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Impairment of Regulatory Capacity of CD4⁺CD25⁺ Regulatory T Cells Mediated by Dendritic Cell Polarization and Hyperthyroidism in Graves' Disease

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Graves' disease (GD) is one of the most common autoimmune diseases. The immune dysfunction in GD involves the generation of thyroid-stimulating hormone receptor (TSHR) autoantibodies that presumably arise consequent to interactions among dendritic cells (DCs), T cells, and regulatory T (Treg) cells. However, the immunological mechanisms of interactions between them that lead to the induction and regulation of this autoimmune disease are poorly defined. In this study, we investigated whether DCs are the main cause of the defective activity of Treg cells in GD patients. We found a significant decrease in the percentage of circulating CD4⁺CD25⁺FOXP3⁺ Treg cells in untreated GD patients (uGD), which was negatively correlated with the concentration of TSHR autoantibodies. uGD-derived DCs were polarized to increase the number of plasmacytoid DCs (pDCs) and conferred the ability to abrogate the suppressive function of Treg cells through inducing apoptosis of CD4⁺CD25⁺ Treg cells in an IFN- α -dependent manner, and elevated thyroid hormones further exacerbated the effect. The nucleotide UDP, which inhibits IFN- α secretion of pDCs through P2Y6 receptor signaling, restored the suppressive function of CD4⁺CD25⁺ Treg cells. Collectively, uGD-derived DCs through pDC polarization and elevated thyroid hormones act in concert to impair the regulatory capacity of Treg cells, facilitating the production of TSHR autoantibodies in the pathogenesis of GD. *The Journal of Immunology*, 2011, 186: 000–000.

Graeves' disease (GD) is a common organ-specific autoimmune disease characterized by the production of agonistic autoantibodies against the thyroid-stimulating hormone receptor (TSHR), which mimic the stimulatory effects of

TSH, leading to hyperthyroidism and diffuse hyperplasia of the thyroid gland (1, 2). Besides environmental, endocrine factors, and genetic predisposition, immune dysfunction also plays an important role in the pathogenesis of GD. Although it has been established that the presence of TSHR autoantibodies in the sera of GD patients is Th cell dependent, the underlying mechanisms of the breakdown of self-tolerance to TSHR with autoantibody production have not been fully elucidated.

The breakdown of central and peripheral immune tolerance to autoantigens in autoimmune diseases, including GD, is largely due to deficient immune regulation. It has been documented that regulatory T (Treg) cells play an important role in preventing autoimmune responses and are involved in the maintenance of homeostatic mechanisms that suppress autoreactive T cell proliferation and promote autoreactive T cell anergy (3–5). Natural CD4⁺CD25⁺ Treg cells, which are the major component of Treg cells, develop in the thymus and represent a subpopulation of ~10% of the peripheral CD4⁺ T cells (6). These Treg cells suppress the proliferation of autoreactive CD4⁺ Th cells, Ab production from autoreactive B cells, and the maturation of dendritic cells (DCs) (6–8). Consequently, defects in the number and/or function of Treg cells may contribute to the loss of peripheral self-tolerance, leading to the occurrence of autoimmune diseases such as experimental autoimmune arthritis (3), experimental gastritis (9), experimental autoimmune thyroiditis (10, 11), and multiple sclerosis (12). In contrast, administration of CD4⁺CD25⁺ Treg cells has been shown to protect against some autoimmune diseases, including experimental autoimmune encephalitis (13), systemic lupus erythematosus (8), diabetes (14), and colitis (15, 16). Recently, it is reported that the depletion of Treg cells increases the incidence of hyperthyroidism and enhances disease severity in a mouse model, indicating that Treg cells have a central role in the pathogenesis of GD (17).

Although recent studies have focused on the roles of Treg cells, APCs, especially DCs, have also long been recognized to play a piv-

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Abbreviations used in this article: 7-AAD, 7-amino-actinomycin D; BDCA, blood dendritic cell Ag; CD, control donor; DC, dendritic cell; eGD, euthyroid Graves' disease patients; eHT, euthyroid Hashimoto's thyroiditis patients; FCM, flow cytometry; GD, Graves' disease; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; T3, 3,3',5-triiodo-L-thyronine; TdR, thymine deoxyriboside; TH, thyroid hormone; Treg, regulatory T; TSHR, thyroid-stimulating hormone receptor; uGD, untreated GD patient.

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otol role in autoimmune diseases because they are capable of either triggering autoreactive T cells or activating Treg cells, depending on the maturation stimuli and/or DC subsets (18, 19). Circulating human DCs, which typically constitute 0.5–2% of PBMCs, are classified into two different subsets with distinct surface markers: CD11c⁺CD123^{lo} or CD11c^{lo}CD123⁻ myeloid DCs (mDCs) and CD11c⁻CD123^{hi} plasmacytoid DCs (pDCs) (20, 21). pDCs are found primarily in the peripheral blood and lymphoid organs. They possess the capacity to respond to self-nucleic acids or viruses via TLR7 and TLR9 by producing large amounts of IFN- α and other proinflammatory cytokines, which participate in autoimmunity and innate antiviral immunity (22–24). In contrast, mDCs mainly produce proinflammatory cytokines, such as IL-12 and TNF- α , in response to microbial stimulation (25). It has been suggested that DCs have the ability to control the suppressive function, expansion, and/or differentiation of Treg cells in mouse models of autoimmune diseases (26–28). Human DCs are able to partially abrogate the inhibition by Treg cells (29). More recent studies suggest that a feedback regulatory loop modulates the number of Treg cells and DCs in vivo. This regulatory circuit is essential for the steady-state balance between immunity and tolerance (30, 31). However, under the pathology conditions, particularly in the context of elevated thyroid hormones (THs), as the functional cross talk between immune and endocrine systems, the regulatory mechanism of DCs, Treg cells, and THs in the pathogenesis of GD remains uncertain.

We therefore mainly focused our study on the interaction between naturally occurring CD4⁺CD25⁺ Treg cells and DCs in the GD patients. We provide evidence that the pDC subset was polarized in untreated GD patients (uGD), leading to a reduction in the number and regulatory capacity of Treg cells through induction of apoptosis. THs further aggravated the apoptosis of Treg cells. Furthermore, the effect of uGD-derived DCs was reversed by the nucleotide UDP through P2Y6 receptor signaling, which restored the regulatory activity of Treg cells.

Materials and Methods

Subjects

The patients in this study were recruited from the Outpatient Department of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, and the Outpatient Department of Affiliated Hospital of Jiangsu University. Seventy-seven uGD, 13 euthyroid GD patients (eGD) from the conversion of GD patients due to antithyroid drug therapy, and 15 euthyroid Hashimoto's thyroiditis patients (eHT) were diagnosed through standard clinical and laboratory examinations. Control donors (CD) ($n = 74$) were selected based on normal physical examination and endocrine function tests. Demographic and clinical information of the study subjects is presented in Table I. The serum levels of FT3, FT4, TSH, and TPOAb were measured by automated chemiluminescent immunoassays (Architect i2000SR; Abbott). The serum level of TSHR Abs was measured by a radioreceptor assay with a commercial kit (Dia Sorin). Informed consent was obtained from all participants before the samples for the study were collected. The study protocol was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

Cell isolation and purification

Peripheral blood was collected in sodium-heparin vacutainer tubes, and PBMCs were isolated with a Ficoll density gradient (Sigma-Aldrich). DCs (>90% purity) were separated by negative and positive selection using the Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions. The kit allows the concurrent isolation of pDCs and mDCs by magnetic labeling of CD303, CD1c, and CD141, respectively. The remaining cells were used to further isolate CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells by negative and positive selection using the human CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec). In this study, to remove autoreactive CD4⁺CD25^{lo} T cells from the CD4⁺CD25⁺ Treg cells, CD25^{hi} T cells were isolated from the CD4⁺ populations by using 50% of the manufacturer's recommended concentration of anti-CD25 microbeads, because CD4⁺CD25⁺ Treg cells

have previously been shown to express a high level of CD25 marker (32). The isolated CD4⁺CD25⁺ T cells (>90% purity) and CD4⁺CD25⁻ T cells (>90% purity) were used in both the proliferation and suppression assays. In some experiments, isolated DCs were further sorted into mDCs (CD11c⁺CD123⁻) and pDCs (CD11c⁻CD123⁺) with >90% purity of both by FACSaria cytometer (BD Biosciences).

Flow cytometric analysis

PBMCs were freshly isolated by Ficoll density gradient centrifugation. The proportion of CD4⁺CD25⁺FOXP3⁺ T cells in the CD4⁺ T cell population was determined with a commercially available kit (eBioscience) according to the manufacturer's instructions. In brief, after CD4 and CD25 surface staining, cells were washed and fixed using fixation/permeabilization solution. Cells were then stained intracellularly for FOXP3. Human blood DC subsets (mDC and pDC) were analyzed with a DC enumeration kit (Miltenyi Biotec) according to the manufacturer's instructions. In brief, samples were stained with anti-blood DC Ag (BDCA) mixture (containing mAbs specific for the DC markers BDCA1, BDCA2, and BDCA3 along with CD14 and CD19 Abs) or control mixture containing appropriate isotype controls. The percentage of positively stained cells was analyzed using flow cytometry (FCM; BD Biosciences). In some experiments, the expression of cell-surface Ags was assessed with the following mAbs: PE-labeled anti-CD123 (BD Pharmingen); anti-CD25 (Miltenyi Biotec); anti-HLA-DR, anti-CD80, anti-CD86, anti-CD95L, and anti-CD95 (eBioscience); anti-CD40 (R&D Systems); and allophycocyanin-labeled anti-CD11c (BD Pharmingen). The appropriate isotype Abs were used as controls.

Functional responses of lymphocytes and DCs

Freshly isolated CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were cultured in 96-well V-bottom plates containing 200 μ l RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. In the DC-free assay, CD4⁺CD25⁻ T cells (5×10^4 cells/well) and CD4⁺CD25⁺ T cells (5×10^4 cells/well) were stimulated with anti-CD3 mAb (OKT3, 1 μ g/ml; eBioscience) and anti-CD28 mAb (CD28.2, 1 μ g/ml; eBioscience). In the DC-based assay, CD4⁺CD25⁻ T cells (5×10^4 cells/well) were incubated with or without CD4⁺CD25⁺ T cells (5×10^4 cells/well) in the presence of irradiated (30 Gy) autologous DCs (2×10^4 cells/well) and anti-CD3 mAb (1 μ g/ml) for 3 d. [³H]thymine deoxyriboside (TdR) incorporation was measured during the last 12–16 h of the incubation. Triplicate wells were cultured for each group. In some experiments, recombinant human IFN- α (100 U/ml; PBL Biomedical), neutralizing anti-human IFN- α mAb (5 μ g/ml; Chemicon International), anti-human IL-12 mAb (5 μ g/ml; R&D Systems), anti-human TNF- α mAb (5 μ g/ml; R&D Systems), or 3,3',5-triiodo-L-thyronine (T3) (5, 15, and 30 nM; Sigma-Aldrich) were added to the culture. In some experiments, the isolated DCs were pretreated with the nucleotide UDP (1 μ M; Sigma-Aldrich) or suramin (0.1 μ M; Sigma-Aldrich) for 24 h before addition to the culture.

Apoptosis assay

Apoptosis detection for T cells in an FCM assay was adapted from Godoy-Ramirez et al. (33). First, freshly isolated DCs were prelabeled with 2.5 μ M CFSE (Sigma-Aldrich) for 10 min, then freshly isolated CD4⁺CD25⁺ T cells (1×10^5 cells/well) were cocultured with labeled autologous DCs (2×10^4 cells/well) in 96-well V-bottom plates containing 200 μ l RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. In the DC-free assay, anti-CD3 mAb (1 μ g/ml) and anti-CD28 mAb (1 μ g/ml) were added to the culture. In the DC-based assay, anti-CD3 mAb (1 μ g/ml) was added to the culture. In some experiments, recombinant human IFN- α (100 U/ml), neutralizing anti-human IFN- α mAb (5 μ g/ml), or T3 (5, 15, and 30 nM) were added to the culture. In some experiments, the isolated DCs were pretreated with the nucleotide UDP (1 μ M) or suramin (0.1 μ M) for 24 h before addition to the culture. After 3 d of culture, the cells were harvested and stained with the Apoptosis Detection Kit 1 (BD Pharmingen) according to the manufacturer's instructions. In brief, cells were stained with Annexin V-PE and 7-amino-actinomycin D (7-AAD) for 15 min at room temperature and analyzed by FCM immediately. Gating for CFSE-negative population, cells with Annexin V-PE-positive and 7-AAD-negative staining or both Annexin V-PE- and 7-AAD-positive staining were regarded as apoptotic cells.

Measurement of cytokine level

Supernatants from cell culture and serum from GD patients were stored at -80°C until use. The levels of serum and supernatant IFN- α , TNF- α , and IL-12 were then measured using an ELISA kit according to the manufacturer's instructions (R&D Systems).

Statistical analysis

Results are expressed as the means \pm SD. Comparison between two groups was performed by Student *t* tests. Data from more than two groups were compared using one-way ANOVA with the Tukey–Kramer multiple comparison test. Correlation analysis was determined by Spearman's rank correlation coefficient. The *p* values <0.05 were considered to be statistically significant.

Results

Proportion of CD4⁺CD25⁺FOXP3⁺ Treg cells in the peripheral blood of uGD is reduced

To determine the number of Treg cells in GD patients, we measured the proportion of circulating CD4⁺CD25⁺ Treg cells in CD4⁺ T cells in the peripheral blood from 48 CD, 48 uGD, 10 eGD, and 10 eHT (Table I). As FOXP3 is thought to be the most unambiguous marker available to identify Treg cell populations (34), a combination of the markers CD4⁺, CD25⁺, and FOXP3⁺ was used to identify Treg cells in this study. As shown in Fig. 1A and 1B, compared with that in CD (3.36 \pm 0.96%), there was a lower proportion of CD4⁺CD25⁺FOXP3⁺ T cells over CD4⁺ T cells in the peripheral blood of uGD (1.57 \pm 0.67%; *p* < 0.001), but not in eGD (2.98 \pm 0.88%) or eHT (3.09 \pm 1.02%). Approximately 70–80% of FOXP3⁺ T cells fell within the CD25^{hi} T cell gate, whereas ~5–10% of FOXP3⁺ T cells fell within the CD25^{lo} T cell gate from either patients or CD (Fig. 1C). Further analysis indicated that, compared with that in CD (2.69 \pm 0.77%), there was a lower proportion of CD4⁺CD25^{hi}FOXP3⁺ T cells over CD4⁺ T cells in uGD (1.26 \pm 0.54%; *p* < 0.001), but not in eGD (2.38 \pm 0.71%) or eHT (2.47 \pm 0.81%). However, there was no significant difference in the proportion of CD4⁺CD25^{lo}FOXP3⁺ T cells over CD4⁺ T cells in uGD (0.26 \pm 0.10%), eGD (0.31 \pm 0.10%), or eHT (0.34 \pm 0.12%) as compared with that in CD (0.32 \pm 0.11%). There was no significant difference in the proportion of CD4⁺CD25⁺FOXP3⁻ T cells over CD4⁺ T cells in uGD (2.88 \pm 0.76%), eGD (3.09 \pm 0.81%), or eHT (3.61 \pm 0.92%) as compared with that in CD (3.23 \pm 0.85%). Taken together, results with markers of CD25^{hi}FOXP3⁺ or CD25^{lo}FOXP3⁺ for the assessment of CD4⁺CD25⁺ Treg cells yielded a similar conclusion. Interestingly, the serum level of TSHR Abs in uGD (21.27 \pm 11.74 U/l) was markedly increased as compared with that in CD (0.50 \pm 0.62 U/l; *p* < 0.001). Statistical analysis showed that the proportion of CD4⁺CD25⁺FOXP3⁺ T cells was strongly negatively correlated with the TSHR Ab concentration in uGD (*r* = -0.735, *p* < 0.001; Fig. 1D), suggesting that CD4⁺CD25⁺ Treg cells may be associated with the production of TSHR Abs.

uGD-derived DCs impair the immunosuppressive activity of CD4⁺CD25⁺ Treg cells

To uncover the mechanism of impaired CD4⁺CD25⁺ Treg cells in uGD, we analyzed the immunosuppressive effect of CD4⁺CD25⁺

Treg cells from 15 CD, 15 uGD, 3 eGD, and 3 eHT. First, the purities of isolated CD4⁺CD25⁺ T cells and DCs were determined. The results showed that the purity of isolated CD4⁺CD25⁺ T cells was >90%, and most of them coexpressed FOXP3 (*n* = 5; 81.1 \pm 4.1%); the purity of these isolated DCs was >90% (Fig. 2A). To determine the immunosuppressive effect of isolated CD4⁺CD25⁺ T cells, we assessed the proliferation of CD4⁺CD25⁻ T cells alone, CD4⁺CD25⁺ T cells alone, and the coculture of equal amounts of the two populations, with or without stimulation by anti-CD3 and anti-CD28 in the culture. As shown in Fig. 2B, CD4⁺CD25⁻ T cells from CD, uGD, eGD, or eHT displayed significant proliferation in response to anti-CD3 and anti-CD28 mAb stimulation. However, CD4⁺CD25⁺ T cells from all of the groups possessed the characteristics of CD4⁺CD25⁺ Treg cells, as indicated by their lower proliferation rate and suppressive effect on the proliferation of autologous CD4⁺CD25⁻ T cells. These results suggest that CD4⁺CD25⁺ T cells themselves have an immunosuppressive effect despite whether they derive from CD, uGD, eGD, or eHT.

Considering that the intrinsic function of CD4⁺CD25⁺ Treg cells in GD remained normal, we then examined whether DCs control the function of CD4⁺CD25⁺ Treg cells. As shown in Fig. 2C, except for uGD-derived DCs, CD-, eGD-, or eHT-derived DCs initiated slight proliferation of autologous CD4⁺CD25⁺ T cells. uGD-, CD-, eGD-, or eHT-derived DCs initiated significant proliferation of autologous CD4⁺CD25⁻ T cells. However, compared with CD-, eGD-, or eHT-derived DCs, uGD-derived DCs had a stronger stimulatory effect on CD4⁺CD25⁻ T cells. When DCs were cocultured with autologous CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells, we found that these T cells had higher proliferation rates in response to uGD-derived DCs than that in CD-, eGD-, or eHT-derived DCs. Thus, it is likely that uGD-derived DCs have the ability to reverse the suppressive effect of CD4⁺CD25⁺ T cells, whereas CD-, eGD-, or eHT-derived DCs do not. Taken together, these results indicate that uGD-derived CD4⁺CD25⁺ Treg cells have normal immunosuppressive effect; however, uGD-derived DCs impair the effect of CD4⁺CD25⁺ Treg cells.

pDCs are increased in the uGD-derived DC population

To determine the reason for impaired activity of CD4⁺CD25⁺ Treg cells by uGD-derived DCs, the characteristics of uGD-derived DCs were investigated. First, using identical conditions for DC isolation, we compared the absolute numbers of isolated DCs from CD, uGD, eGD, or eHT. As shown in Fig. 3A, the absolute numbers of isolated DCs from the peripheral blood had no significant difference in CD (*n* = 20; 0.45 \pm 0.17 \times 10⁵/ml), uGD (*n* = 20; 0.61 \pm 0.38 \times 10⁵/ml), eGD (*n* = 8; 0.42 \pm 0.09 \times 10⁵/ml), or eHT (*n* = 8; 0.53 \pm 0.24 \times 10⁵/ml). We then analyzed the phenotypes of the DC population. As shown in Fig. 3B, the expression of CD80, CD86, and CD40 in uGD slightly increased as

Table I. Clinical characteristics of the study subjects

	CD	uGD	eGD	eHT
<i>n</i>	74	77	13	15
Female, <i>n</i> (%)	60 (81.1)	65 (84.4)	11 (84.6)	15 (100)
Age (y)	40.7 \pm 10.5	41.1 \pm 12.7	35.8 \pm 8.8	38.5 \pm 9.4
FT3 (pmol/l)	3.74 \pm 0.90	19.41 \pm 5.72	4.75 \pm 1.27	3.75 \pm 0.84
FT4 (pmol/l)	13.34 \pm 2.50	43.03 \pm 12.03	15.80 \pm 2.09	13.17 \pm 2.20
s-TSH (mIU/l)	2.09 \pm 1.02	0.013 \pm 0.008	1.12 \pm 0.72	3.72 \pm 0.67
TPOAb (IU/ml)	3.52 \pm 2.03	90.55 \pm 65.33	67.77 \pm 37.69	726.29 \pm 241.86
TRAb (U/l)	0.49 \pm 0.33	26.06 \pm 12.17	3.25 \pm 1.17	1.19 \pm 0.84

Values are expressed as means \pm SD unless indicated otherwise.

FT3, free triiodothyronine; FT4, free thyroxine; s-TSH, sensitive TSH; TPOAb, Ab against thyroperoxidase; TRAb, TSH receptor Ab.

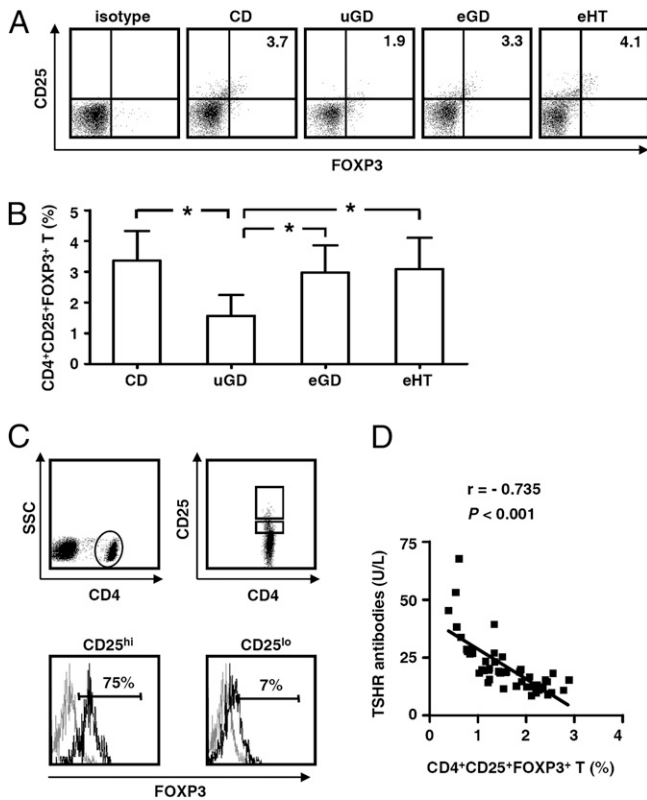


FIGURE 1. Proportion of $CD4^+CD25^+FOXP3^+$ T cells in the peripheral blood of GD patients. *A*, Freshly isolated PBMCs from CD, uGD, eGD, and eHT were stained for intracellular FOXP3 after CD4 and CD25 surface staining. Representative FCM graphs of the proportion of $CD4^+CD25^+FOXP3^+$ T cells from a CD, a uGD, an eGD, and an eHT are depicted. *B*, Cumulative data of the proportions of $CD4^+CD25^+FOXP3^+$ T cells over $CD4^+$ T cells in the 48 CD, 48 uGD, 10 eGD, and 10 eHT are shown. Results are expressed as means \pm SD. *C*, The expression of FOXP3 in $CD4^+CD25^{hi}$ and $CD4^+CD25^{lo}$ T cells was analyzed. Representative FCM graphs from a uGD are depicted. *D*, The correlation between the proportion of $CD4^+CD25^+FOXP3^+$ T cells and the concentration of TSHR Abs from 48 uGD was analyzed. $*p < 0.001$.

compared with that in CD, but had no significant difference as compared with that in eGD or eHT. DCs in uGD showed distinct expression of HLA-DR, different from CD, eGD, or eHT ($n = 5$). Interestingly, the proportion of $CD11c^+CD123^+$ pDC in the isolated DC population from uGD ($n = 20$; $41.95 \pm 8.39\%$) was significantly higher as compared with that from CD ($n = 20$; $32.85 \pm 8.02\%$; $p < 0.01$), eGD ($n = 8$; $31.63 \pm 7.07\%$; $p < 0.01$), or eHT ($n = 8$; $28.50 \pm 3.96\%$; $p < 0.001$) (Fig. 3C, 3D), indicating increased proportion of pDC in uGD. To further substantiate the conclusion, we compared the ratio of pDC/DC from PBMCs using FCM analysis. As shown in Fig. 3E and 3F, the ratio of pDC/DC in uGD ($n = 5$; $37.56 \pm 5.94\%$) was significantly higher than that in CD ($n = 5$; $24.30 \pm 3.96\%$; $p < 0.01$), eGD ($n = 5$; $25.62 \pm 3.07\%$; $p < 0.05$), or eHT ($n = 5$; $23.90 \pm 3.55\%$; $p < 0.01$), further demonstrating increased pDC proportion in uGD.

Previous studies have demonstrated that pDCs are the major producers of IFN- α (24, 25); thus, the enhanced number of pDCs in uGD could increase the production of IFN- α . For this reason, the concentration of serum IFN- α was measured. As shown in Fig. 3G, there was a higher concentration of serum IFN- α in uGD ($n = 35$; 518.7 ± 368.7 pg/ml) relative to that in CD ($n = 31$; 16.38 ± 7.56 pg/ml; $p < 0.001$), eGD ($n = 10$; 21.2 ± 12.2 pg/ml; $p < 0.001$), or eHT ($n = 10$; 20.9 ± 5.2 pg/ml; $p < 0.001$). Statistical analysis suggested that the concentration of serum IFN- α was

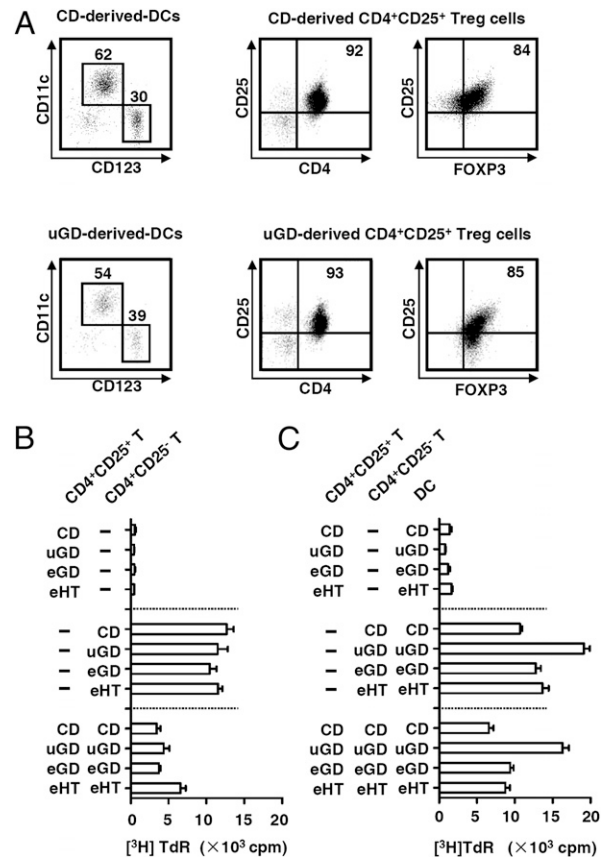


FIGURE 2. Impaired immunosuppressive function of $CD4^+CD25^+$ Treg cells in the presence of uGD-derived DCs. $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells, and DCs from PBMCs were isolated by MACS. *A*, Representative FCM graphs of the purities of isolated DCs and isolated $CD4^+CD25^+$ T cells that coexpress FOXP3 from PBMCs of a CD and a uGD are depicted, respectively. *B*, Isolated $CD4^+CD25^-$ T cells (5×10^4 cells/well) and/or $CD4^+CD25^+$ T cells (5×10^4 cells/well) were cultured in 96-well V-bottom plates for 3 d in the presence of anti-CD3 mAb (1 μ g/ml) and anti-CD28 mAb (1 μ g/ml). *C*, Isolated $CD4^+CD25^-$ T cells (5×10^4 cells/well) and/or $CD4^+CD25^+$ T cells (5×10^4 cells/well) were cocultured for 3 d with irradiated autologous DCs (2×10^4 cells/well) in 96-well V-bottom plates in the presence of anti-CD3 mAb (1 μ g/ml). $[^3H]$ TdR incorporation was measured during the last 12–16 h of the incubation. Results are expressed as means \pm SD in cpm of triplicate wells. The data are representative of at least three independent experiments.

significantly positively correlated with the ratio of pDC/DC ($r = 0.825$, $p < 0.001$; Fig. 3H) in uGD, whereas the ratio of pDC/DC had a significant negative correlation with the proportion of $CD4^+CD25^+FOXP3^+$ T cells in uGD ($r = -0.689$, $p < 0.001$; Fig. 3I). These results suggest that there is a significant skewing to pDCs in uGD, which might contribute to impairing the activity of $CD4^+CD25^+$ Treg cells in these patients.

pDCs and THs are the main contributors in impairing $CD4^+CD25^+$ Treg cell-mediated immunosuppression

To explore the mechanism of increased pDCs in uGD-derived DCs in impairing the activity of $CD4^+CD25^+$ Treg cells, IFN- α and autologous DCs with or without related functional neutralizing Abs were added to the culture system of $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells from CD or uGD. As compared with controls, the addition of IFN- α did not increase T cell proliferation (Fig. 4A), suggesting that IFN- α does not directly impair the inhibitory activity of $CD4^+CD25^+$ Treg cells. By contrast, the addition of uGD-derived DCs to the culture system markedly impaired the

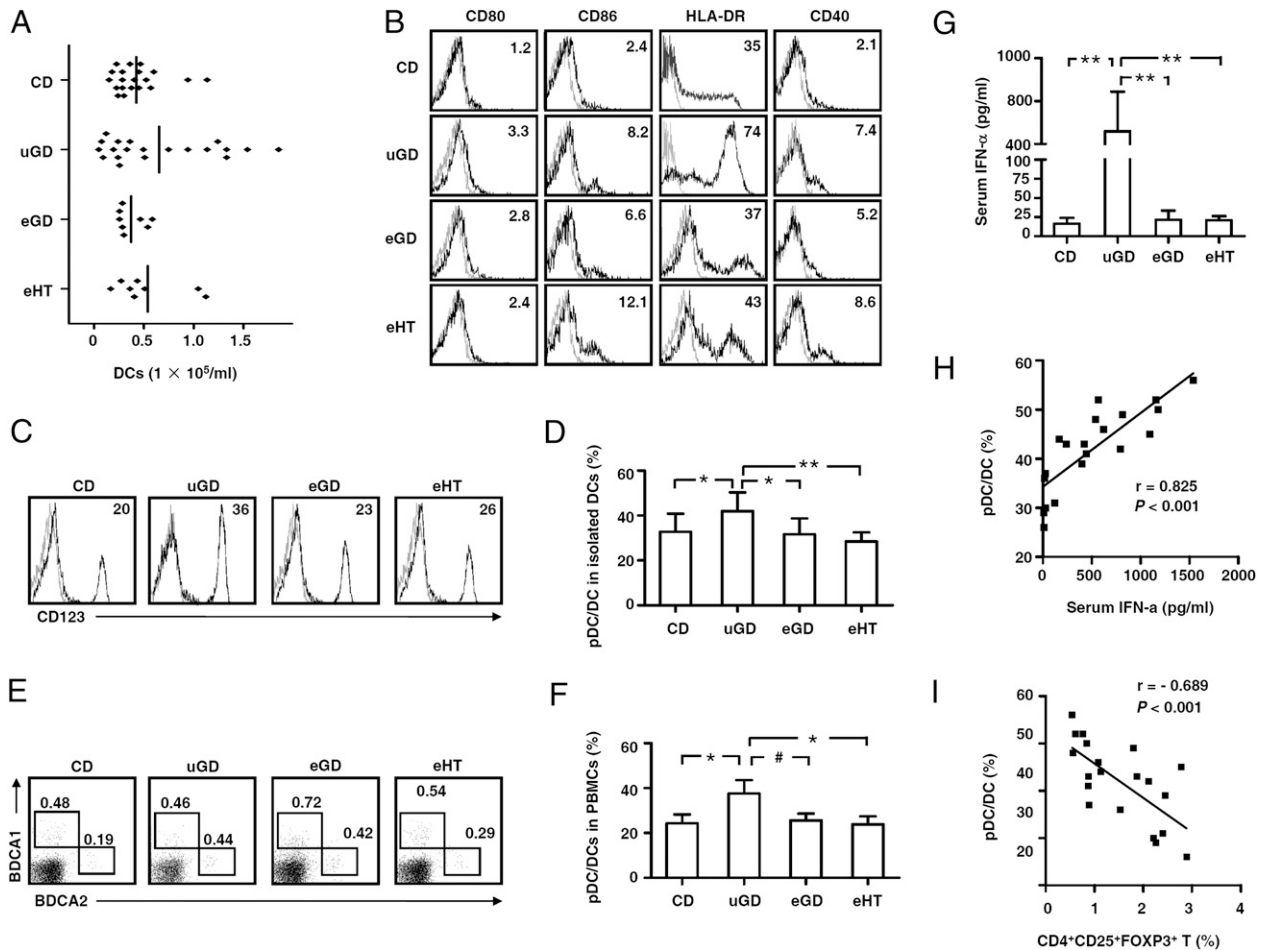


FIGURE 3. Increased number of pDCs in the uGD-derived DC population. Using identical conditions for DC isolation, DCs from PBMCs were isolated by MACS. *A*, The absolute number of DCs in the unit volume was calculated and compared among 20 CD, 20 uGD, 8 eGD, and 8 eHT. *B*, The phenotypes of the isolated DCs were determined by FCM ($n = 5$). Representative histograms from a CD, a uGD, an eGD, and an eHT are depicted. *C* and *D*, CD11c⁻CD123⁺ pDCs in the isolated DCs were determined by FCM. The ratios of pDC/DC from 20 CD, 20 uGD, 8 uGD, and 8 eHT were analyzed. Results are expressed as means \pm SD. *E* and *F*, BDCA2⁺ pDCs and BDCA1⁺ mDCs in the PBMCs were directly determined by FCM. The ratios of pDC/DC from 5 CD, 5 uGD, 5 eGD, and 5 eHT were analyzed. *G*, The levels of serum IFN- α were measured from 31 CD, 35 uGD, 10 eGD, and 10 eHT using ELISA. Results are expressed as means \pm SD. *H*, The correlation between the concentration of serum IFN- α and the ratio of pDC/DC in the isolated DCs from 20 uGD was analyzed. *I*, The correlation between the ratio of pDC/DC in the isolated DCs and the proportion of CD4⁺CD25⁺FOXP3⁺ T cells from 20 uGD was analyzed. # $p < 0.05$, * $p < 0.01$, ** $p < 0.001$.

inhibitory activity of CD4⁺CD25⁺ Treg cells and strikingly promoted T cell proliferation ($p < 0.001$), but not that of CD-derived DCs. This suggests that the stimulatory effect may be confined to uGD-derived DCs. When uGD-derived DCs together with anti-IFN- α mAb, but not anti-TNF- α mAb or IL-12 mAb, were added to the culture system, proliferation was largely reduced as compared with uGD-derived DCs ($p < 0.01$), suggesting that uGD-derived DCs need their secreted IFN- α to work together. To further determine whether the effect of uGD-derived DCs is driven by pDC subset, not mDC subset, pDCs and mDCs were sorted for further analysis. The results showed the addition of uGD-derived pDCs to the culture system markedly impaired the inhibitory activity of CD4⁺CD25⁺ Treg cells and strikingly promoted T cell proliferation, as compared with that of uGD-derived mDCs, CD-derived mDCs, or pDCs ($p < 0.01$), strongly indicating that the pDC subset is a contributor in the effect of uGD-derived DCs on CD4⁺CD25⁺ Treg cells.

As elevated THs occurred in the uGD patients, the effect of THs on the interaction between DCs and Treg cells was also determined. As shown in Fig. 4*B*, T3 alone did not display a direct impact on the suppressive effect of CD-derived or uGD-derived CD4⁺CD25⁺

Treg cells as compared with controls. However, uGD-derived DCs and T3 together significantly increased the proliferation of T cells as compared with uGD-derived DCs alone ($p < 0.05$). T3 and CD-derived DCs together slightly increased the proliferation of T cells; however, the extent of proliferation was significantly lower than uGD-derived DCs alone or with T3 together ($p < 0.001$). Collectively, these results suggest that THs exert an indirect impact on the immunosuppressive effect of CD4⁺CD25⁺ Treg cells, dependent on uGD-derived DCs.

uGD-derived DCs and THs render CD4⁺CD25⁺ Treg cells more susceptible to apoptosis

To explore the mechanism of DCs on CD4⁺CD25⁺ T cells, DCs were cocultured with CD4⁺CD25⁺ T cells for 3 d, and the apoptosis of CD4⁺CD25⁺ T cells was analyzed. Fig. 5*A* showed apoptosis measurements for gated T cells and representative FCM graphs from a CD and an uGD. As shown in Fig. 5*B*, there was no marked difference in spontaneous apoptosis of CD4⁺CD25⁺ T cells either from CD or uGD. However, when CD4⁺CD25⁺ T cells were cocultured with DCs, uGD-derived DCs significantly promoted the apoptosis of CD4⁺CD25⁺ T cells as compared with

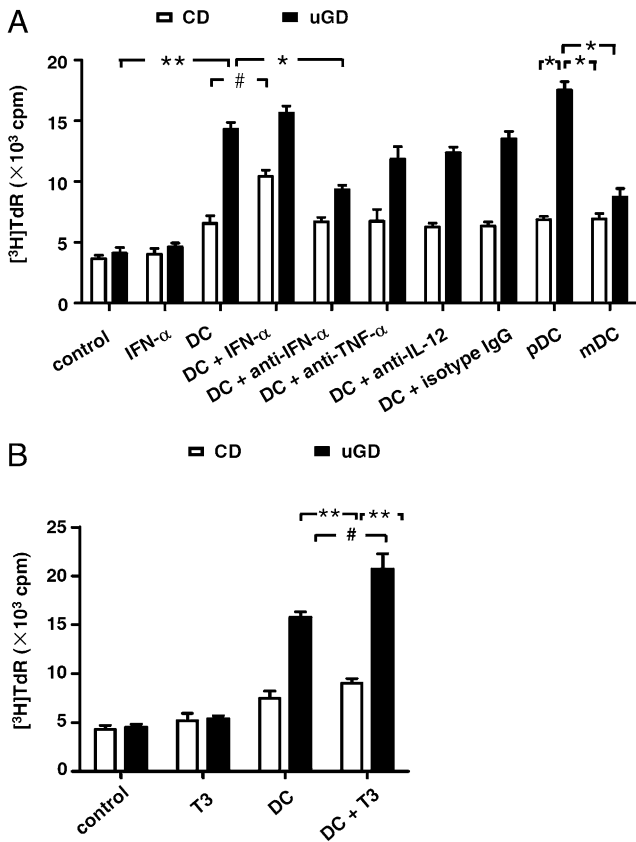


FIGURE 4. uGD-derived DCs and THs together impair the immunosuppression of CD4⁺CD25⁺ Treg cells. *A* and *B*, Isolated CD4⁺CD25⁻ T cells (5×10^4 cells/well) and CD4⁺CD25⁺ T cells (5×10^4 cells/well) from CD or uGD were cocultured in 96-well V-bottom plates for 3 d in the presence or absence of IFN- α (100 U/ml), T3 (15 nM), autologous DCs (2×10^4 cells/well), autologous DCs + IFN- α , autologous DCs + anti-IFN- α mAb (5 μ g/ml), autologous DCs + anti-TNF- α mAb (5 μ g/ml), autologous DCs + anti-IL-12 mAb (5 μ g/ml), autologous DCs + isotype Ab (5 μ g/ml), autologous pDCs (2×10^4 cells/well), and autologous mDCs (2×10^4 cells/well) as indicated. In the DC-free assay, anti-CD3 mAb (1 μ g/ml) and anti-CD28 mAb (1 μ g/ml) were added to the culture. In the DC-based assay, anti-CD3 mAb (1 μ g/ml) was added to the culture. [³H]TdR incorporation was measured during the last 12–16 h of culture. Results are expressed as means \pm SD in cpm of triplicate wells. The data are representative of five independent experiments. #*p* < 0.05, **p* < 0.01, ***p* < 0.001.

CD-derived DCs ($n = 5$; $16.52 \pm 3.10\%$ versus $5.72 \pm 1.58\%$; $p < 0.01$). However, anti-IFN- α mAb treatment significantly inhibited the apoptosis of CD4⁺CD25⁺ T cells induced by uGD-derived DCs as compared with isotype control ($n = 5$; $10.36 \pm 0.89\%$ versus $15.26 \pm 1.95\%$; $p < 0.01$). Of note, the addition of exogenous IFN- α not only slightly promoted DC-induced apoptosis of CD4⁺CD25⁺ T cells in uGD-derived DCs, but also produced a significantly elevated apoptosis in CD-derived DCs ($n = 5$; $8.48 \pm 1.22\%$ versus $5.72 \pm 1.58\%$; $p < 0.05$). To further determine whether uGD-derived DC-induced apoptosis of CD4⁺CD25⁺ Treg cells is driven by pDC subset, not mDC subset, pDCs and mDCs were sorted for further analysis. The results showed uGD-derived pDCs strikingly promoted apoptosis of CD4⁺CD25⁺ T cells ($19.28 \pm 2.45\%$; $p < 0.01$) as compared with uGD-derived mDCs ($9.74 \pm 0.61\%$), CD-derived mDCs ($5.56 \pm 0.43\%$), or CD-derived pDCs ($6.04 \pm 1.01\%$). As elevated THs occurred in the uGD, the effect of THs on DC-induced apoptosis of Treg cells was also determined. As shown in Fig. 5C, T3 did not directly induce apoptosis of CD-derived or uGD-derived CD4⁺CD25⁺

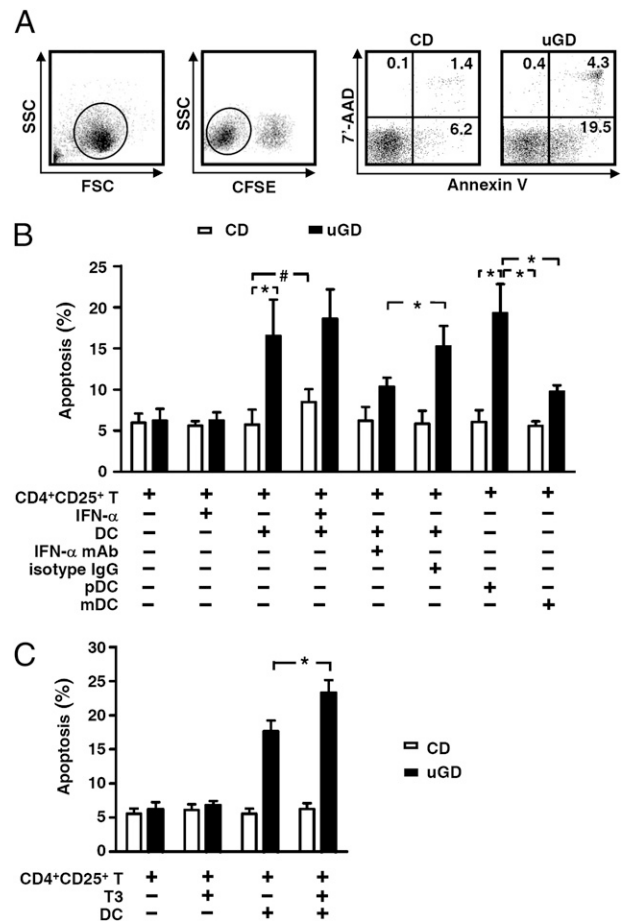


FIGURE 5. uGD-derived DCs and THs induce apoptosis of CD4⁺CD25⁺ Treg cells. uGD-derived and CD-derived DCs were isolated by MACS and prelabeled with 2.5 μ M CFSE. Isolated CD4⁺CD25⁺ T cells (1×10^5 cells/well) from CD or uGD were cocultured in 96-well V-bottom plates in the presence or absence of IFN- α (100 U/ml), T3 (15 nM), autologous DCs (2×10^4 cells/well), autologous DCs + IFN- α , autologous DCs + anti-IFN- α mAb (5 μ g/ml), autologous DCs + isotype Ab (5 μ g/ml), autologous pDCs (2×10^4 cells/well), and autologous mDCs (2×10^4 cells/well) as indicated. In the DC-free assay, anti-CD3 mAb (1 μ g/ml) and anti-CD28 mAb (1 μ g/ml) were added to the culture. In the DC-based assay, anti-CD3 mAb (1 μ g/ml) was added to the culture. After 3 d of culture, cells were washed and stained using the Apoptosis Detection Kit. *A*, Gating for CFSE-negative population, apoptotic cells with 7-AAD-negative and Annexin V-PE-positive staining or both annexin V-PE and 7-AAD-positive staining were analyzed by FCM. *B* and *C*, Cumulative data on apoptosis of CD4⁺CD25⁺ Treg cells are depicted. The data are expressed as means \pm SD of three independent experiments. #*p* < 0.05, **p* < 0.01, ***p* < 0.001.

Treg cells as compared with controls. However, T3 and uGD-derived DCs together significantly increased apoptosis of CD4⁺CD25⁺ Treg cells as compared with uGD-derived DCs alone ($p < 0.01$). Of note, T3 and CD-derived DCs together did not significantly alter apoptosis of CD4⁺CD25⁺ Treg cells as compared with CD-derived DCs alone. Taken together, these findings suggest that uGD-derived DCs induce the apoptosis of CD4⁺CD25⁺ Treg cells in an IFN- α -dependent mechanism, whereas elevated THs exacerbate the apoptotic effect.

Activity of CD4⁺CD25⁺ Treg cells is restored through regulating the P2Y6 receptor on uGD-derived DCs

The approach by which uGD-derived DCs are negatively regulated to restore the activity of CD4⁺CD25⁺ Treg cells is an interesting

subject. Recent studies have shown that extracellular nucleotides, such as UDP, can modulate the function and IFN- α production of human pDCs through the P2Y6 receptor, which is mainly expressed on pDCs, not T cells (35, 36). Thus, it is likely that the function of uGD-derived DCs is reversed by the P2Y6 ligand. To test this possibility, we treated uGD-derived DCs with the P2Y6 ligand UDP and assessed whether it affected CD4⁺CD25⁺ Treg cells by regulating DC function. As shown in Fig. 6A, uGD-derived DCs resisted the suppressive function of autologous CD4⁺CD25⁺ T cells. However, after treatment with UDP, T cell proliferation was markedly decreased ($p < 0.001$), accompanied

by a decrease in supernatant IFN- α , indicating that the suppressive activity of CD4⁺CD25⁺ Treg cells was restored through UDP on pDCs. To further confirm that this effect was mediated by the P2Y6 receptor, suramin, an inhibitor of the P2Y6 receptor, was used to block P2Y6 receptor signaling. As expected, we found that T cell proliferation was restored significantly ($p < 0.01$) along with increased IFN- α secretion, but not TNF- α or IL-12, indicating that suramin may abrogate the effect of UDP on P2Y6 receptor signaling. In addition, it was observed that the effect of UDP or suramin on uGD-derived DC-induced apoptosis of CD4⁺CD25⁺ Treg cells had a similar corresponding trend as T cell proliferation ($p < 0.01$, Fig. 6B). Thus, these results collectively demonstrate that UDP, which downregulates the function of uGD-derived DCs through regulation of P2Y6 receptor signaling, restores the suppressive function of CD4⁺CD25⁺ Treg cells and inhibits DC-mediated apoptosis of CD4⁺CD25⁺ Treg cells.

Discussion

The mechanisms by which Treg cells lose suppressive activity in autoimmune diseases have attracted increasing attention. Treg cells are recognized as an important player in a variety of immunoregulatory processes, and defective activity of Treg cells typically contributes to the development of autoimmune diseases. However, the proportion of Treg cells in peripheral blood has been previously reported to be increased, decreased, or unchanged in several autoimmune disorders, such as autoimmune thyroid diseases (37), multiple sclerosis (12), and systemic lupus erythematosus (38). Our results showed that the proportion of CD4⁺CD25⁺ Treg cells was reduced in the peripheral blood of primary uGD, which is consistent with the conclusion from Nakano et al. (39), but in contrast to the reports from Pan et al. (40) and Wang et al. (41). One possible cause for this discrepancy is related to selected marker differences for the detection of Treg cells. For example, Pan et al. (40) selected CD4⁺CD25^{int-hi}CD127^{lo} as Treg cell markers, whereas Wang et al. (41) selected CD4⁺CD25⁺, different from the CD4⁺CD25⁺FOXP3⁺ markers for our study. In addition, our functional assays demonstrated that CD4⁺CD25⁺ Treg cells from uGD still exhibited suppressive function, suggesting that these Treg cells are not intrinsically defective in function. These findings are in agreement with some (32, 38, 42, 43) but not with others that examine functionally defective Treg cells themselves (37, 44, 45). In contrast, it is possible that besides the pathological differences in disease types, the disagreement among studies may be associated with differences in race, disease status, clinical course, treatment, and site of specimen collection as well as use of different Treg cell markers. These differences impact the assessment of Treg cells in number and function. For example, the purity of isolated CD4⁺CD25⁺ Treg cells is a critical factor for assessment of the function of Treg cells, though there is so far a lack of specific markers for Treg cells. CD25 is one of the characteristic markers of CD4⁺CD25⁺ Treg cells; it is an activation marker and also expresses on the surface of autoreactive CD4⁺ T cells at a low level in autoimmune diseases. As a result, the coisolated CD4⁺CD25⁺ Treg and CD4⁺CD25^{lo} effector T cells could affect the assessment of the suppressive function of Treg cells.

A question raised from these findings is what attenuated the regulatory capacity of Treg cells in the pathogenesis of GD. Environmental, endocrine, and genetic factors play etiological roles in the development of GD (46). It has been well established that DCs in the periphery capture and process Ags, express lymphocyte costimulatory molecules, migrate to lymphoid organs, and secrete cytokines to initiate adaptive immune responses, including autoimmunity (47). Our data showed that uGD-derived DCs abrogate the regulatory function of CD4⁺CD25⁺ Treg cells through pDC

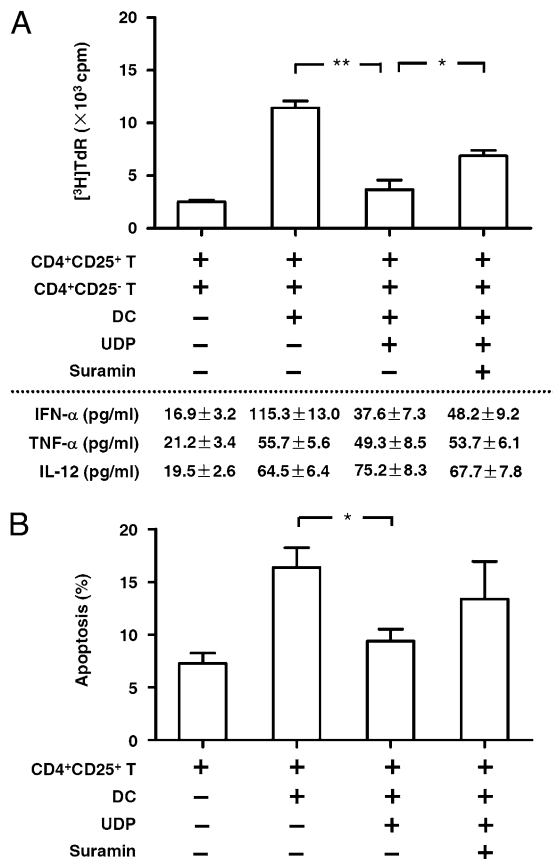


FIGURE 6. The nucleotide UDP restores the suppressive function of CD4⁺CD25⁺ Treg cells. *A*, Isolated CD4⁺CD25⁺ T cells (5×10^4 cells/well) and CD4⁺CD25⁻ T cells (5×10^4 cells/well) from uGD were cocultured in 96-well V-bottom plates in the presence or absence of autologous DCs (2×10^4 cells/well), UDP (1 μ M), and/or suramin (0.1 μ M). In the DC-free assay, anti-CD3 mAb (1 μ g/ml) and anti-CD28 mAb (1 μ g/ml) were added to the culture. In the DC-based assay, anti-CD3 mAb (1 μ g/ml) was added to the culture. After 3 d of assay, 100 μ l supernatant was removed from each well and stored for IFN- α , TNF- α , and IL-12 measurement, and 100 μ l fresh medium containing 1 μ Ci [³H]TdR was added for the last 12–16 h of culture. Results are expressed as means \pm SD in cpm of triplicate wells. The data are representative of three independent experiments. *B*, uGD-derived DCs were isolated and prelabeled with 2.5 μ M CFSE. Isolated CD4⁺CD25⁺ T cells (1×10^5 cells/well) from uGD were cultured in 96-well V-bottom plates in the presence or absence of autologous DCs (2×10^4 cells/well), UDP (1 μ M), and/or suramin (0.1 μ M). In the DC-free assay, anti-CD3 mAb (1 μ g/ml) and anti-CD28 mAb (1 μ g/ml) were added to the culture. In the DC-based assay, anti-CD3 mAb (1 μ g/ml) was added to the culture. After 3 d of culture, the cells were collected and stained using the Apoptosis Detection Kit. Gating for CFSE-negative population, the apoptotic cells with 7-AAD-negative and Annexin V-PE-positive staining, or both Annexin V-PE and 7-AAD-positive staining were analyzed by FCM. The data are expressed as means \pm SD of three independent experiments. * $p < 0.01$, ** $p < 0.001$.

polarization and IFN- α production. The result provides evidence that changes in the subpopulation and/or function of DCs can regulate the activity of Treg cells, and pDC polarization in the GD patients may be associated with immune system dysfunction and GD development. In addition, it is reported that cross talk between pDCs and mDCs through IFN- α leads to the maturation of mDCs, which subsequently induces a strong autoimmune response (48, 49). Hence, more studies are necessary to further clarify the effect of DC subsets on Treg cells in the context of autoimmune disorders.

Under steady-state conditions, it has been reported in recent studies that the immune system maintains immune homeostasis through a feedback regulatory loop between Treg cells and DCs in vivo, in which the number of DCs plays a critical role (30, 31). However, under pathological conditions, it is unclear whether DCs break the regulatory loop to initiate autoimmunity. In the current study, we demonstrated that the increased pDCs from uGD induced the apoptosis of Treg cells, suggesting their possible role in the breakdown of Treg-mediated regulatory loop. In conclusion, in the development of GD, DC migration and division in lymphoid organs may lead to pDC polarization after encountering the autoantigen TSHR. The polarization of pDCs and secretion of IFN- α impair Treg cell-mediated regulatory effect in lymphoid organs and consequently triggers the activation and interaction of autoreactive Th cells and B cells, resulting in the generation of large amounts of autoantibodies to TSHR and the development of GD (Fig. 7).

Notably, evidence indicates functional cross talk between immune and endocrine systems in the modulation of innate and adaptive immunity, such as the immunosuppressive effects of thyroxine on T and B cells in C57BL/6 mice (50), suppression of peripheral blood NK cell activity by excess thyroid hormone (51),

modulation of differential homing of CD4⁺ T cells and CD8⁺ T cells to peripheral lymphoid organs (52), and control of maturation and function of DCs by triiodothyronine (53, 54). Because the polarization and effect of uGD-derived DCs are found in uGD patients with elevated THs, it is necessary to determine the effect of elevated THs on DCs and Treg cells. Our study revealed that T3 could not directly increase apoptosis of CD4⁺CD25⁺ Treg cells, which is different from uGD-derived DCs. However, it could largely promote the apoptosis of CD4⁺CD25⁺ Treg cells induced by uGD-derived DCs, suggesting that it is favorable for the generation of TSHR autoantibodies (Fig. 7). These findings are in agreement with previous published observations indicating that THs exert an effect on the immunological function of DCs (53, 54). Our results further provide experimental evidence that the interaction between the endocrine and immune systems occurs under GD pathological conditions.

It is interesting to note the relation between polarized DCs and elevated THs in the pathogenesis of GD. In general, in the pathogenesis of GD, production of TSHR stimulatory autoantibodies is a precondition that leads to the production of elevated THs. Thus, Graves' hyperthyroidism is a consequence of GD development. Decreased numbers of Treg cells and immune dysfunction are beneficial to the generation of TSHR autoantibodies. As polarized uGD-derived DCs directly induce the apoptotic effect of Treg cells and THs promote the effect, DC polarization may be an initial factor compared with THs in the pathogenesis of GD. The hypothesis also partially explains why in patients with toxic nodular goiter, hyperthyroidism arises in the absence of thyroid autoimmunity, due to lack of polarized pDCs in these patients.

Recent studies suggest that the apoptosis-induced decrease in Treg cells is an important mechanism that leads to the progressive breakdown of self-tolerance and the development of some autoimmune diseases (39, 55). Our data demonstrated that uGD-derived pDCs and elevated THs conspire to induce apoptosis of CD4⁺CD25⁺ Treg cells, suggesting that the apoptotic mechanism may be one of the main causes for the decrease of CD4⁺CD25⁺ Treg cells in GD patients. Our results provide evidence that the apoptosis of Treg cells was associated with IFN- α from uGD-derived pDCs, but not Fas-FasL signaling pathway (data not shown); thus, the molecular mechanisms of pDC-induced apoptosis need to be further defined.

The capacity of pDCs to induce the loss of CD4⁺CD25⁺ Treg cell activity suggests that depletion or downregulation of pDCs could be favorable for controlling the development of autoimmune disease. An alternative strategy would be to manipulate TLR signaling in pDCs, for example, with hydroxychloroquine treatment, which may inhibit pDC function and production of IFN- α (56), much like treatment with anti-BDCA-2/4 mAb and corticosteroids (57, 58). A more recent report suggests that nucleotide UDP and UDP-glucose strongly suppress IFN- α production and function of human pDCs through P2Y6 receptor signaling (35), indicating that UDP administration may serve as a potential approach to inhibit IFN- α production and the function of pDCs in GD patients. It is possible that the application of UDP for regulating pDC function is more desirable compared with other methods because it is a common component of glycometabolism in the body. In the current study, we demonstrated that UDP inhibited pDC-induced apoptosis of CD4⁺CD25⁺ Treg cells, which may provide a target for modulating of the suppressive capacity of Treg cells in GD patients.

In summary, uGD-derived pDCs and elevated THs impair the activity of CD4⁺CD25⁺ Treg cells in GD patients. Manipulating pDCs by P2Y6 receptor agonists protects CD4⁺CD25⁺ Treg cells in GD patients. Accordingly, these findings have important impli-

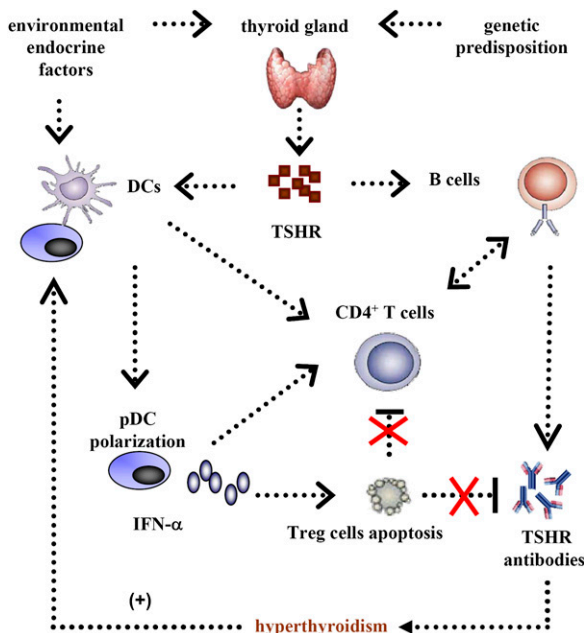


FIGURE 7. A hypothetical diagram of the production of TSHR Abs in GD patients. Triggered by multiple pathological factors, DC polarization leads to an increase in the number of pDC and IFN- α secretion, which plays a central role in the induction of apoptosis of CD4⁺CD25⁺ Treg cells in GD patients. Consequently, the loss of CD4⁺CD25⁺ Treg cells enhances the activities of autoreactive CD4⁺ T and B cells, which contributes to the production of TSHR autoantibodies and hyperthyroidism. Elevated THs further aggravate the effect of DC-induced apoptosis of Treg cells and promote the production of TSHR autoantibodies.

cations in the understanding of immunopathogenesis of GD and in the therapy potential for GD patients.

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Disclosures

The authors have no financial conflicts of interest.

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