunction was confirmed by FMD with cutoff at 7% or less [19].

Immunophenotyping analysis of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Hypaque (GE Healthcare, Sweden) and analyzed by flow cytometry. PBMCs were stained with fluorochrome-conjugated monoclonal antibodies against surface antigens for 30 min at 4 °C. The

antibodies used included anti-CD3-PE-Cy7, anti-CD4-Horizon V500, anti-CD8-APC-Cy7, anti-CD31-FITC, anti-CXCR4-PerCP-Cy5.5, and anti-CD28-APC-H7 (all from BD Biosciences, San Jose, California, USA). Phenotypic characterization of $CD28^{null}$-T_{ang} and $CD28^{+}$-T_{ang} subsets was performed by extracellular staining with anti-CD57-APC, anti-CD27-APC, anti-CCR7-APC, or antihTERT-APC or the corresponding isotype controls (all from BD Biosciences). Multicolor flow cytometry was performed using an CytoFlex S (Beckman Coulter), and FlowJo V10 software (Treestar, San Carlos, California, USA) was used to analyze the data.

Intracellular cytokine staining

PBMCs were stimulated with anti-CD3 antibody (0.1 μg/ml) for 6 h. After 1 h of incubation, brefeldin A and monensin (BD Biosciences) were added to stimulate intracellular cytokine protein accumulation. Following surface staining with anti-CD3-PE-Cy7, anti-CD4-Horizon V500, anti-CD8-APC-Cy7, anti-CD31-FITC, anti-CXCR4-PerCP-Cy5.5, and anti-CD28-APC-H7, the cells were fixed and permeabilized using the Fixation/Permeabilization Buffer Kit and further stained for intracellular cytokines with anti-TNF-α-FITC, anti-IFN-γ-FITC, anti-IL-6-PE, and anti-IL-10-APC (all from BD Biosciences).

Cytokine quantification

IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-1α, IL-1β, and MCP-1 were analyzed by Cytometric Bead Arrays (BD Biosciences) in accordance with the manufacturer's instructions. ELISA kits were used for TNF-α (PeproTech, Rocky Hill, New Jersey, USA), IFN-γ (BD Biosciences), CRP (Invitrogen, ThermoFisher Scientific) and CX3CL (R&D systems).

Statistical analysis

Continuous variables were reported as the means ± SD. Categorical variables were summarized by the percentage of the group total. All reported probability values were two-sided, and a P less than 0.05 was considered statistically significant. All the parameters were tested for normal distribution using the Kolmogorov–Smirnov test. An independent t tests were used for two group continuous variables. A Mann–Whitney test was performed for two group continuous, nonparametric variables. Discrete variables were compared using the chi-square test. Paired data comparisons were summarized using the paired t-test. Correlation between FMD or cytokines levels and frequency of T_{ang}-cell subsets were tested by Pearson's correlation test. Multivariate logistic regression analysis was performed to identify independent predictors of endothelial dysfunction. Statistical analyzes and graphs were performed with SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA) and Graphpad Prism version 8 (GraphPad Software Inc., San Diego, California, USA).

RESULTS

Characteristics of participants

The present study included 80 essential hypertensive patients without any treatment for high blood pressure enrolled at the First Affiliated Hospital of Sun Yat-sen University from June 2019 to August 2019. Endothelial dysfunction was confirmed by FMD with cutoff at 7% or less [19] and 32 in 80 hypertensive patients present normal endothelial function. Demographic and clinical characteristics of patients were summarized in Table 1. Baseline characteristics were not significantly different between hypertensive patients without endothelial dysfunction and hypertensive patients with endothelial dysfunction with the exception of FMD.

$CD4^{+}$ T_{ang} cells from hypertensive patients with endothelial dysfunction exhibit characteristics of senescence

T_{ang}, a specific T-cell subset, have been identified to be involved in the repair of damaged endothelium. To evaluate the prevalence of the circulating T_{ang} cells and subpopulations in hypertensive patients with or without endothelial dysfunction, the percentages of T_{ang} ($CD3^{+}CD31^{+}CXCR4^{+}$) and $CD4^{+}/CD8^{+}$ subsets were analyzed by flow cytometry. As shown in Fig. 1, panel a, no significant differences were observed in the frequency of T_{ang} or $CD4^{+}/CD8^{+}$ subsets between hypertensive patients with endothelial dysfunction and hypertensive patients without endothelial dysfunction.

Loss of CD28 expression is the most consistent biologic indicator of T-cell senescence. We showed significantly decreased level of cellular surface CD28 in $CD4^{+}$ T_{ang} cells (Fig. 1 panel f) in hypertensive patients with endothelial dysfunction, whereas the level of CD28 in $CD8^{+}$ T_{ang}-cell subset showed no significant alteration, supporting a disease-associated immunosenescence of $CD28^{null}CD4^{+}$ T_{ang} cells.

Therefore, $CD4^{+}$ T_{ang} cells exhibit characteristic of senescence accumulated in hypertensive patients with endothelial dysfunction and relationship between $CD28^{null}CD4^{+}$ T_{ang} cells and endothelial dysfunction deserves further study.

Senescent $CD4^{+}$ T_{ang} cells from hypertensive patients are correlated with defect endothelial function

T_{ang} cells have been reported participated in repair of endothelial damage and senescence induces dysregulated function of T cells. However, the possible relationship between senescent $CD4^{+}$ T_{ang} cells and endothelial dysfunction in hypertensive patients has not been studied so far.

Our data revealed that FMD was only associated with the frequency of $CD28^{null}CD4^{+}$ T_{ang} cells (Fig. 2, panel e), whereas the frequency of $CD28^{null}$ cells in the $CD8^{+}$ T_{ang} cell subset showed no significant association with endothelial function (Fig. 2, panel d). Moreover, by means of ROC curve analysis, there was good sensitivity and specificity of $CD28^{null}CD4^{+}$ T_{ang} cells (AUC = 0.9118, $P < 0.001$; cutoff value <9.3%, sensitivity% = 87.5%, specificity% = 81.25%) for endothelial dysfunction (Fig. 2, panel f).

Furthermore, multivariate logistic regression analysis revealed that $CD28^{null}CD4^{+}$ T_{ang}-cell subset was an independent risk factor of endothelial dysfunction controlled for age, sex, and SBP (Table 2). Therefore, $CD28^{null}CD4^{+}$ T_{ang} cells from hypertensive patients exhibit a potential

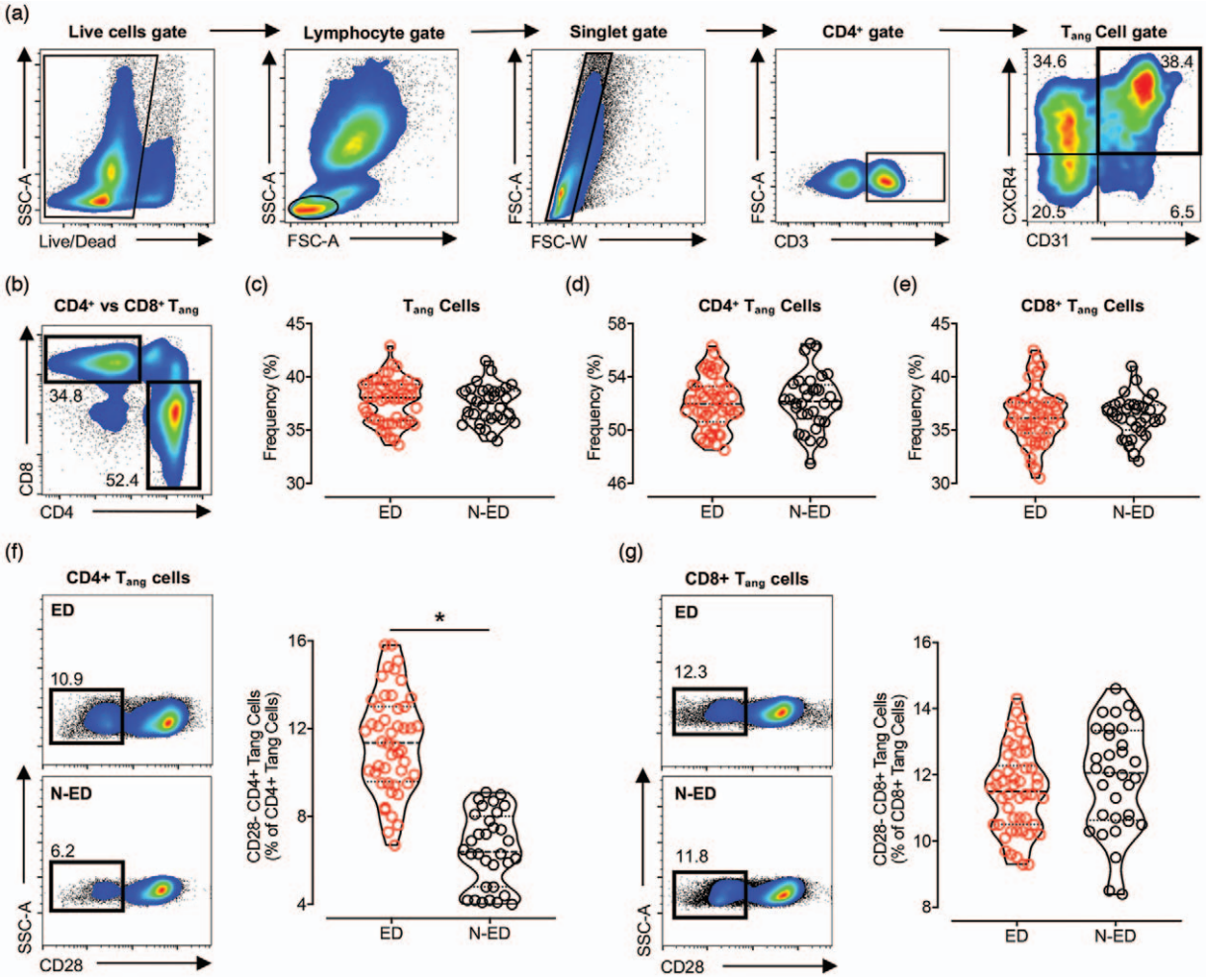

FIGURE 1 The frequency of T_{ang}-cell subsets in patients with hypertension. PBMNCs isolated from hypertensive patients (HT) with endothelial dysfunction (ED) ($n = 48$) or without endothelial dysfunction (N-ED) ($n = 32$) were stained for CD3, CD4, CD31, CXCR4, and CD28 and analyzed by flow cytometry. (a) Gating strategy of T_{ang} cells. (b) Gating strategy of $CD4^+$ or $CD8^+$ T_{ang} cells. (c–e) The frequency of total T_{ang} cells (c) or $CD4^+$ T_{ang} cells (d) or $CD8^+$ T_{ang} cells was compared between HT patients with ED and HT patients without ED. (f) Representative cytometry plots and cumulative data of CD28 expressing level in $CD4^+$ T_{ang} cells. (g) Representative cytometry plots and cumulative data of CD28 expressing level in $CD8^+$ T_{ang} cells. $^{**}P < 0.01$. ED, hypertensive patients with endothelial dysfunction; HT, hypertension; N-ED, hypertensive patients without endothelial dysfunction; PBMNCs, peripheral blood mononuclear cells; T_{ang}, angiogenic T cells.

linkage with endothelial function in hypertensive patients. $CD28^{null}CD4^+$ T_{ang} cells were deserved a focus of further analyses.

$CD28^{null}CD4^+$ T_{ang} cells from hypertensive patients exhibit senescent profile and pro-inflammatory functional phenotype

Next, we wanted to determine whether the downregulation of CD28 expression in $CD4^+$ T_{ang} cells was accompanied by other senescent features. Thus, we performed a phenotypic characterization of $CD28^{null}$ and $CD28^+$ subsets in $CD4^+$ T_{ang} cells by analyzing the expression of a range of senescent markers usually present in $CD28^{null}$ T cells. Increased expression of CD57 (Fig. 3, panel a) and decreased expression of CCR7 (Fig. 3, panel b) and CD27 (Fig. 3, panel c) supported the prominent senescent profile of $CD28^{null}CD4^+$ Tang cells. Decreased expression of human telomerase reverse transcriptase (hTERT) is a typical feature of T-cell senescence (Fig. 3, panel d). Thus, we analyzed the expression of hTERT within $CD28^{null}$ and $CD28^+$ subsets in $CD4^+$ T_{ang} cells and found that $CD28^{null}$ subset exhibits marked decline in the expression of hTERT compared with $CD28^+$ subsets (Fig. 3, panel d).

The dramatically elevated capacity to secrete pro-inflammatory cytokines is a prominent characteristic of senescent $CD4^+$ T cell. PBMCs were stimulated with anti-CD3 antibody for 6 h, and intracellular staining for IFN-γ, TNF-α, IL-6, and IL-10 was performed. The frequency of IFN-γ, TNF-α, IL-6 or IL-10-secreting cells in the $CD28^{null}CD4^+$ T_{ang} cell population was compared with those in the paired $CD28^+CD4^+$ subpopulation. In this regard, we found that while the frequency of IFN-γ, TNF-α, or IL-6-secreting cells in the $CD28^{null}CD4^+$ T_{ang} cell population was significantly greater than that in the $CD28^+$ subpopulation, there was no difference in the frequency of IL-10-secreting cells between the two subsets (Fig. 3, panels e–h).

We also evaluated the cytotoxic capacity of $CD28^{null}CD4^+$ T_{ang} cells vs. $CD28^+CD4^+$ T_{ang} cells by intracellular staining of cytotoxic granule proteins, such as granzyme B and perforin, and we found that these

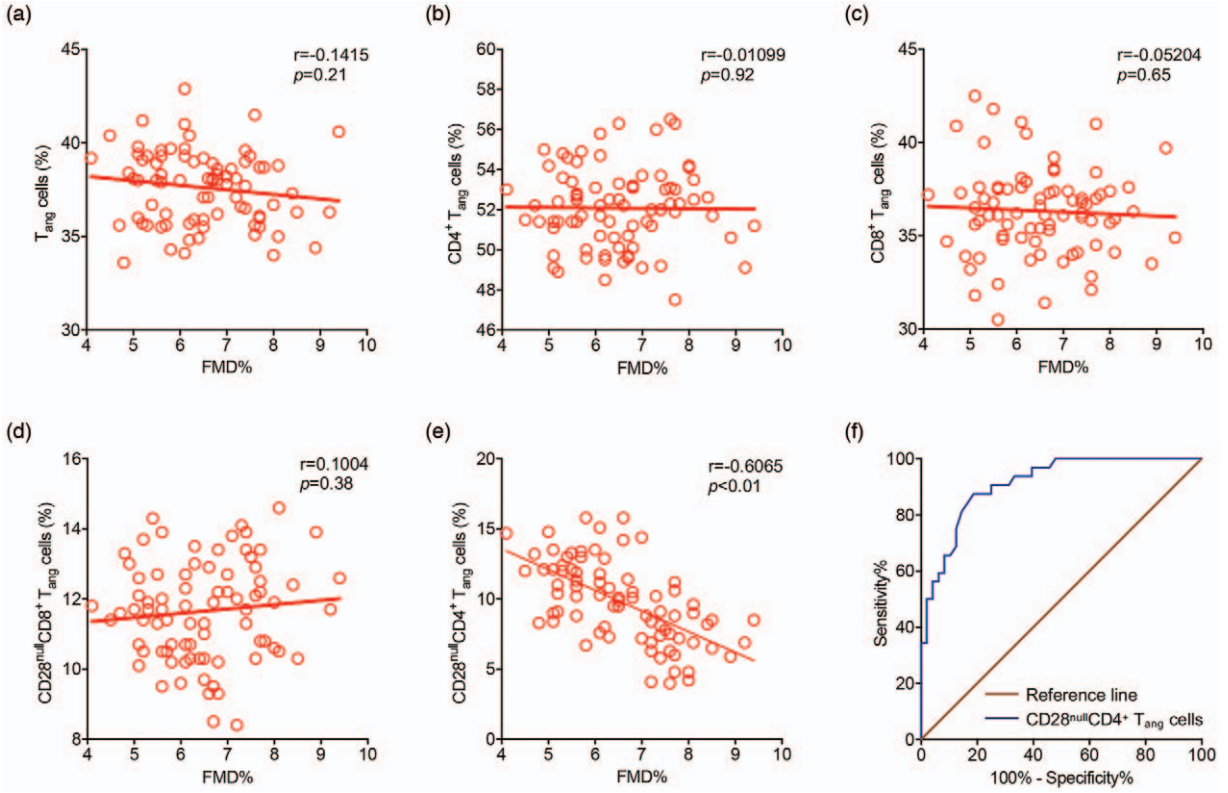

FIGURE 2 Correlation between flow-mediated dilation and frequency of T_{ang}-cell subsets in patients with hypertension and receiver-operating characteristic curve. (a–e) Correlation between FMD and frequency of T_{ang}-cell subsets were tested using Pearson's correlation test. (f) Receiver-operating characteristic (ROC) Curves of $CD28^{null}CD4^{+}$ T_{ang} cells for endothelial dysfunction. FMD, flow-mediated dilation; T_{ang}, angiogenic T cells.

TABLE 2. Multivariate logistic regression analysis for endothelial dysfunction controlled for age, sex and SBP

		95% CI		
	Odds ratio	Lower	Upper	*P* value
T_{ang} cells				
Age	0.982	0.926	1.042	0.549
Male sex	0.654	0.236	1.811	0.414
SBP	0.944	0.895	0.995	0.032*
T_{ang} cells	1.152	0.901	1.473	0.261
$CD4^{+}$ T_{ang} cells				
Age	0.982	0.927	1.041	0.549
Male sex	0.695	0.256	1.889	0.476
SBP	0.947	0.899	0.998	0.043*
$CD4^{+}$ T_{ang} cells	0.955	0.753	1.211	0.705
$CD8^{+}$ T_{ang} cells				
Age	0.984	0.929	1.042	0.578
Male sex	0.687	0.252	1.872	0.463
SBP	0.947	0.898	0.998	0.043*
$CD8^{+}$ T_{ang} cells	0.994	0.811	1.217	0.951
$CD28^{null}CD4^{+}$ T_{ang} cells				
Age	1.017	0.929	1.112	0.718
Male sex	0.924	0.219	3.897	0.914
SBP	0.966	0.902	1.035	0.326
$CD28^{null}CD4^{+}$ T_{ang} cells	2.551	1.677	3.880	<0.001*
$CD28^{null}CD8^{+}$ T_{ang} cells				
Age	0.983	0.927	1.042	0.567
Male sex	0.722	0.264	1.976	0.526
SBP	0.946	0.896	0.998	0.041*
$CD28^{null}CD8^{+}$ T_{ang} cells	0.813	0.580	1.14	0.23

CI, confidence interval; T_{ang}, angiogenic T cells.
*$P < 0.05$ is considered significant.

cytotoxic granule proteins were highly expressed in the $CD28^{null}CD4^{+}$ T_{ang} cells (Fig. 3, panels i and j).

Taken together, these data suggest that $CD28^{null}CD4^{+}$ T_{ang} cells, presented typical T-cell senescent profile, might secrete pro-inflammatory cytokines and display higher cytotoxic function. Thus, they might participate in the pathophysiology of endothelial dysfunction through regulation of inflammation.

Increased systemic inflammation correlates with the accumulation of senescent T_{ang} cells

Chronic low-grade inflammation is one of the most important physiopathologic mechanism of endothelial dysfunction. Low-grade inflammation mediated by production of pro-inflammatory cytokines is linked to dysfunctional senescence features of T cells. The senescent profile and pro-inflammatory functional phenotype of $CD28^{null}CD4^{+}$ T_{ang} cells from hypertensive patients suggests they might exert a pro-inflammatory, rather than a protective, effect on endothelial cells. However, the linkage between senescent $CD4^{+}$ T_{ang} cells and systemic inflammation in hypertensive patients remains largely unknown.

Thus, we performed a serological analysis for some common pro-inflammatory cytokines in hypertensive patients. Hypertensive patients with endothelial dysfunction had increased serum concentrations of IL-6, IL-17, IFN-γ, and TNF-α (Table 3 and Fig. 4, panels a–d). Linear regression analyses adjusted by age, sex and SBP revealed

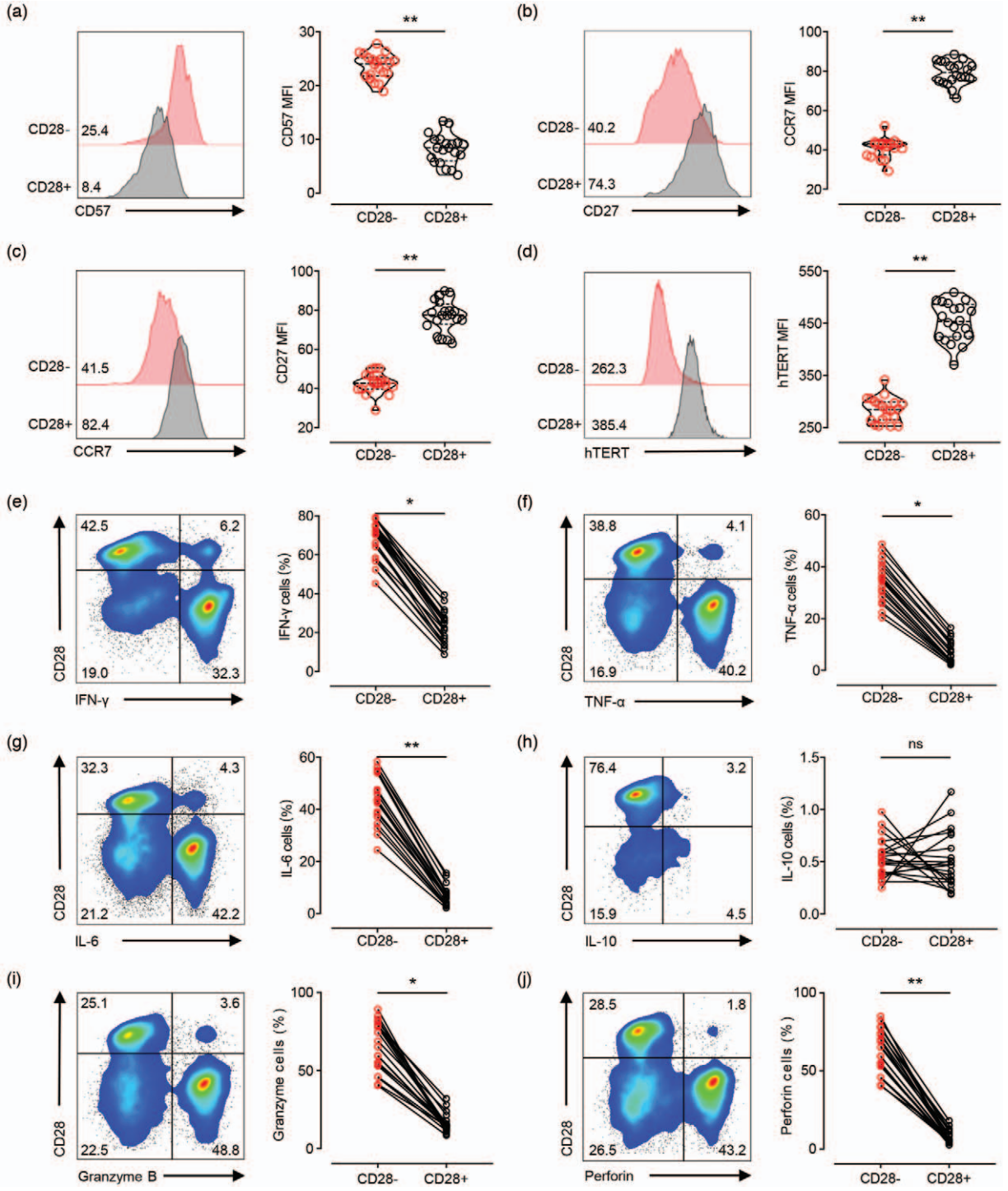

FIGURE 3 Senescent profile and pro-inflammatory secretory of $CD28^{null}CD4^{+}$ T_{ang} cells from hypertensive patients. (a–c) Expression of senescence markers in $CD28^{null}CD4^{+}$ T_{ang} cells and $CD28^{+}CD4^{+}$ T_{ang} cells. Numbers in plots indicate the median fluorescence intensity of each marker staining, with the matched irrelevant control value subtracted. (d) PBMCs were stimulated with 0.5 μg/ml of anti-CD3 antibody for 72 h. Representative cytometry plots and cumulative data of telomerase reverse transcriptase (hTERT) MFI of $CD28^{null}CD4^{+}$ T_{ang} cells and $CD28^{+}CD4^{+}$ T_{ang} cells. (e–h) PBMCs were stimulated with anti-CD3 antibody for 6 h, and intracellular cytokine staining for IFN-γ, TNF-α, IL-6, and IL-10 was performed. The frequency of IFN-γ-, TNF-α- or IL-17A-secreting cells in the $CD28^{null}CD4^{+}$ T_{ang}-cell subpopulation was compared with those in the paired $CD28^{+}CD4^{+}$ T_{ang}-cell subpopulation. (i–j) Intracellular staining for cytotoxic granule proteins was performed. The frequency of granzyme B+ or perforin+ cells in either the $CD28^{null}CD4^{+}$ or $CD28^{+}CD4^{+}$ T_{ang}-cell populations was assessed. $^{*}P < 0.05$; $^{**}P < 0.01$. MFI, mean fluorescent intensity; PBMNCs, peripheral blood mononuclear cells; hTERT, human telomerase reverse transcriptase.

positive correlation of these proinflammatory cytokines in hypertensive patients with the accumulation of senescent $CD4^{+}$ T_{ang}-cell subsets (Table 4 and Fig. 4, panels e–h).

It has been reported that $CD4^{+}CD28^{-}$ T cells does not possess the ability to secrete IL-17, the elevated IL-17 level in hypertensive patients with endothelial dysfunction might be a consequence of memory $CD4^{+}$ T cells activated by IL-

TABLE 3. Serum levels of cytokines in hypertensive patients

Cytokine	HT without ED ($N=32$)	HT with ED ($N=48$)	*P* value
CRP (mg/l)	0.76 (0.72–0.81)	0.88 (0.78–1.04)	0.0108
IL-2 (pg/ml)	2.67 (2.32–2.97)	2.72 (2.49–2.98)	0.5900
IL-4 (pg/ml)	1.83 (1.59–2.04)	1.85 (1.58–2.11)	0.8000
IL-6 (pg/ml)	0.93 (0.77–1.10)	1.68 (1.47–1.10)	<0.0001
IL-8 (pg/ml)	5.39 (4.97–5.60)	5.53 (4.75–5.86)	0.6218
IL-10 (pg/ml)	0.79 (0.60–0.98)	0.86 (0.69–0.99)	0.1698
IL-12 (pg/ml)	1.53 (1.29–1.84)	1.48 (1.13–1.79)	0.5746
IL-17 (pg/ml)	4.22 (3.58–4.80)	5.46 (4.97–6.18)	<0.0001
IFN-γ (pg/ml)	0.58 (0.38–0.75)	1.20 (0.99–1.44)	<0.0001
TNF-α (pg/ml)	1.72 (1.50–1.96)	1.99 (1.29–1.93)	<0.0001
IL-1α (pg/ml)	0.59 (0.45–0.74)	0.56 (0.39–0.77)	0.6762
IL-1β (pg/ml)	0.54 (0.46–0.62)	0.57 (0.41–0.74)	0.5257
MCP-1 (pg/ml)	106.31 (92.83–119.09)	109.60 (93.07–123.58)	0.5268

Data presented as median (interquartile range). Differences between groups were evaluated by the Mann–Whitney *U* test. CRP, C-reactive protein; ED, endothelial dysfunction; HT, hypertension.
*$P<0.05$ is considered significant.

TABLE 4. Association of $CD28^{null}CD4^{+}$ T_{ang} cells with serum cytokine serum levels in hypertensive patients

Variable	β coefficient	95% CI Lower	95% CI Upper	*P* value
$CD28^{null}CD4^{+}$ T_{ang} cells				
IL-6	5.110	4.147	6.072	0.036*
IL-17	1.548	1.028	2.068	0.012*
IFN-γ	5.543	4.451	6.636	<0.01*
TNF-α	3.503	2.518	4.488	<0.01*

Multiple backward linear regression analyses performed with each cytokine as the dependent variable and age, sex, SBP as covariates.
*$P<0.05$ is considered significant.

6. The frequency of $IL\text{-}17^{+}$ subpopulation in memory $CD4^{+}$ T cells ($CD4^{+}CD45RO^{+}$) were analyzed by flow cytometry. Data showed that frequency of $IL17^{+}$ subpopulation in $CD4^{+}CD45RO^{+}$ T cells were elevated in hypertensive patients with endothelial dysfunction (Online Supplemental Figure 2, http://links.lww.com/HJH/B511).

Therefore, $CD28^{null}CD4^{+}$ T_{ang} cells with notable senescent and pro-inflammatory phenotype were associated with systemic inflammatory environment, which seem to be closely related to endothelial dysfunction in hypertension.

DISCUSSION

The present study provides the first evidence for the presence that the frequency of $CD28^{null}$ cells in the $CD4^{+}$ T_{ang}-cell population is associated with endothelial dysfunction in patients with hypertension. $CD28^{null}CD4^{+}$ T_{ang} cells presents senescent profile and high pro-inflammatory capacity, which might contribute to the immunopathologic mechanism of endothelial dysfunction and be considered as a novel biomarker.

Hypertension appears to have a complicated association with endothelial dysfunction, a phenotypical alteration of the vascular endothelium considered as the initial step in the morbidity of atherosclerosis [2]. In addition, endothelial dysfunction has been shown to be an independent predictor of cardiovascular events [3]. A recently discovered subpopulation of T cells, termed angiogenic T cells (T_{ang}), possesses the ability to facilitate the repair of damaged endothelium and formation of new blood vessels [12]. Reduced T_{ang} population has been previously described in rheumatoid arthritis [13], type 2 diabetes mellitus [15], hypertension-related cerebral small vessel disease [20] and other cardiovascular conditions. By contrast, levels were not reduced in people with SLE, only in the those with

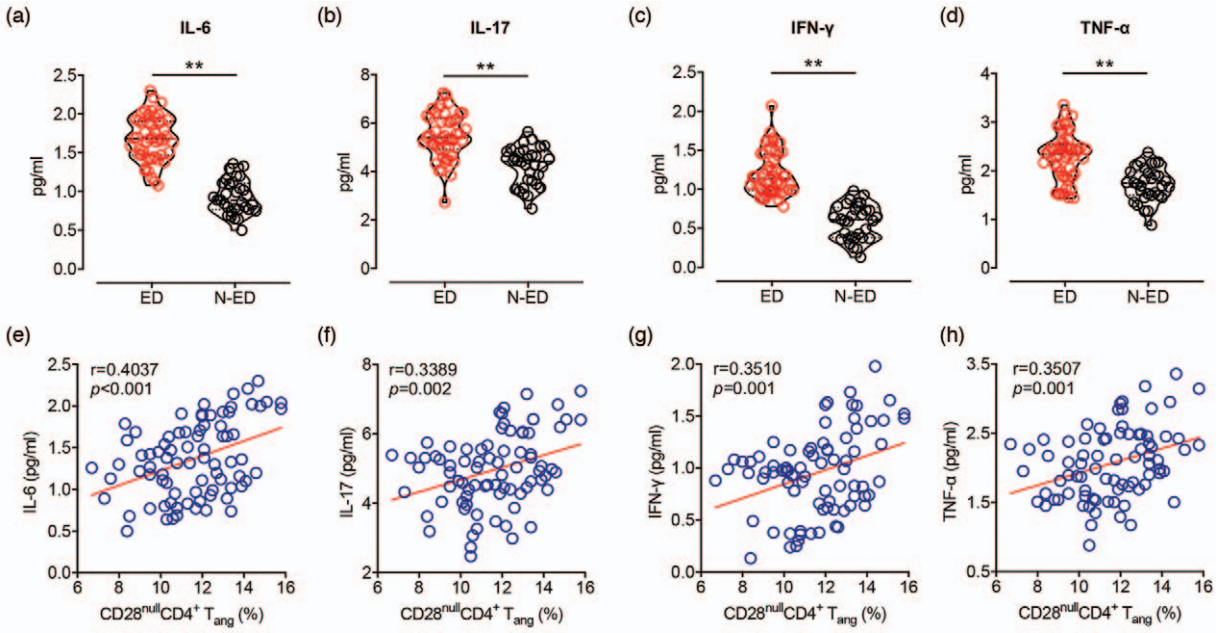

FIGURE 4 Inflammatory profile is exhibited in the serum of hypertensive patients with endothelial dysfunction and correlates with frequency of $CD28^{null}CD4^{+}$ T_{ang} cells. (a–d) Serological analysis of IL-6, IL-17, IFN-γ and TNF-α in hypertensive patients with endothelial dysfunction (ED) ($n=48$) and without endothelial dysfunction (N-ED) ($n=32$). (e–h) Correlation between serum cytokines s and frequency of $CD28^{null}CD4^{+}$ T_{ang} cells were tested using Pearson's correlation test. **$P<0.01$. ED, hypertensive patients with endothelial dysfunction; N-ED, hypertensive patients without endothelial dysfunction; T_{ang}, angiogenic T cells.

rheumatoid arthritis and cardiovascular risk factors [14]. Decreased T_{ang}-cell levels were observed in hypertensive individuals with cerebral small vessel disease [20]. Miao *et al.* [14] reported increased $CD8^+$ T_{ang}-cell levels in people with SLE, but not $CD4^+$ T_{ang} or total $CD3^+$ T_{ang} cells. However, we did not find any changes in $CD4^+$ or $CD8^+$ T_{ang} or total T_{ang} cells between hypertensive patients with or without endothelial dysfunction. These data highlight the complexity of T_{ang} cells in different pathophysiological states.

Loss of CD28 costimulatory molecule expression is a well known, senescent event of T cells, and $CD28^{null}$ T cells accumulate with age [21]. $CD28^{null}$ phenotype of T cells has been considered a marker of immunosenescence [10,21]. A role for senescent T cells in atherosclerotic diseases has been reported in several studies. Previous studies examining $CD28^{null}CD4^+$ T cells as a senescent T-cell population suggested a role in promoting vascular inflammation in atherosclerotic diseases [22]. Lopez *et al.* [23] used CD28 expression to redefine the angiogenic T-cell population and found that $CD28^{null}CD4^+$ T_{ang} cells were notably increased in the SLE patients. In our study, we revealed that $CD28^{null}CD4^+$ subset of T_{ang} cells was remarkedly elevated in hypertensive patients with endothelial dysfunction. Furthermore, FMD, as the most widely used technique for the assessment of endothelial function in humans [24], was negatively associated only with the frequency of this subset. ROC curve indicates great diagnostic value of this subset in T_{ang} cells for endothelial dysfunction. Logistic regression showed that level of $CD28^{null}CD4^+$ T_{ang} cells was an independent risk factor of endothelial dysfunction controlled for age, sex and SBP. As $CD4^+CD28^-$ T cells have been reported to express C-X3-C Motif Chemokine Receptor 1 (CX3CR1) and endothelial cells are known to express its ligand C-X3-C Motif Chemokine Ligand 1 (CX3CL1), elevated level of plasma CX3CL1 in hypertensive patients with endothelial dysfunction suggested that CXC3CL1/CXC3CR1 axis might involve in regulating the harmful effect of $CD4^+CD28^-$ T_{ang} cells on endothelium, which is worth to be studied in our further studies (Supplementary Figure 1, http://links.lww.com/HJH/B510). Together, these data suggest that $CD28^{null}CD4^+$ T_{ang} cells might be functionally relevant to the pathogenesis of endothelial dysfunction. For these reasons, we focused on elucidating the functional capacity of $CD28^{null}CD4^+$ T_{ang} cells in hypertensive patients.

$CD4^+$ subset within the $CD28^+$-T_{ang} in SLE patients presented with a senescent profile has been previously described recently [23]. In line with this, $CD28^{null}$ subset of $CD4^+$ T_{ang} cells in hypertensive patients showed a significant increase in CD57 expression and a remarked decline in CCR7 and CD27 expressions. Moreover, it was shown that $CD28^{null}CD4^+$ T_{ang} cells have reduced hTERT expression. These data indicate prominent immunosenescent profile.

Elevated production of abundant atypical pro-inflammatory cytokines is a typical feature of senescent T cells [6,25]. Previous publications suggested that they could enhance autoimmunity through the production of pro-inflammatory cytokines that might activate autoreactive T cells [26]. It also has been shown that $CD28^{null}CD4^+$ T cells, which produced large amounts of inflammatory cytokines, can be isolated from atherosclerotic plaque [27]. Thus, we attempted to investigate the pro-inflammatory cytokines produced by $CD28^{null}CD4^+$ T_{ang} cells. We hypothesized that the $CD28^{null}CD4^+$ T_{ang}-cell subset reported in the present study might also exhibit abnormal secretory. Multiparameter flow cytometric analysis revealed that high frequencies of IFN-γ, TNF-α and IL-6-secreting cells were observed in the $CD28^{null}$ subset of $CD4^+$ T_{ang} cells rather than $CD28^+$ subpopulation.

Chronic inflammation is one the most critical mechanisms of endothelial dysfunction [4,8]. Patients with chronic inflammatory diseases, such as rheumatoid arthritis [28], inflammatory bowel diseases [29], lupus [30] and others, manifest endothelial dysfunction, often in the early phase of the disease. Increased inflammatory markers have been reported linked with decreased FMD and linked to a wide variety of major adverse cardiovascular events [8]. According to our experiments, elevated levels of pro-inflammatory mediators, IL-6, IL-17, IFN-γ, and TNF-α are observed systemically in hypertensive patients with endothelial dysfunction. Systemic production of pro-inflammatory mediators is linked to the gathering of phenotypic and dysfunctional senescence features of T cells. Increased systemic levels of pro-inflammatory cytokines in hypertension mentioned above correlated significantly with the accumulation of $CD28^{null}CD4^+$ T_{ang} cells.

Limitations

Although we demonstrated a relationship between the frequency of senescent $CD4^+$ T_{ang} cells and endothelial dysfunction in hypertension, the current data must be cautiously interpreted. First, the present study was cross-sectional and observational. We could not examine the frequency of $CD28^{null}CD4^+$ T_{ang} cells prior to occurrence of endothelial dysfunction. The frequencies of $CD28^{null}CD4^+$ T_{ang} cells before and after occurrence of endothelial dysfunction must be compared in the future. Second, further basic experiments exploring the inflammatory injury effect of $CD28^{null}CD4^+$ T_{ang} cells on vascular endothelium would provide a more valuable perspective for the role of this T_{ang} cell subset in hypertension and endothelial dysfunction. Third, the small size of the study population restricted a precise analysis on the independent incremental value of $CD28^{null}CD4^+$ T_{ang} cells as a prognostic factor for endothelial dysfunction over other traditional risk factors in hypertensive patients.

In conclusion, we identified a link between the frequency of $CD28^{null}CD4^+$ T_{ang} cells and endothelial dysfunction in patients with hypertension. $CD28^{null}CD4^+$ T_{ang} cells exhibit senescent profile and pro-inflammatory property. This specific subset of $CD4^+$ T_{ang} cells might contribute to endothelial injury via producing high level of pro-inflammatory cytokines. Identification of a pathogenic $CD4^+$ T_{ang}-cell subset lacking CD28 may offer new opportunities for the prevention and treatment of endothelial dysfunction.

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Conflicts of interest

There are no conflicts of interest.

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