Enhancement of soluble CD28 levels in the serum of Graves' disease

ZHONGWEN SUN, LIXIAN YI, HONG TAO, JINGFANG HUANG, ZHENGHONG JIN, YANG XIAO, CAIYUN FENG, JING SUN

Suzhou Health College, Suzhou, PR China

Abstract

Graves' disease is an autoimmune disease of the thyroid gland mediated by T cells. CD28, a member of costimulatory molecules, plays a pivotal role in regulating T-cell responses. Plasma-soluble CD28 is one form of CD28 in peripheral blood. To investigate the concentrations of soluble CD28 in patients with Graves' disease, we used a sensitive dual monoclonal antibody sandwich enzyme-linked immunosorbent assay (ELISA) to detect the soluble form of CD28. Our results suggested that mean concentrations of soluble CD28 in plasma of patients with Graves' disease were 1.79 ±1.52 ng/ml, and levels of soluble CD28 in healthy subjects were only 0.83 ±1.35 ng/ml. Concentrations of soluble CD28 detected in patients with Graves' disease were significantly higher than those of healthy subjects ($p < 0.01$). Moreover, there was a significant positive correlation between the concentrations of soluble CD28 in plasma and levels of FT3 ($r = 0.663$), FT4 ($r = 0.624$) and TRAb ($r = 0.728$) in serum, but a negative correlation was found between sCD28 levels and TSH ($r = -0.726$). Through in vitro experiments we observed that engagement of soluble CD28 protein and B7-1/B7-2 molecules expressed on dendritic cells could exert the secretion of cytokine IL-6, which may promote the production of autoantibody and aggravate Graves' disease. Therefore, aberrant elevation of plasma-soluble CD28 in patients with Graves' disease may reflect the dysregulation of immune system, and may serve as a useful biomarker in Graves' disease diagnosis.

Key words: *soluble CD28, membrane CD28, correlation analyses, IL-6, Graves' disease.*

Introduction

Graves' disease (GD), a well-known multi-systemic autoimmune disorder, is characterised by the presence of autoantibodies that bind to the thyroid follicular cells to induce unregulated production of thyroid hormone, and by the infiltration of inflammatory cells into the thyroid tissue. An autoantibody specific for thyroid-stimulating hormone receptor (TSH-R) induces thyroid follicular cells to overproduce thyroid hormones. Moreover, in clinical diagnosis patient follow-up, prediction of relapse and GD treatment monitoring, a test that specifically and sensitively detects TSH-R autoantibodies (TRAb) is a very useful tool.

In the initiation of the adaptive immune response, T-cell activation is a critical event . Costimulation is necessary for T cells to achieve maximal activation by T-cell receptor (TCR) interacting with major-histocompatibility-complex (MHC complex). To achieve the full effect, TCR and CD28 costimulatory molecules are needed to be temporally coincident and spatially proximal. Physiological engagement of CD28 alone is insufficient to induce T cells to proliferate or conduct effective function. En-

gagement of CD28 expressed on T cells with its ligands B7-1 (CD80) or B7-2 (CD86) on the antigen presenting cells plays a central role in the early stage of T cells activation [1]. In naďve T cells, CD28 costimulation enhances cell cycle entry and potently stimulates the production of IL-2, the activation of an anti-apoptotic program and the formation of immune synapse [2-4]. Some studies have shown that CD28-deficient mice reduce responses to infectious pathogens as well as allograft or contact hypersensitivity antigens [5, 6]. The B7-CD28/CTLA-4 costimulation can regulate T cell chemotaxis and differentiation, probably through the activation of inflammatory cytokines and chemokines [7, 8]. However, the above reports about the functions of CD28 molecules were all based on studies of membrane-bound forms on the cell surface.

Previous studies indicated that costimulatory molecules have two forms *in vivo*. As well as the membrane-bound form, a soluble form had been found and investigated in plasma from patients with autoimmune diseases [9-15]. Therefore, the dysregulation of T-cell functions by aberrant elevation of soluble costimulatory molecules and proin-

flammatory cytokines and chemokines may lead to inflammation, particularly in systemic lupus erythematosus (SLE) patients with more severe disease [16]. Elevated plasma levels of soluble CD40L have been shown to be associated with disease activity in SLE or unstable angina, and serum levels of 4-1BBL are prognostic factors for progression of myelodysplastic syndrome [17-20]. Similar findings have been noted in subjects with several chronic liver diseases, in terms of sCD95L concentrations [21]. Apart from these findings, recent studies have demonstrated that there is a significant increase of serum soluble CD28 (sCD28) concentration in SLE patients compared with healthy control subjects [22]. Aberrant elevation of sCD28 form in patients with inactive and active disease concurs with the results reported from another study [16]. However, it is still not clear that the concentration and functional capacity of plasma soluble CD28 in the patients with GD.

In present study, we established enzyme-linked immunosorbent assay (ELISA) for quantitative detection to evaluate plasma soluble CD28 (sCD28) levels in patients with Graves' diseases, for the first time, to our knowledge, and analysed its correlation with clinical parameters. To explore the biological significance of soluble CD28 in patients with Graves' disease, we also measured IL-2 and IL-6 secretion in the culture supernatant of dendritic cells at 24 hours after using soluble CD28 fusion protein to interact with B7-1 or B7-2 molecules, on immature dendritic cells *in vitro*. Soluble CD28 may be involved in the development of GD and may be considered as a biomarker of GD.

Material and methods

Blood donors and blood samples

Sixty-nine Chinese patients with GD (51 women, 18 men, mean age: 43.7 ±15.8 years) were recruited from Suzhou municipal hospital. All patients with GD were diagnosed by ultrasonography of thyroid volume (patients with thyroid goitre size greater than 5 cm were included), measurement of circulating thyrotropin receptor antibodies (TRAb), suppression of serum TSH (less than 3.5 mIU/l), elevation 3,5,3',5'-tetraiodothyronine (thyroxin, T4) (higher than 25 pM) and 3,5,3'-triiodothyronine (T3) (higher than 5.5 nM) was processed. Biochemical, immunological assays and clinical characteristics of the probands were performed according to the previous report [23, 24]. Fifty-seven sex- and age-matched healthy Chinese donors (34 women, 21 men, mean age: 35.7 ±12.6 years) were recruited from Suzhou Blood Centre as control subjects, and all volunteers with disease status were excluded. Ten millilitres of venous peripheral blood were collected in heparin from all the patients and control subjects. The plasma samples were centrifuged, collected and stored at –70°C before determination. This study was approved by the Ethical Committee of the hospital.

Detection of soluble CD28 by ELISA

The concentration of sCD28 in plasma was evaluated by ELISA. Briefly, 96-well ELISA Plates (Costar, USA) were coated with 4 µg/ml anti-CD28 mAb (prepared in our lab, clone 2F5) and incubated at 4°C overnight. Unbinding sites on the plates were blocked with 1% BSA in phosphate buffer saline (PBS) at 37°C for 2 hours. After washing, recombinant human CD28 protein (R&D Systems, Abingdon, UK) and samples were triplicately added and incubated for 2 hours at 37°C. After washing twice, soluble CD28 captured by coating the antibody was incubated with biotin-conjugated anti-CD28 mAb (prepared in our lab, clone 2D5), followed by HRP-streptavidin (Sigma, MO, USA) incubation for 1 hour at 37°C and then reacted with the substrate TMB (KPL, UK). Absorbance values were measured at 450 nm. In addition, stability was analysed by the detection with four known soluble CD28 concentrations (1 ng/ml, 5 ng/ml, 10 ng/ml and 15 ng/ml) after plate coating and preservation at 4°C for 60 days. Measurement precision was determined by spiking four serial two-fold dilutions of sCD28 into four different levels of known sCD28 concentrations. Specificity was analysed by the measurement of recombinant human CD80/Fc, CD86/Fc, CTLA-4/Fc and OX40L/Fc fusion proteins (all from R&D Systems, UK). The results are expressed as ng/mL, mean ± SD.

The effect of soluble CD28 on dendritic cell response

Dendritic cells (DCs) were generated as described previously [25]. Human peripheral blood was obtained from Suzhou Blood Centre. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated as described above and were allowed to adhere in six-well plate at a density of 5×10^6 cells/ml at 37°C in RPMI 1640 with 10% FCS. Two hours later, non-adherent cells were removed. The adherent cells were cultured in 3 ml RPMI 1640 with 10% FCS, 750IU GM-CSF and 500 IU IL-4 (Essex Pharma, Nürnberg, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin in each well for seven days to generate DCs. The culture media along with the cytokines was changed every three days. After 7 days' culturing, clusters of large non-adherent cells displaying the cytological morphology of DCs appeared in the wells. Cytokines secreted by DCs were analysed at 24 hours after interacting the dendritic cells with sCD28-Ig fusion proteins (20 µg/ml) or control IgG.

Measurement of the cytokines

Culture supernatants from dendritic cells were sampled at 24 hours after interaction of sCD28 and DCs. IL-2 and IL-6 ELISA were performed by commercially available kits according to the manufacturer's instructions (Diaclone, France).
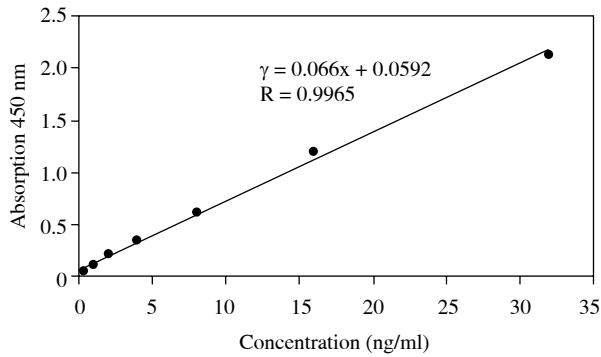
$$\gamma = 0.066x + 0.0592$$
$$R = 0.9965$$

Fig. 1. Establishment of sandwich ELISA for detecting soluble CD28. sCD28-Ig samples of four serials of two-fold dilution were added to an ELISA plate precoated with 2F5 mAb. After sufficient washing, 2D5 mAb labelled with biotin and streptavidin-HRP was added. Then results were revealed with TMB substrate and stop reagent (H_2SO_4) at 450 nm and 620 nm as reference. The detection linear range of sCD28 was from 0.25 to 32 ng/ml. Dots represented the mean value of three parallel well of sCD28

Statistical analyses

Since Gaussian distribution was not seen on levels of sCD28, the Mann-Whitney rank sum test was used to analyse the differences on sCD28 concentrations between patients and controls. The linear regression test was used to compare sCD28 concentration with optical density value. Student's *t* test was used for comparison of two groups, and the Pearson or Spearman rank was used to assess correlations. All analyses were performed using the Statistical Package for Social Sciences (SPSS) statistical software for windows, version 9.0 (SPSS, IL, USA). A *p* value < 0.05 was considered as statistically significant.

Results

Establishing soluble CD28 detection

For quantitative detection of sCD28, a sandwich ELISA method was successfully established by using 2F5 mAb as the capturing antibody and 2D5 mAb for detection. To draw the best linear curve of sCD28, we calculated the average absorbance values for each set of triplicates and finally created a linear work curve by regression analysis of the mean absorbance values for each standard concentration on the ordinate against the sCD28 concentration on the abscissa. From our results, the ELISA method for detecting sCD28, prepared by ourselves, showed that the detection range of ELISA permitted reliable sCD28 concentration from 0.25ng/ml up to 32ng/ml, and showed that a sensitivity of approximately 0.06 ng/ml based upon results of tests with commercial standard concentration of CD28/Fc fusion protein (Fig. 1). To extensively explore

the practical application of this established sCD28 ELISA, we also analysed the stability, precision and specificity of the method. After pre-coating with 2F5, the plates were preserved at 4°C for 0, 20, 40 or 60 days, and four known sCD28 concentrations (1 ng/ml, 5 ng/ml, 10 ng/ml and 15 ng/ml) were analysed, respectively. Our results showed that the mean sCD28 concentration and the coefficient of variation were not significantly decreased in single sample concentrations at different times during 60 days at 4°C (CV % < 7.61%). It demonstrated that storage of plates coated with 2F5 mAb over prolonged periods did not affect the detection of sCD28. The spike recovery ranged from 93% to 109% through spiking four serial two-fold dilutions of sCD28 into four different levels of known sCD28 concentrations. To further evaluate the specificity, four related soluble proteins (CD80/Fc, CD86/Fc, CTLA-4/Fc and OX40L/Fc) were respectively added to recombinant CD28/Fc positive control. Our results revealed that the above-mentioned costimulatory molecules did not have significant cross-reactivity or interference with detection of sCD28. Therefore, the quantitative detection method of sCD28 can be applied for the evaluation of concentrations of sCD28 in samples.

Soluble CD28 increased in plasma of GD patients

Using the above-mentioned sandwich ELISA for detecting sCD28 concentrations, we measured sCD28 levels in plasma from healthy donors and patients with GD. Our results suggest that mean concentrations of sCD28 in the plasma of patients with GD were 1.79 ±1.52 ng/ml, but levels of sCD28 in healthy subjects were 0.83 ±1.35 ng/ml. Concentrations of sCD28 detected in patients with GD were significantly higher than those detected in healthy subjects ($p < 0.001$). Four patients with GD had very high levels of sCD28 (> 5 ng/ml) (Fig. 2). These patients had significant goitre, proptosis and exophthalmos. Moreover, there was a positive correlation between the concentrations of sCD28 in plasma and FT3 ($r = 0.663$, $p < 0.01$), FT4 ($r = 0.624$, $p < 0.01$) and TRAb ($r = 0.728$, $p < 0.01$) levels in serum, but a negative correlation was found between sCD28 levels and TSH ($r = –0.726$, $p < 0.01$) (Fig. 3). Interestingly, for treated GD patients, sCD28 concentrations in patients with GD were gradually decreased. In addition, concentrations of sCD28 in culture supernatants of T cells activated by PHA were significantly higher than those of resting T cells (0.67 ±0.15 vs. 0.34 ±0.11 ng/ml, $p < 0.05$).

IL-6 secretion was induced by soluble CD28 *in vitro*

To investigate whether CD28-Ig fusion protein could display a functional response to human dendritic cells, we assessed the production of IL-2 and IL-6 in culture supernatants of dendritic cells treated with CD28-Ig fusion protein and control IgG at 24 hours. Our results suggested that the concentration of IL-6 induced by sCD28 was much greater than that of control groups (870.52 ±73.61
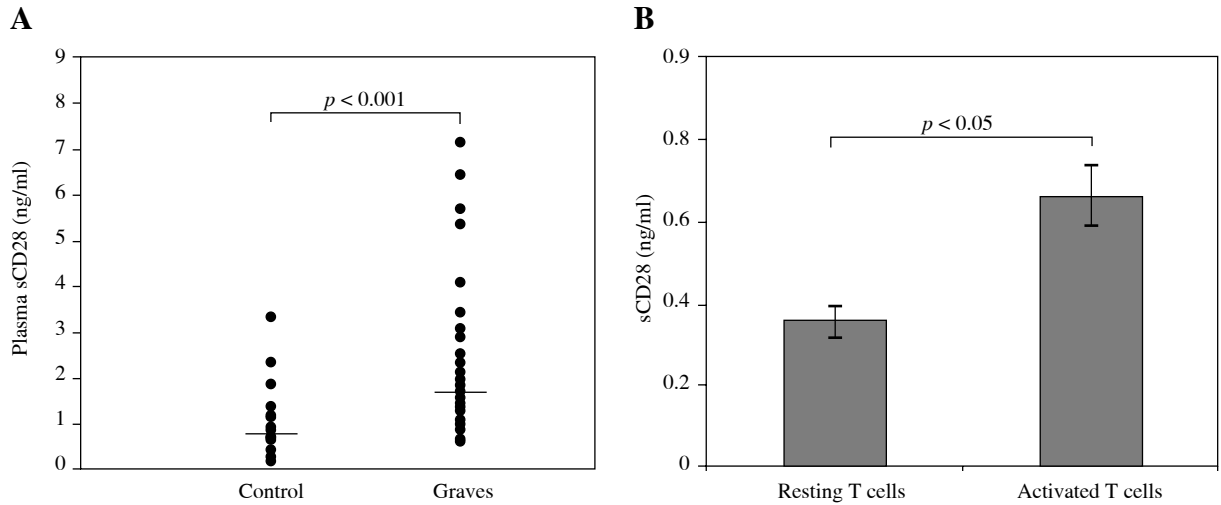
A

B

Fig. 2. Detection of sCD28 in patients and healthy control samples. **A)** Plasma of patients with Graves' disease and healthy donors was collected; concentrations of sCD28 were measured by the above-established sandwich ELISA. The level of sCD28 from each sample was calculated in terms of separated and triplicated experiments. The differences between Graves' disease patients and control subjects were determined by non-parametric Mann-Whitney rank sum test; $p < 0.01$. **B)** sCD28 was detected in culture supernatants of resting T cells and activated T cells with PHA; $p < 0.05$
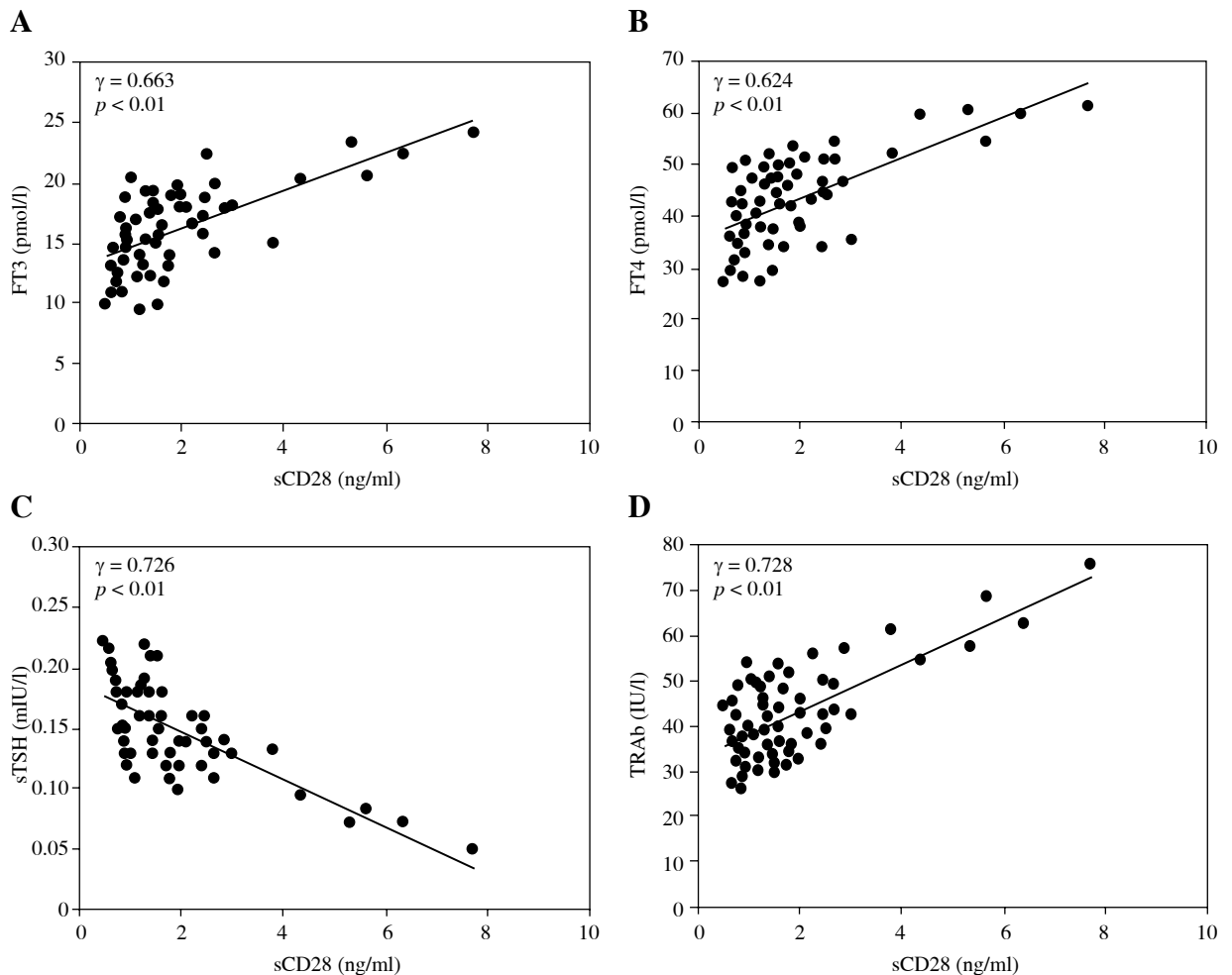
A

B

C

D

Fig. 3. Correlation between concentration of plasma sCD28 and FT3, FT4, sTSH and TRAb in patients with Graves' disease

pg/ml vs. 4.25 ±2.83 pg/ml), but the level of IL-2 secreted by sCD28 and control IgG showed no significant difference (Fig. 4). Similarly, concentrations of cytokine IL-6 in plasma were also significantly higher than those in healthy donors (73.46 ±9.18 pg/ml vs. 8.27 ±3.24 pg/ml), but compared with control subjects, IL-2 levels were found to decrease in plasma of GD patients (11.54 ±1.72 pg/ml vs. 15.36 ±1.48 pg/ml) Thus, sCD28 fusion protein could induce dominant production of cytokine IL-6, which may be involved in GD development.
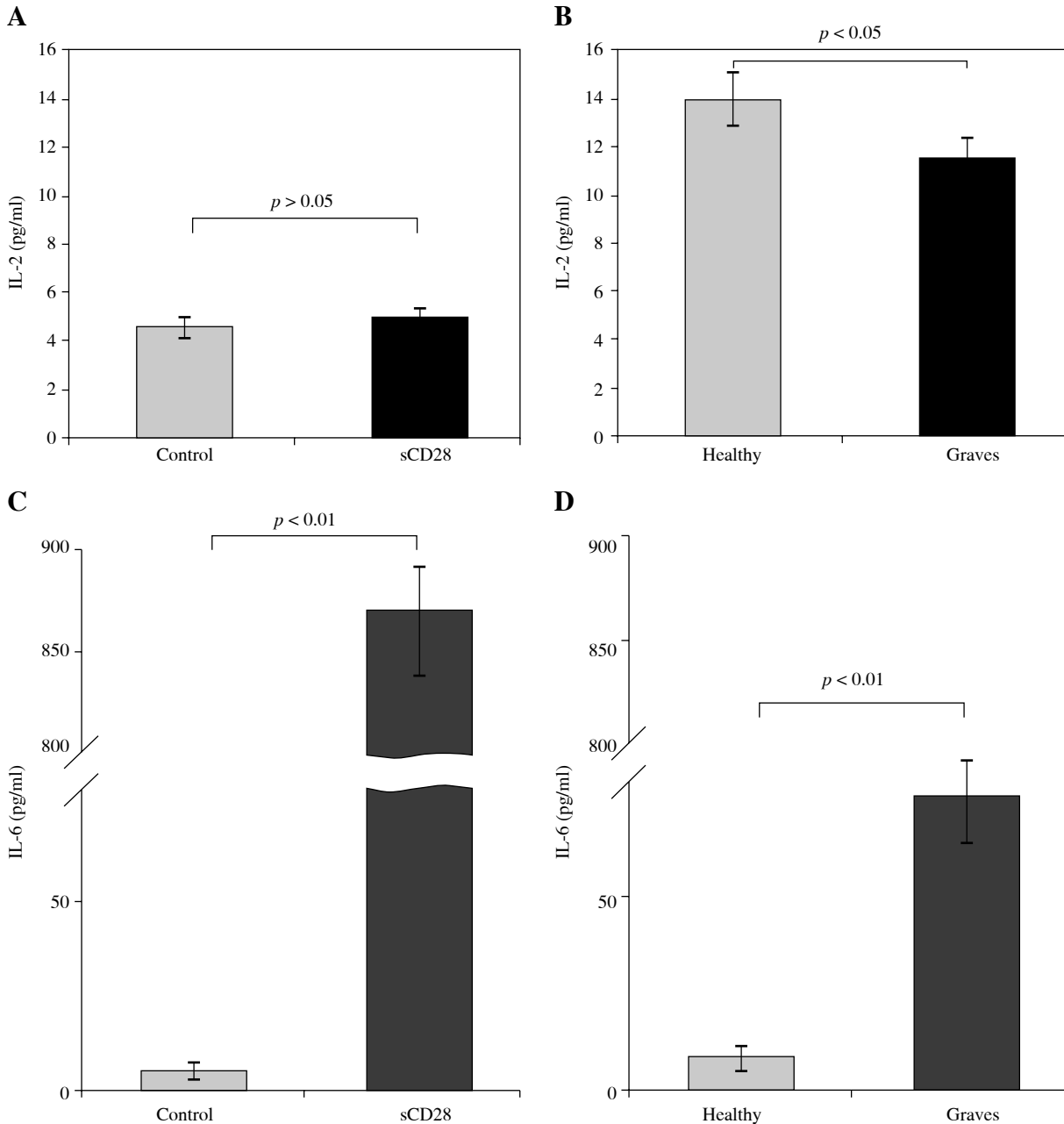

Fig. 4. Cytokine concentrations of Graves' disease patients and culture supernatants induced by sCD28 on dendritic cells. Monocyte-derived dendritic cells were exposed to sCD28 fusion protein and IgG (control). After 24 hours, the supernatants were harvested to measure the IL-2 (**A**, $p > 0.05$) and IL-6 (**B**, compared with control, $p < 0.01$) concentrations. In addition, plasma of healthy donors and patients with Graves' disease was also collected to detect levels of IL-2 (**C**, compared with healthy subjects, $p < 0.05$) and IL-6 (**D**, compared with Graves patients, $p < 0.01$) according to the kit instructions. All data represented by the mean concentrations of cytokines was from three separate experiments

Discussion

In autoimmunity, recent efforts have focused on identifying novel disease biomarkers and their biological characteristics. The B7 family of costimulatory molecules is critical in regulating T-cell responses. CD28, the ligand of B7.1 or B7.2, has been demonstrated in previous studies to have costimulatory functions, and this protein has sCD28 could be produced by shedding of the membrane form or could result from alternative mRNA splicing, but recently RT-PCR analysis has shown that only the full-length CD28 transcript can be detected in T cells from SLE patients exhibiting sCD28, suggesting that the elevation of sCD28 found in patients may be attributed to the shedding of the membrane [26, 27]. In accordance with the results, our data revealed that detection of membrane CD28 expression on T cells from GD patients who had a high circulating sCD28 level but showed a decreased expression, was comparable with the control subjects. Moreover, the exclusive full-length soluble form of CD28 in serum was confirmed by western blot. In addition, RT-PCR analyses confirmed that exclusive full-length CD28 expression is detected in activated T lymphocytes, as previously reported [22]. sCD28 in culture supernatants of T cells activated with PHA was demonstrated by ELISA method, but sCD28 forms were undetectable in culture supernatants of resting T cells. These results might give some insight into the close relationship between the elevation of sCD28 and the activated state of peripheral T cells.

B7-CD28/CTLA-4 costimulation can regulate T-cell chemotaxis and differentiation, probably through the activation of inflammatory cytokines and chemokines [7, 8]. Therefore, the dysregulation of T-cell functions by the elevation of sCD28 and proinflammatory cytokines and chemokines is involved in the development of airway inflammation and airway hyperresponsiveness [28] and may lead to inflammation, particularly in SLE patients with more severe autoimmune disease [16]. However, it is not clear from the present studies whether aberrant expression of costimulatory molecules, such as sCD28, in GD patients represents a primary existence in autoimmune diseases or only represents the self-regulation mechanism of GD.

As is already known, the interaction of B7 and CD28 can exert a positive effect on the initiation and proliferation of T cells, but our results and previous studies demonstrated that sCD28 could lead to dendritic-cell-induced IL-6 production. Similar results were demonstrated by using mouse CD28-Ig to treat mouse dendritic cells [29, 30]. However, it is not clear from present studies whether elevation of sCD28 in GD represents a primary defect that directly contributes to disease or just represents a secondary event. In addition, our data showed that aberrant expression of sCD28 in plasma has a significantly positive correlation with IL-6 production ($\gamma = 0.792$, $p < 0.01$). We speculated that with the development of disease, the shedding of membrane CD28 molecules on T cells might promote the production of sCD28, which can interact with B7-1/B7-2 on the cell surface of DCs. The reverse signal produced by the ligation of sCD28 and B7-1/B7-2 can lead to the production of IL-6, which plays an important role as a cytokine in the development of B cells and in the production of thyroid stimulating hormone receptor antibodies (TRAb). The ligation of anti-TRAb and thyroid stimulating hormone (TSH) receptors may result in a decreased level of TSH and increased level of free T3 and T4. Therefore, in any event, aberrant production of sCD28 represents a hallmark in GD and may provide a useful biomarker.

In summary, we established sandwich ELISA for detecting sCD28 in biological samples, and demonstrated elevated concentrations of plasma sCD28 in GD patients, which had a significant association with laboratory parameters. Simultaneously, ligation of sCD28 fusion protein and B7-1/B7-2 expressed on DCs could promote DC activation and IL-6 secretion. Based on the above findings, sCD28 molecules in plasma were functional and had a reverse correlation with positive percentages of membrane CD28 on T cells of GD patients.

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