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Tolerogenic Semimature Dendritic Cells Suppress Experimental Autoimmune Thyroiditis by Activation of Thyroglobulin-Specific CD4+CD25+ T Cells[1]

Panayotis Verginis, Haiyan S. Li, and George Carayanniotis[2]

Ex vivo treatment of bone marrow-derived dendritic cells (DCs) with TNF-α has been previously shown to induce partial maturation of DCs that are able to suppress autoimmunity. In this study, we demonstrate that i.v. administration of TNF-α-treated, semimature DCs pulsed with thyrogloblin (Tg), but not with OVA Ag, inhibits the subsequent development of Tg-induced experimental autoimmune thyroiditis (EAT) in CBA/J mice. This protocol activates CD4+CD25+ T cells in vivo, which secrete IL-10 upon specific recognition of Tg in vitro and express regulatory T cell (Treg)-associated markers such as glucocorticoid-induced TNFR, CTLA-4, and Foxp3. These CD4+CD25+ Treg cells suppressed the proliferation and cytokine release of Tg-specific, CD4+CD25− effector cells in vitro, in an IL-10-independent, cell contact-dependent manner. Prior adoptive transfer of the same CD4+CD25+ Treg cells into CBA/J hosts suppressed Tg-induced EAT. These results demonstrate that the tolerogenic potential of Tg-pulsed, semimature DCs in EAT is likely to be mediated through the selective activation of Tg-specific CD4+CD25+ Treg cells and provide new insights for the study of Ag-specific immunoregulation of autoimmune diseases. *The Journal of Immunology,* 2005, 174: 7433–7439.

Apart from being highly immunogenic (1), dendritic cells (DC)[3] have been shown to play an important role in peripheral tolerance via various mechanisms, including activation of T regulatory (Treg) cells, induction of T cell anergy, and Th1/Th2 polarization (2, 3). Although the exact mechanisms involved in the decision of a DC to become immunogenic or tolerogenic have not been elucidated, increasing evidence suggests that DC function is dependent on their maturation stage (4, 5). Although immature DCs have been implicated for anergy induction, fully mature DCs are efficient activators of naive T cells (6). An intermediate stage of DC maturation was recently described, in which DCs express high levels of MHC class II and costimulatory molecules, but do not secrete proinflammatory cytokines (7). This stage of maturation was achieved upon exposure of immature DCs to TNF-α ex vivo, and the cells were termed semimature DCs. These DCs were shown to induce tolerance through the generation of IL-10-secreting Treg cells whose profile was not characterized (7).

CD4+CD25+ Treg cells arise normally in naive mice and constitute ~10% of the peripheral CD4+ T cells. Treg cells are generated in the thymus upon high avidity interactions with self peptides (8–10) and they participate in the maintenance of peripheral self-tolerance (11, 12). Their development has been shown to be programmed by the transcription factor Foxp3 (13–15) and, in addition to IL-2R α-chain (CD25), they constitutively express CTLA-4 (16) and glucocorticoid-induced TNFR (GITR) (17, 18) on their surface. Although accumulating evidence suggests a major role of this subset in the maintenance of self-tolerance (12), the Ag specificity and the exact mechanism(s) of action of the CD4+CD25+ T cells remain unresolved. Meanwhile, ample evidence indicates that DCs play an important role in expansion/induction of Treg cells (2) that could be specific for self Ags (19). However, a clear profile of DC involved in this process has yet to be identified.

Experimental autoimmune thyroiditis (EAT), a murine model of Hashimoto's thyroiditis in humans, can be induced upon challenge of susceptible animals with thyroglobulin (Tg) in CFA (20). The disease is mediated by CD4+ T cells and is characterized by lymphocytic infiltration of the thyroid gland (21). Several reports have suggested a major role of the CD4+CD25+ T cells in immunoregulation of EAT. First, elimination of Treg cells in mice resulted in development of multiorgan autoimmune diseases, including thyroiditis, whereas reconstitution of CD4+CD25+ T cells inhibited development of autoimmunity (22). Second, it was recently reported that CD4+CD25+ T cells, isolated from GM-CSF-treated mice, were able to suppress Tg-specific T cell responses in vitro (23). The authors suggested differential activation of DCs by GM-CSF that induces Treg cells. In addition, Kong and colleagues (24) showed that CD4+CD25+ T cells from Tg-tolerized mice can suppress mouse Tg-specific responses in vitro.

In this study, we sought direct evidence for the induction of CD4+CD25+ Treg cells, with the ability to suppress Tg-induced EAT. To investigate this, TNF-α-treated DCs isolated from CBA/J mice were pulsed with Tg and transferred into syngeneic mice. Splenic CD4+CD25+ T cells from these recipients were then tested for their ability to suppress Tg-specific effector T cell responses in vitro as well as development of EAT.

Materials and Methods

Animals and Ags

Female CBA/J (H-2^k) mice were purchased from The Jackson Laboratory and were used in experiments at 6–8 wk of age. All experimental procedures were reviewed and approved by the Animal Care Committee at Memorial University of Newfoundland. Tg was extracted from thyroid glands

of outbred ICR mice (Harlan Bioproducts for Science), as previously described (25). OVA was purchased from Sigma-Aldrich. PE-labeled hamster anti-CD11c (clone HL3), FITC-labeled rat mAbs specific for I-A^k (clone 10-3.6), CD86 (clone GL1), CD40 (clone 3/23), CD137 (clone 1AH2), CD62L (clone MEL-14) or FITC-labeled hamster mAbs specific for CD69 (clone H1.2F3), or CD80 (clone 16-10A1), and appropriately labeled isotype-matched control mAbs were purchased from BD Pharmingen. FITC-labeled rat anti-GITR (clone 108619) and isotype control were purchased from R&D Systems. Purified hamster anti-mouse CD152 (clone 9H10), FITC-labeled goat anti-hamster, and isotype-matched control mAbs were purchased from BD Pharmingen.

Generation of bone marrow-derived DCs (BM-DCs)

Dendritic cells were generated from bone marrow (BM) progenitors, as described by Lutz et al. (26). Briefly, BM was prepared from femurs and tibias of CBA mice, and RBC were lysed with NH$_4$Cl. On day 0, BM cells were seeded at 2×10^6 per 100-mm dish (Corning Glass) in 10 ml of RPMI 1640 medium supplemented with 10% FBS (Cansera), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen Life Technologies), and 5×10^{-5} M 2-ME (Sigma-Aldrich). The culture medium was supplemented with 10% supernatant from a cell line (X63Ag8; kindly provided by B. Stockinger, National Institute of Medical Research, London, U.K.) transfected with the murine GM-CSF gene (27). On days 3, 6, and 8, cultures were supplemented with fresh medium containing 10% XG6 supernatant. After 9 days of culture, semimature DCs or mature DCs were generated by the addition of 40 ng/ml TNF-α (PeproTech) or 1 μg/ml LPS (Sigma-Aldrich), respectively, and 24 h later, nonadherent cells were harvested by gentle dislodging for further study. The generation of DCs was assessed based on CD11c expression by flow cytometry. The fluorescence of 10^4 cells was analyzed by a FACStar^{Plus} flow cytometer and CellQuest software (BD Biosciences).

RT-PCR for detection of Foxp3 expression

Total RNA was extracted from 2×10^5 purified CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ T cells and was reverse transcribed to cDNA, as previously described (28). The primer sequences used for RT-PCR amplification of Foxp3 were: 5′-CAG CTG CCT ACA GTG CCC CTA G-3′ (sense) and 5′-CAT TTG CCA GCA GTG GGT AG-3′ (antisense), as described by Hori et al. (14). The GADPH gene was amplified using the following primers: 5′-CCC ATC ACC ATC TTC CAG GAG-3′ (sense) and 5′-CCT GCT TCA CCA CCT TCT TG-3′ (antisense). All primers were synthesized by Qiagen. RT-PCR was performed, as previously described (28). RT-PCR products were visualized by agarose gel electrophoresis, and the relative expression of Foxp3 gene to that of GADPH was assessed by densitometry.

Isolation of CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells

On day 9 of DC culture, cells were pulsed with Tg (100 μg/ml) or OVA (100 μg/ml) for 6 h and were subsequently treated with TNF-α (40 ng/ml) for 24 h. TNF-α-treated DCs were then washed (three times) with PBS, adjusted at 2.5×10^7 cells/ml, and i.v. injected into CBA/J mice (100 μl/mouse) on days 1, 3, and 5. Twenty-one days later, splenic cell suspensions were prepared and CD4$^+$CD25$^+$ T cells were purified using a T cell isolation kit (Miltenyi Biotec), based on magnetic cell sorting. The flow through contained >90% of CD4$^+$CD25$^-$ T cells, whereas the column-retained and subsequently eluted cells were >80% CD4$^+$CD25$^+$ T cells by flow cytometric analysis.

T cell proliferation assays

CBA mice were immunized s.c. with 100 μg of Tg in CFA (with *Mycobacterium butyricum*; Difco Laboratories), and 9 days later, CD4$^+$CD25$^-$ T cells (effector cells) were purified from draining lymph nodes, as described above. The CD25$^-$ effector cells were cultured (1×10^5 cells/well) in the presence or absence of Tg (100 μg/well) in flat-bottom 96-well plates for 4 days at 37°C in a 10% CO$_2$, 90% air-humidified incubator. Syngeneic splenocytes (2×10^5 cells/well), treated for 15 min with 50 μg/ml mitomycin C (Sigma-Aldrich), were used as APC. Mixing experiments were performed by the addition of equal numbers (1×10^5 cells/well) of CD4$^+$CD25$^+$ T cells, isolated from DC-challenged CBA mice, and CD4$^+$CD25$^-$ effector T cells. Eighteen hours before harvesting, 1 μCi of [^{3}H]thymidine (6.7 Ci/mol; DuPont Pharmaceuticals) was added to each well in 25 μl of complete medium. Cell harvesting and radioactivity measurements were performed, as previously described (25). Stimulation index is defined as: (cpm in the presence of Ag)/(cpm in the absence of Ag). In experiments performed in 24-well transwell plates (0.22 μm pore size; Costar), 6×10^5 CD4$^+$CD25$^-$ effector T cells were cultured in the bottom chamber with 1.2×10^6 APC, 100 μg/ml Tg, and 6×10^5 CD4$^+$CD25$^+$ T cells. When CD4$^+$CD25$^+$ T cells were cultured in the top chamber, APC and Tg were similarly added, as above. After 3 days of culture, the transwell chamber was removed, and 1 μCi of [^{3}H]thymidine was added to the lower well. Cells were harvested 18 h later, and incorporated thymidine was measured, as described above.

Detection of cytokines by ELISA

Detection of IL-2, IL-4, IL-10, IL-12, IL-1β, IL-6, and IFN-γ in culture supernatants harvested at the indicated time was performed by sandwich ELISA using the BD OptEIA mouse ELISA set (BD Biosciences) following the manufacturer's recommendations. TGF-β1 was measured by the TGF-β Emax Immuno Assay System (Promega). Light absorbance at 450 nm was measured using a Vmax plate reader (Molecular Devices).

EAT induction

EAT was induced in CBA mice upon challenge with Tg (100 μg/mouse) in CFA (with *M. butyricum*; Difco Laboratories). Thyroid glands were removed 18–21 days after Tg challenge to be assessed for thyroid pathology. Fixation, embedding, and sectioning of thyroids were performed, as previously described (25). Histological sections were stained with H&E, and the mononuclear cell infiltration index (I.I.) was scored as follows: 0 = no infiltration; 1 = interstitial accumulation of cells between two or three follicles; 2 = one or two foci of cells at least the size of one follicle; 3 = extensive infiltration 10–40% of total area; 4 = extensive infiltration 40–80% of total area; and 5 = extensive infiltration >80% of total area.

Suppression of EAT

CBA/J mice received three i.v. injections (days 1, 3, and 5) of 2.5×10^6 TNF-α-treated DCs pulsed with 100 μg of Tg or OVA. Control mice received three i.v. injections of PBS. Two days after the last DC injection, some mice were immunized with 100 μg of Tg in CFA, and 21 days later, all mice were sacrificed and their thyroids were collected to assess EAT severity. For the adoptive transfer assays, 5×10^5 splenic CD4$^+$CD25$^+$ T cells isolated from CBA/J primed with Tg- or OVA-pulsed DCs as above were administered i.p. into syngeneic naive recipients. Control mice received one i.p. injection of PBS. One day later, some mice were immunized s.c. with 100 μg of Tg in CFA, and 21 days after Tg challenge, all mice were sacrificed for assessment of thyroiditis.

Results

Generation of semimature DCs

To generate semimature DCs, we treated BM-DCs with TNF-α for 24 h. Untreated and LPS-treated DCs were used as immature and completely mature DCs, respectively. The surface marker phenotype of the DC populations was assessed by flow cytometry. Both TNF-α-treated and LPS-treated DCs expressed higher levels of cell surface MHC class II, CD80, CD86, and CD40 molecules, as compared with untreated DCs (Fig. 1A). It has been shown previously that TNF-α-treated DCs express low levels of proinflammatory cytokines at the mRNA level and do not secrete significant amounts of IL-12 (7). To confirm this finding, we performed sandwich ELISA in DC culture supernatants collected upon 24-h TNF-α treatment. Low levels of IL-12, IL-1β, and IL-6 were secreted by TNF-α-treated DC, similarly to those secreted by immature DCs. In contrast, all cytokines tested were highly produced by LPS-treated DCs (Fig. 1B). Collectively, these results confirmed the generation of semimature DCs upon TNF-α treatment.

Induction of Tg-specific, IL-10-secreting CD4$^+$CD25$^+$ T cells by Tg-pulsed semimature DCs

To assess a possible tolerogenic role of semimature DCs in EAT, DCs were pulsed with Tg or OVA for 6 h and were subsequently treated with TNF-α for additional 24 h. CBA/J mice received three i.v. injections of DCs (2.5×10^6 cells/mouse) on days 1, 3, and 5 and were sacrificed 21 days after the last challenge. Splenocyte-derived CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were cultured with Tg or OVA and mitomycin C-treated syngeneic splenic APCs, as described in *Materials and Methods*. Both T cell subpopulations did not proliferate in the presence of Ag (data not
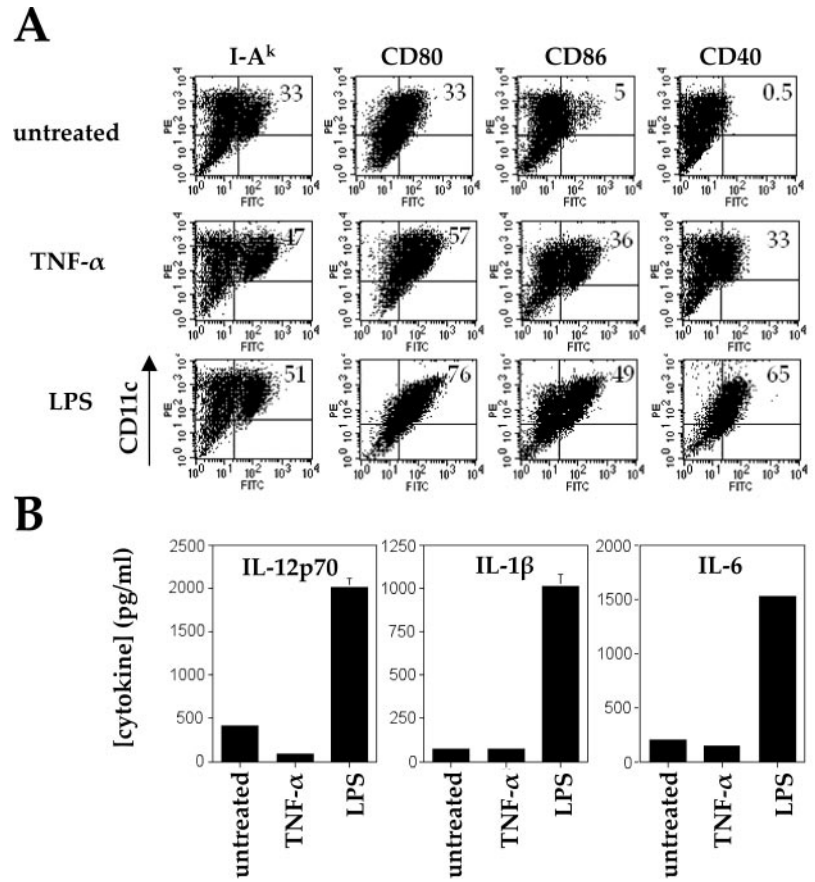
FIGURE 1. Cell surface markers and cytokine secretion profile of semimature DCs induced by TNF-α. DCs were derived from bone marrow cells of CBA mice cultured in the presence of GM-CSF. On day 9, cells were incubated with TNF-α (40 ng/ml) and LPS (1 μg/ml), or remained untreated for 24 h. *A*, FACS analysis of DCs stained with CD11c PE mAb, and FITC-conjugated Abs to MHC class II, CD80, CD86, and CD40. *B*, Supernatants of the DC cultures were collected 24 h after stimulation and tested by cytokine ELISA for the presence of IL-12, IL-1β, and IL-6. The results are representative of two independent experiments for *A* and *B*.

shown) and they did not produce detectable amounts of IL-2 or IFN-γ in culture supernatants (Table I). However, CD4$^+$CD25$^+$ T cells, isolated from mice challenged with Tg-pulsed DCs (Tg/DC), secreted significant amounts of IL-10 in response to Tg stimulation in vitro, and this effect was Tg-specific because it was not detected in response to OVA. Interestingly, OVA-pulsed DCs (OVA/DC) were not able to induce OVA-specific CD4$^+$CD25$^+$ T cells with the ability to produce IL-10 (Table I). Additionally, no IL-4 was detected in supernatants of any of the above cell cultures (data not shown), indicating the activation of T cell subpopulation(s) distinct from Th2. These results demonstrate that Tg/DCs have the ability to induce CD4$^+$CD25$^+$ T cells that produce IL-10 in response to Tg in vitro.

Expression of a Treg phenotype by CD4$^+$CD25$^+$ T cells

To examine whether the CD4$^+$CD25$^+$ T cells obtained from mice challenged with Tg/DC express surface markers associated with Treg, we assessed their phenotype by FACS. The CD4$^+$CD25$^+$ T cells were CD62L^{high} and CD69^{low}, indicating that the cells were not in an activated stage and their expression of GITR, CTLA-4, and CD137 molecules was higher as compared with that of the CD25$^-$ T cell subpopulation (Fig. 2*A*). Similarly, Foxp3 expression on CD25$^+$ T cells was 5-fold higher than that in the CD25$^-$ population at the transcriptional level (Fig. 2*B*). These data indicated that the CD4$^+$CD25$^+$ T cells isolated from Tg/DC-treated

Table I. *Induction of IL-10-secreting CD4$^+$CD25$^+$ T cells following challenge of mice with Tg-pulsed semimature DCs^a*

In Vivo Treatment	In Vitro Activation		Cytokine (pg/ml)		
			IL-2	IFN-γ	IL-10
DC/Tg	CD4$^+$CD25$^-$	Tg	31 ± 0.3	105 ± 5.3	39 ± 0.4
	CD4$^+$CD25$^+$	Tg	33 ± 0.1	81 ± 2.1	461 ± 0.4
	CD4$^+$CD25$^-$	OVA	31 ± 0.1	96 ± 2.1	28 ± 0.7
	CD4$^+$CD25$^+$	OVA	34 ± 0.2	97 ± 7.2	27 ± 4.3
DC/OVA	CD4$^+$CD25$^-$	Tg	31 ± 0.2	109 ± 8.4	36 ± 1.8
	CD4$^+$CD25$^+$	Tg	34 ± 0.2	119 ± 14.6	28 ± 1.7
	CD4$^+$CD25$^-$	OVA	32 ± 0.3	132 ± 20.6	32 ± 0.2
	CD4$^+$CD25$^+$	OVA	37 ± 0.4	77 ± 18.2	35 ± 0.8

^{a} TNF-α-treated DCs pulsed either with Tg or OVA were i.v. injected three times (days 1, 3, and 5) into CBA/J mice, as described in *Materials and Methods*. Twenty-one days later, spleen-derived CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells (1 × 10^5 cells/well/200 μl) were stimulated with Tg or OVA in the presence of mitomycin C-treated, syngeneic splenocytes (2 × 10^6 cells/well/200 μl) as APC. Culture supernatants were collected after 48 h and assayed for the presence of IL-2, IL-10, and IFN-γ by ELISA. Results are representative of two independent experiments.
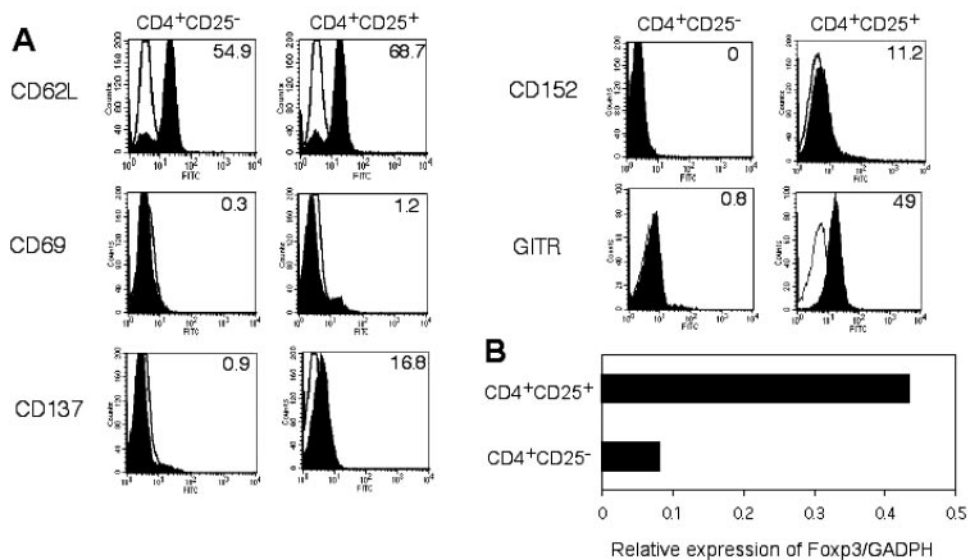
FIGURE 2. CD4$^+$CD25$^+$ T cells from Tg/DC-primed mice express the characteristic markers of Treg cells. CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ splenic T cells were isolated from CBA mice that received Tg-pulsed TNF-α-treated DCs, as described in *Materials and Methods*. *A*, Cells were stained and analyzed for cell surface expression of CD62L, CD69, CD137, CD152, and GITR (filled histograms). Results obtained with isotype-matched control Abs are indicated with open histograms. *B*, RNA was extracted from 5×10^5 cells and reverse transcribed to cDNA. The Foxp3 as well as the constitutively expressed GADPH gene were amplified by RT-PCR, using gene-specific primers.

mice possess a phenotype similar to that described for Treg cells (17).

CD4$^+$CD25$^+$ T cells from Tg/DC-challenged mice suppress Tg-specific T cell responses in vitro

The functional characteristics of the CD25$^+$ T cell subpopulation were subsequently assessed in mixing experiments. CD25$^-$ T cells isolated from the draining lymph nodes of Tg/CFA-immunized CBA mice were used as effector cells. The CD25$^-$ cells proliferated strongly in the presence of Tg and APC and secreted high levels of IL-2 and IFN-γ (Fig. 3). The response was Tg-specific because proliferation or cytokine release was not detected upon culture of CD25$^-$ cells with OVA (data not shown). CD4$^+$CD25$^+$ T cells isolated from mice that received Tg/DC, but not OVA/DC, were able to completely suppress the proliferation and cytokine production of the CD25$^-$ effector cells (Fig. 3). CD4$^+$CD25$^+$ T cells isolated from naive mice were also unable to suppress the Tg-specific response in vitro. None of the CD4$^+$CD25$^+$ T cell populations proliferated in response to Tg, and only CD25$^+$ T cells from Tg/DC-treated mice secreted IL-10 upon culture with Tg and APC, indicating efficient activation of this subset (Fig. 3). These

data highlighted the ability of Tg/DCs to induce a CD4$^+$CD25$^+$ T cell population that suppresses Tg-specific T cell responses.

CD4$^+$CD25$^+$ T cells mediate suppression in a cell-cell contact-dependent manner

To investigate the mechanism by which CD25$^+$ T cells mediate suppression, Tg-specific CD4$^+$CD25$^-$ effector cells were stimulated with Tg and APC in a 24-well transwell plate. Tg-specific CD25$^+$ T cells, isolated as above, were placed in the same well or in the upper chamber at a 1:1 ratio, in the presence (or absence) of Tg. Inhibition of proliferation was observed only when effector cells and CD25$^+$ cells were cocultured in the same well (Fig. 4), and this inhibition correlated well with the suppression of IL-2 and IFN-γ secretion by the effector cells. As expected, significant amounts of IL-10 were detected only in wells in which CD25$^+$ T cells were cultured in the presence of Tg and APC. These data also suggested that while contact between effector cells and CD25$^+$ cells is required to mediate suppression, it is not needed for the production of IL-10 by Tregs. Altogether, the results supported the view that CD25$^+$ T cells suppress the activation of Tg-specific
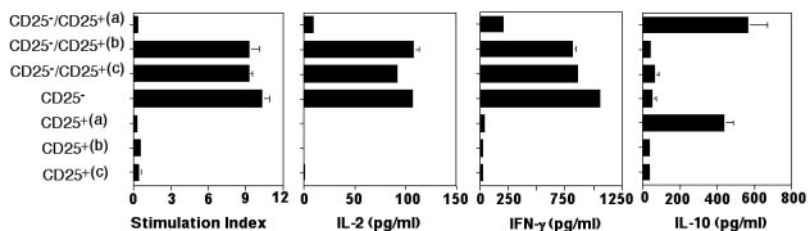

FIGURE 3. Suppression of proliferation of Tg-specific T cells by CD4$^+$CD25$^+$ T cells in vitro. CBA/J mice were immunized s.c. with 100 μg of Tg in CFA, and 9 days later, CD4$^+$CD25$^-$ T cells were isolated and used as effector cells. CD4$^+$CD25$^+$ T cells (Treg) were isolated from spleens of syngeneic mice that had been challenged with *a*, Tg-pulsed- or *b*, OVA-pulsed, TNF-α-treated DCs, or *c*, isolated from spleens of naive CBA/J mice. Effector cells (1×10^5 cells/well) were cultured with equal numbers of Treg cells, in the presence of Tg. Mitomycin C-treated syngeneic splenic cells (2×10^5 cells/well) were used as APC. The stimulation index values (cpm in the presence of Ag/cpm in the absence of Ag) are means of triplicate wells. Secretion of IL-2, IFN-γ, and IL-10 was measured by cytokine ELISA in supernatants collected after 48 h of culture. Data are representative of two independent experiments.
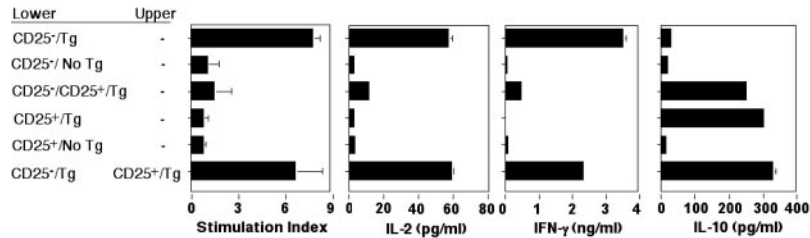
FIGURE 4. Cell-cell contact is required for the suppressive activity of the Tg-specific Treg cells. CD25⁻ effector T cells (6×10^5 cells) were stimulated in the bottom chamber of 24-well transwell plates, in the presence of 100 μg of Tg and 1.2×10^6 splenic APC. A total of 6×10^5 CD25⁺ Treg cells was placed either in the bottom chamber or in the top chamber along with Tg and APC. Proliferation was assessed after 4 days of culture. Supernatants were collected after 48 h of stimulation, and cytokine ELISA was performed to measure the content of IL-2, IFN-γ, and IL-10 in the culture. Data are representative of two independent experiments.

CD25⁻ effector cells via a cell-cell contact-dependent, cytokine-independent mechanism.

Tg/DCs can suppress EAT induction through the generation of CD4⁺CD25⁺ T cells

To assess the tolerogenic potential of Tg/DC in Tg-induced EAT, we challenged CBA mice on days 1, 3, and 5 with Tg/DC, OVA/DCs, or PBS, and 2 days after the last DC challenge, we immunized them with Tg in CFA. Twenty-one days later, the thyroids were removed and examined for mononuclear cell infiltration. As expected, mice that received PBS and were subsequently challenged with Tg showed the highest degree of EAT (I.I. = 2.2) (Fig. 5), whereas mice that received OVA/DCs had a slight decrease of EAT incidence (I.I. = 1.8) that was not statistically significant ($p = 0.613$). In contrast, disease was significantly suppressed (I.I. = 0.5, $p = 0.006$) in mice that received Tg/DCs before Tg challenge. We did not detect any thyroid pathology in mice challenged with Tg/DC or OVA/DCs alone, indicating that the semimature DCs themselves did not contribute to the development of EAT. Based on these observations, we formulated the hypothesis that Tg/DCs, but not OVA/DCs, are able to expand CD4⁺CD25⁺ T cells with the ability to suppress Tg-induced EAT. To directly address this, we adoptively transferred CD25⁺ T cells into naive mice, and 1 day later, we challenged the mice with Tg in adjuvant. Mononuclear cell infiltration of the thyroid was assessed 21 days later, as described in *Materials and Methods*. Mice that received

FIGURE 5. Suppression of Tg-induced EAT by Tg-pulsed semimature DCs. CBA/J mice received three i.v. injections (days 1, 3, and 5) of 2.5×10^6 TNF-α-treated DCs pulsed with 100 μg of Tg or OVA. Control mice received three i.v. injections of PBS. Two days after the last DC injection, some mice were immunized with 100 μg of Tg in CFA. On day 21, all mice were sacrificed and thyroid glands were collected. The mononuclear cell I.I. was scored, as described in *Materials and Methods*. Statistical analysis was performed using the Mann-Whitney nonparametric test.

CD25⁺ cells from Tg/DC-treated donors showed a significant decrease of EAT (I.I. = 0.89, $p = 0.03$) (Table II), as compared with the control group (I.I. = 2.5). In contrast, EAT in mice that received CD25⁺ T cells from OVA/DC-challenged mice was not significantly reduced (I.I. = 2.13, $p = 0.50$), as compared with that of control mice immunized with Tg/CFA alone. Collectively, these data demonstrated the suppressogenic potential of CD4⁺CD25⁺ T cells derived from mice that have been challenged with Tg/DCs, but not OVA/DCs.

Discussion

The present study demonstrates that Tg-pulsed semimature DCs can induce Tg-specific CD4⁺CD25⁺ T cells with the ability to inhibit EAT development. Our data confirm earlier findings in the EAE model that TNF-α-treated DCs, expressing a semimature phenotype, mediate Ag-specific protection against autoimmune disease (7), and extend these observations by highlighting that this protection is likely to be mediated by autoantigen-specific CD4⁺CD25⁺ Treg cells. Tg/DC, but not OVA/DC, may activate and/or expand pre-existing, naturally occurring, Tg-specific CD25⁺ T cells that have been positively selected in the thymus. This hypothesis is in agreement with recent data demonstrating that thymic expression of a self Ag facilitates the development of high numbers of Ag-specific Treg cells (9, 29) and that Tg is known to be expressed intrathymically (30).

Although the critical factors that determine the tolerogenic potential of TNF-α-treated DCs cells remain unknown, the absence of proinflammatory cytokine secretion by this subset has been suggested to contribute to tolerogenicity (4). This hypothesis is supported by our findings because the TNF-α-treated DCs were found to secrete low levels of IL-12, IL-6, or IL-1β. Unlike the immature DC, semimature DCs also express relatively high levels of co-stimulatory molecules such as CD80, CD86, and CD40, which have been implicated to play an important role in homeostasis and expansion of CD4⁺CD25⁺ Treg cells. Blockage of CD80/86 molecules has been known to lead to autoimmunity (31, 32), and CD40-deficient mice exhibit a reduced population of CD25⁺ T cells associated with increased T cell autoreactivity (33). In contrast to the above findings, two groups have recently reported that mature DCs are able to expand CD4⁺CD25⁺ Treg cells both in vitro and in vivo (34, 35). However, the classification of DCs as mature in both studies was based on their surface phenotype expression and not on the cytokines that they secrete.

Apart from TNF-α, several other factors have been described to induce DCs with a semimature-like phenotype, including lactobacilli (36) and cholera toxin (37). It has also been proposed that steady state migrating DCs in vivo (veiled cells) resemble the ex vivo generated semimature DCs (4). Veiled cells circulate through peripheral tissues, where they pick up apoptotic cells and migrate
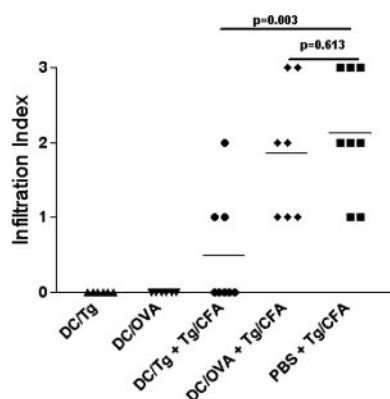
Table II. *Adoptive transfer of CD4$^+$CD25$^+$ T cells from Tg/DC-treated CBA/J mice into naive hosts inhibits EAT development[a]*

T Cell Transfer	Ag In Vivo	I.I. 0	1	2	3	4	No. of Mice with EAT	I.I. Mean	p
CD4$^+$CD25^{+b}	Tg	4	2	1	1	0	4/8	0.89	0.03
CD4$^+$CD25^{+c}	Tg	1	1	2	4	0	7/8	2.13	0.50
None	Tg	1	0	2	4	1	7/8	2.50	
CD4$^+$CD25^{+b}		8	0	0	0	0	0/8	0	
CD4$^+$CD25^{+c}		8	0	0	0	0	0/8	0	

[a] A total of 5×10^5 CD4$^+$CD25$^+$ T cells, isolated from mice primed either with [b]Tg-pulsed or [c] OVA-pulsed. DCs, were adoptively transferred i.p. into syngeneic naive recipients. Control mice received an i.p. injection of PBS. One day after the T cell transfer, some mice were immunized with 100 μg of Tg in CFA. On day 21, all mice were sacrificed and their thyroid glands were collected. The mononuclear cell I.I. was scored, as described in *Materials and Methods*. Statistical analysis was performed using the Mann-Whitney nonparametric test.

to the secondary lymphoid organs (38, 39), where they present self peptides in the context of MHC molecules (40). In the absence of maturation stimuli, however, DCs do not secrete proinflammatory cytokines and induce tolerance (41–43). Other types of DCs have been shown to induce tolerance by distinct mechanisms (2). For example, human IL-10-treated DCs were characterized by low levels of MHC class II and costimulatory molecule expression and induced Ag-specific anergic T cells (44, 45). In addition, mouse pulmonary DCs that secrete IL-10, but not IL-12, upon exposure to Ag, induced tolerance through the development of Treg cells (46). Tolerogenic DCs have been generated by other methods, including treatment with vitamin D3 (47), corticosteroids (48), and low doses of GM-CSF (49), but it was not clear in these studies how modulated DCs mediated tolerance.

The functional and phenotypic characteristics of the Tg-specific CD4$^+$CD25$^+$ T cells are consistent with those described for the naturally arising Treg cells (16–18, 50–53). Yet, Tg-specific CD25$^+$ Treg cells, maintained presumably by the normal physiological levels of Tg in the periphery, could not be functionally detected in the spleen of naive CBA/J mice. This suggests that challenge with Tg/DCs must increase the relative number as well as the efficiency of Tg-specific CD4$^+$CD25$^+$ T cells in a selective manner, because we did not observe a size difference or phenotypic changes in the overall splenic CD4$^+$CD25$^+$ T cell subset between the Tg/DC-challenged mice and the controls (data not shown). A similar expansion and/or activation of Tg-specific, CD25$^+$ Treg cells have been proposed to occur in the induction of tolerance observed following the elevation of Tg levels in the circulation (24).

Our results do not support a role for IL-10 in the suppressive effect of Tg-specific CD25$^+$ cells in vitro, but cannot formally exclude a possible role for this and other cytokines in the observed suppression of EAT. Vasu et al. (23) have recently shown that GM-CSF-treated mice have increased numbers of CD4$^+$CD25$^+$ T cells and fail to develop EAT upon challenge with Tg. CD4$^+$ T cells isolated from GM-CSF-treated mice did not proliferate in response to Tg in vitro, but addition of neutralizing anti-IL-10 mAb increased the Tg-specific T cell response, indicating a possible role for IL-10 and an alternative mode of EAT suppression in this model. Currently, the reasons for these contrasting findings remain unclear; previous studies have provided evidence for (54) or against (53, 55) an involvement of IL-10 in CD4$^+$CD25$^+$ T cell-mediated suppression.

The observation that CD4$^+$CD25$^+$ T cells from Tg/DC-treated mice mediate in vitro suppression by a cell contact-dependent mechanism, and with no apparent requirements for secretion of soluble factors, agrees well with previous findings (11, 41, 52, 53, 56). The CTLA-4 molecule, expressed on the surface of Tg-specific CD4$^+$CD25$^+$ T cells, has been shown to contribute to the cell-cell contact-mediated suppression of effector cells (16). CD4$^+$CD25$^+$ Treg cells are also known to mediate suppression through ligation of the cell surface-bound TGF-β to TGF-βR on target cells (57, 58), and additional costimulatory molecules, such as OX40, ICOS, and 4-1BB, have been reported to be involved in down-regulation of immune responses (59). Finally, in our study, the activation and/or expansion of Treg cells by Tg/DC, but not OVA/DC, suggest that this phenomenon is Tg-specific, but it remains to be established whether suppressor effector function is completely Ag nonspecific, as has been well described in other systems (11).

The generation of Tg-specific CD4$^+$CD25$^+$ Treg cells by semimature Tg/DCs raises new questions about their fine specificity and mode of function. Currently, 13 pathogenic epitopes have been mapped within the Tg molecule (60), and it will be interesting to test whether effector and Treg cells recognize distinct or overlapping regions in mouse Tg as well as examine whether posttranslational modifications of Tg have any role in this process. The delineation of physiological processes that promote the generation of tolerogenic DC in EAT, similar to TNF-α-treated DCs, would contribute greatly to our understanding of the immunoregulation of this disease. Finally, the examination of the effectiveness of CD25$^+$ Treg cells in reversing established disease would aid in the development of new therapeutic approaches in this field.

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Disclosures

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