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Downregulation of IL-12 and a novel negative feedback system mediated by $CD25^+CD4^+$ T cells ☆

Kojiro Sato [a,b,*], Shoko Tateishi [a], Kanae Kubo [a], Toshihide Mimura [c], Kazuhiko Yamamoto [a], Hiroko Kanda [a]

[a] *Department of Allergy and Rheumatology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8655, Japan*
[b] *Department of Cell Signaling, Graduate School, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8549, Japan*
[c] *Division of Rheumatology and Applied Immunology, Department of Medicine, Saitama Medical School, Morohongo 38, Moroyama, Iruma-gun, Saitama 350-0495, Japan*

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Abstract

$CD25^+CD4^+$ regulatory T cells suppress immune responses and are believed to play roles in preventing autoimmune diseases. However, the mechanism(s) underlying the suppression and the regulation of their homeostasis remain to be elucidated. Here we show that these regulatory T cells downregulated $CD25^-CD4^+$ T-cell-mediated production of IL-12 from antigen-presenting cells, which can act as a growth factor for $CD25^-CD4^+$ T cells. We further found that $CD25^+CD4^+$ T cells, despite their well-documented "anergic" nature, proliferate significantly in vitro only when $CD25^-CD4^+$ T cells are present. Notably, this proliferation was strongly dependent on IL-2 and relatively independent of IL-12. Thus, $CD25^+CD4^+$ T cells suppress $CD25^-CD4^+$ T-cell responses, at least in part, by inhibiting IL-12 production while they themselves can undergo proliferation with the mediation of $CD25^-CD4^+$ T cells in vitro. These results offer a novel negative feedback system involving a tripartite interaction among $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells, and APCs that may contribute to the termination of immune responses.

Keywords: T cells; Cell proliferation; Suppression; Anergy; IL-12; IL-2; CD25; CD80; CD86; CTLA-4

Self-reactive lymphocytes can be dangerous to the body as they may attack self and cause autoimmune diseases under certain conditions (a state called 'horror autotoxicus' by Paul Ehrlich). Hence, the immune system has evolved several mechanisms to prevent this from occurring. One and the most well-documented mechanism is the so-called clonal deletion in the thymus where immature T cells that interact strongly with self-peptides in association with MHC molecules undergo apoptosis (reviewed in [1]). Second, passive mechanisms, such as anergy and ignorance, work when self-reactive T cells encounter tissue-specific antigens in the periphery if proper signals from APCs are not provided. Another mechanism attracting much attention recently is that self-reactive T cells are dominantly suppressed by newly identified T-cell subpopulations, among which $CD25^+CD4^+$ T cells have been the most well characterized [2–6].

$CD25^+CD4^+$ T cells not only suppress $CD25^-CD4^+$ T-cell proliferation in vitro but also prevent inflammation in nu/nu mice caused by transferring $CD25^-CD4^+$ T cells [2]. It has been shown that $CD25^+CD4^+$ T cells have to be activated by signals delivered through TCR

☆ *Abbreviations:* CFSE, carboxyfluorescein succinimidyl ester; PI, propidium iodide; MFI, mean fluorescence intensity.

* Corresponding author. Fax: +81 3 3815 5954.
E-mail address: satok-tky@umin.ac.jp (K. Sato).

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and require a direct cell-to-cell contact [3,4]. Experiments using gene-targeted mice and blocking antibodies indicated that so-called suppressive cytokines, such as IL-4, IL-10, and TGF-β, are not likely involved [3,4,7]. Several surface molecules, such as CTLA-4 [8,9] and glucocorticoid-induced TNF receptor (GITR) [10,11], have been proposed to contribute to the suppression, but still require further confirmation. Recently, the existence of human suppressive $CD25^+CD4^+$ T cells has been confirmed, indicating their evolutionary conservation across species [12–17]. These findings not only further underscored the importance of the $CD25^+CD4^+$ T-cell system in the regulation of the immune system but also opened up a possibility of therapeutic applications of these cells.

Here we show that $CD25^+CD4^+$ T cells inhibit IL-12 production from APCs triggered by $CD25^-CD4^+$ T cells. As exogenous IL-12 could restore, albeit partially, the $CD25^-CD4^+$ T-cell proliferation suppressed by $CD25^+CD4^+$ T cells in vitro, the inhibition of IL-12 production may contribute to the $CD25^+CD4^+$ T-cell-mediated suppression.

It is yet to be elucidated why 'anergic' regulatory T cells that constitute only a minor population can effectively suppress immune responses mediated by the prevailing population of $CD25^-CD4^+$ T cells. In this regard, we show here that $CD25^+CD4^+$ T cells proliferate in vitro only when stimulated $CD25^-CD4^+$ T cells are present. We propose herein a novel feedback mechanism by which conventional $CD4^+$ T cells are suppressed by regulatory T cells and simultaneously help their expansion thereby further downregulating immune responses.

Materials and methods

Mice. Female BALB/c mice and SCID mice were obtained from CLEA Japan (Tokyo, Japan). DBA/2 mice were purchased from Charles River Japan (Tokyo, Japan). DO11.10 Tg mice were originally obtained from Jackson Laboratory (Bar Harbor, ME) and bred in our own facilities.

Medium, reagents, and antibodies. All cells were grown in the RPMI1640 medium containing 10% heat-inactivated FCS, 0.1 mg/ml each of ampicillin and kanamycin, 0.3 mg/ml L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME (culture medium). Anti-CD4 mAb-FITC (RM4-5), anti-CD5.1 mAb-FITC (H11-86.1), anti-CD25 mAb-PE (3C7), biotinylated anti-CD25 mAb (7D4), biotinylated anti-CD40L mAb (MR1), anti-CD3 mAb (145-2C11), anti-IL-12 mAb (C15.6), anti-IL-2 mAb (JES6-5H4), anti-IL-12 mAb (C15.6), and streptavidin-PE were purchased from BD PharMingen (San Diego, CA). Clonotype-TCR-specific KJ1-26 mAb was purchased from Caltag (Burlingame, CA). Recombinant murine IL-12 was purchased from Genzyme-Techne (Cambridge, MA). Propidium iodide (PI) was purchased from Molecular Probe (Eugene, OR).

Cell purification. Spleens were harvested from 8- to 10-week-old female mice. They were mashed through a wire mesh into the culture medium to prepare single cell suspensions. To separate $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells from other cells, first, a Mouse Regulatory T Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to separate $CD25^+CD4^+$ T cells according to the manufacturer's instruction. The remaining $CD25^-CD4^+$ T-cell-rich population was further selected using BD IMag Mouse CD4 Particles-MSC (BD PharMingen). The purities of the sorted cells were more than 85% for $CD25^+CD4^+$ T cells and more than 90% for $CD25^-CD4^+$ T cells.

Thymidine-uptake assay. Cells were plated in 96-well U-bottomed microtiter plates at a density of 3×10^6/ml in a total volume of 100 μl culture medium. Proliferation was measured after a pulse with 1 μCi of [^{3}H]thymidine.

ELISA. OptEIA Mouse IL-2 SET and OptEIA Mouse IL-12 SET (BD PharMingen) were used to detect cytokine according to the manufacturer's instructions.

CFSE staining. $CD4^+$ T cells were stained with fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Wako Pure Chemical, Osaka, Japan) by incubating them in PBS containing 10 μM CFSE for 10 min at room temperature before washing twice in ice-cold PBS.

Flow cytometric analysis. Nonspecific staining was blocked with anti-CD16/CD32 mAb (Fc block, 2.4G2). Cells were incubated with appropriate antibodies for 20 min, after which cells were washed twice with PBS containing 0.5% bovine serum albumin. In the case of biotinylated anti-CD40L mAb, 20-min incubation with PE-streptavidin and washing twice were carried out. Cells were analyzed on a FACSCalibur using CellQuest software (Becton–Dickinson Japan, Tokyo, Japan). Viable cells were gated on the basis of forward and side scatters, and by PI staining.

Bone marrow-derived dendritic cells. Bone marrow-derived dendritic cells (BMDCs) were generated using the method described in [18]. Briefly, BALB/c mouse bone marrow was sieved through a 70-μm nylon mesh into the culture medium. Red blood cells were then lysed using ammonium chloride, and plated at 7.5×10^6 per 10 cm plate, supplemented with 20 ng/ml murine recombinant GM-CSF (PeproTech EC, London, UK). The medium was replaced on days 3 and 6. BMDCs were harvested on day 8 by gentle pipetting.

Results

First we analyzed whether regulatory T cells have some effects on APCs in vitro. We sorted $CD4^+$ T cells into $CD25^+CD4^+$ cells and $CD25^-CD4^+$ cells (hereafter referred to as $CD25^+$ T cells and $CD25^-$ T cells, respectively) (Fig. 1A), and confirmed that $CD25^+$ T cells inhibited $CD25^-$ T-cell proliferation in a dose-dependent manner in the presence of APCs and soluble anti-CD3 mAb (Fig. 1B). It is known that when $CD4^+$ T cells interact with APCs in the presence of antigens, these T cells release IL-2 and the APCs release IL-12, respectively [19]. We cultured the sorted T cells together at a 50:50 ratio or separately and analyzed the amounts of released IL-2 and IL-12 by ELISA after 16 h of incubation. A small amount of IL-12 and no IL-2 were detected in the medium in the case of $CD25^+$ T cells cultured alone. In contrast, in the case of $CD25^-$ T cells cultured alone, both IL-2 and IL-12 were easily detected. Interestingly, when $CD25^+$ T cells and $CD25^-$ T cells were cocultured at a 50:50 ratio, while IL-2 was readily detected (at an amount about one-half of that in the case of $CD25^-$ T cells cultured alone), only a very small amount of IL-12 was released into the medium (Fig. 1C).

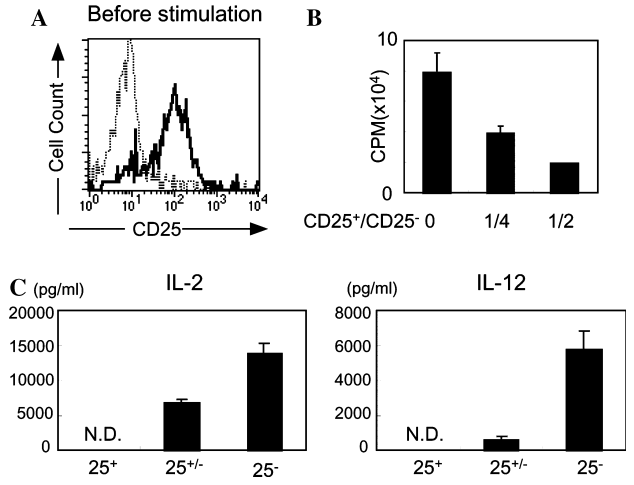

Fig. 1. Regulatory T cells inhibit IL-12 release from APCs. (A) $CD4^+$ T cells from wild-type BALB/c mice were sorted into $CD25^-$ and $CD25^+$ cells. The dotted line represents $CD25^-CD4^+$ T cells and the solid line represents $CD25^+CD4^+$ T cells; the purities of sorted cells were more than 90% and 85%, respectively. (B) $CD25^+CD4^+$ T cells, added to the culture system of 1.5×10^6/ml each of $CD25^-CD4^+$ T cells, APCs (splenocytes from SCID mice), and 1 μg/ml soluble anti-CD3 mAb inhibited thymidine uptake on day 3 in a dose-dependent manner. (C) $CD25^+CD4^+$ T and $CD25^-CD4^+$ T cells were cultured separately or together at a 50:50 ratio (1.5×10^6/ml each) in the presence of 1.5×10^6/ml APCs and 1 μg/ml soluble anti-CD3 mAb for 16 h. Culture supernatant was subjected to ELISA for IL-2 and IL-12.

To clarify the mechanism underlying these observations, we analyzed the expression level of CD40L on T cells in this system, because signaling through CD40 on APCs via CD40L on $CD4^+$ T cells is required for APC activation and IL-12 release [20,21]. Indeed, CD40L that was induced on $CD25^-$ T cells after 8 h of stimulation was barely induced on $CD25^+$ T cells, although both cells came to express CD25. After 16 h of stimulation, however, both groups expressed little CD40L (Figs. 2A and B). This transient expression of CD40L agrees with the data of Maruo et al. [19], in which IL-12 activity in the culture supernatant reached the peak 9–15 h after stimulation; after which it rapidly decreased. When we used sorted T cells derived from DO11.10 Tg mice and stimulated them with the specific OVA peptide (323–339) instead of anti-CD3 mAb, we found a similar difference in CD40L expression level (Fig. 1C), indicating that this phenomenon was not restricted to T cells stimulated with an antibody and APCs. In this experiment alone, we gated KJ1-26^+ cells instead of $CD4^+$ cells, because it has been shown that in conventional DO11.10 mice, $CD25^+$ T cells contain clonotype-negative cells that will not respond to the peptide stimulation [22]. In order to analyze the effect of $CD25^+$ T cells on CD40L expression of $CD25^-$ T cells, we utilize Ly-1.1 $(CD5.1)^+CD25^-$ T cells derived from DBA/2 mice. When cocultured with Ly-1.1^-CD25^+ T cells obtained from BALB/c mice, the CD40L expression was clearly depressed (Fig. 2D).

These data strongly suggest that $CD25^+$ T cells regulate APC activation. If so, is the inhibition of this 'pulsatile IL-12 release' by these regulatory cells merely a result of the abortion of APC activation, or is it playing

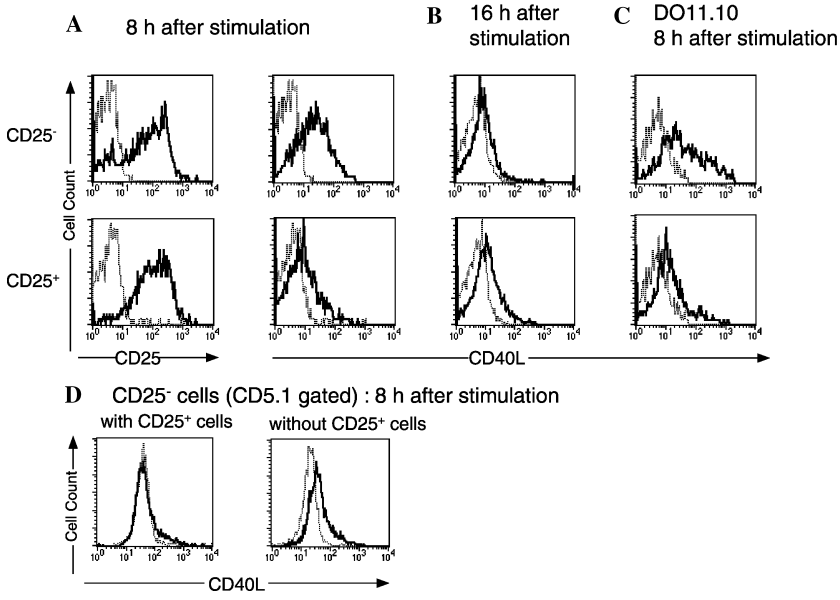

Fig. 2. Regulatory T cells do not express CD40L after stimulation. (A) Eight hours after stimulation, CD4-positive and PI-negative cells were gated and analyzed by FACS for CD25 and CD40L expressions. The dotted lines represent control staining. (B) Sixteen hours after stimulation, CD40L expression was analyzed as in (A). (C) Similar experiments were performed using DO11.10 Tg mice instead of wild-type BALB/c mice, stimulated with APCs and 0.3 μM OVA peptide. After 8 h of stimulation, CD40L expression was analyzed. Clonotype-TCR-specific KJ1-26 mAb-positive and PI-negative cells were gated in this experiment. (D) $CD25^-CD4^+$ T cells from DBA/2 mice $(CD5.1^+)$ were stimulated with APCs and 1 μg/ml soluble anti-CD3 mAb with or without the presence of $CD25^+CD4^+$ T cells from BALB/c mice $(CD5.1^-)$ for 8 h. $CD5.1^+CD4^+$ cells were gated and CD40L expression was analyzed. Data shown are representative of three independent experiments.

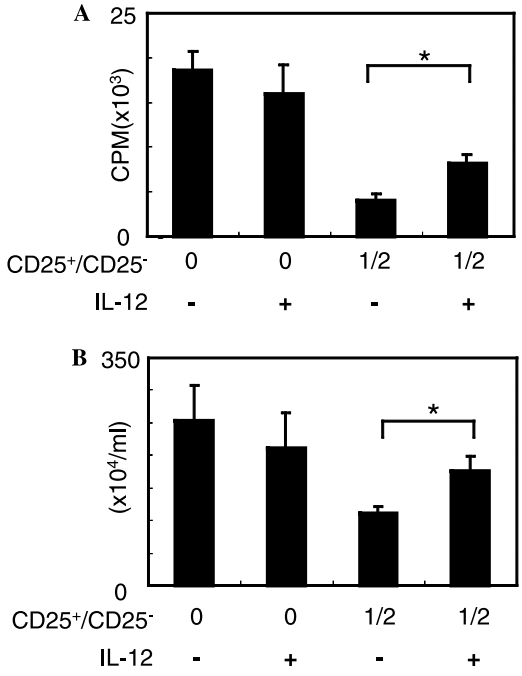

Fig. 3. Adding exogenous IL-12 partially rescues regulatory-T-cell-mediated inhibition of cell proliferation. 1.5×10^6/ml $CD25^-CD4^+$ T cells alone or 1.5×10^6/ml each of $CD25^+CD4^+$ T cells were stimulated with 1.5×10^6/ml APCs and 1 μg/ml soluble anti-CD3 mAb for 3 days with or without adding 10 ng/ml IL-12. Thymidine uptake of last 4 h was analyzed as well as the number of viable cells. $*p < 0.05$.

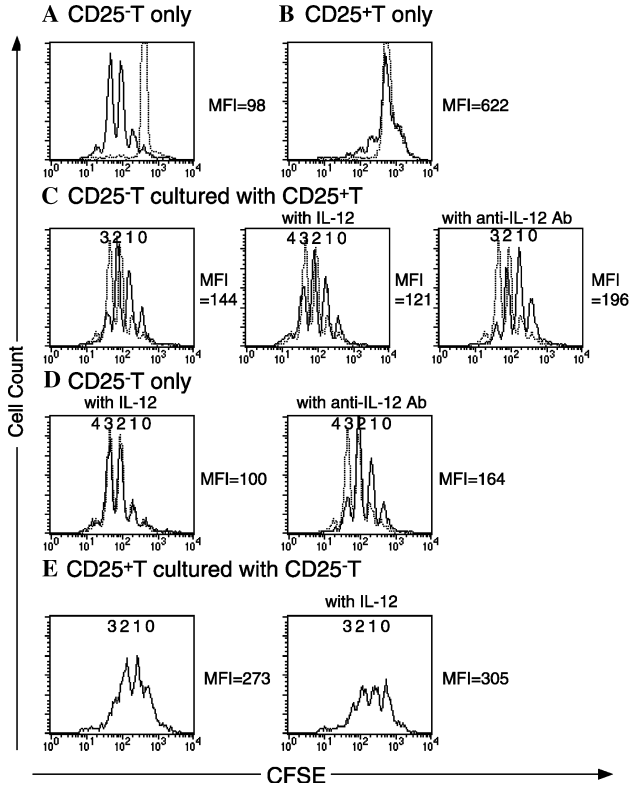

Fig. 4. CFSE-staining method also indicates that IL-12 is important for suppressing $CD4^+$ T-cell division by regulatory T cells. (A) 1.5×10^6/ml CFSE-stained $CD25^-CD4^+$ T cells were stimulated with 1.5×10^6/ml APCs and 1 μg/ml soluble anti-CD3 mAb for 3 days. The number of cell divisions was analyzed by FACS. The dotted line represents control cells without anti-CD3 mAb stimulation. (B) Similar experiments were performed using $CD25^+CD4^+$ T cells. (C) $CD25^-CD4^+$ T cells were stained with CFSE, mixed with the same number of $CD25^+CD4^+$ T cells, and stimulated with or without 10 ng/ml IL-12 or 10 μg/ml of anti-IL-12 mAb. $CFSE^+CD4^+$ cells were gated. The dotted line represents the control $CD25^-CD4^+$ T cells stimulated without the presence of $CD25^+CD4^+$ T cells. (D) CFSE-stained $CD25^-CD4^+$ T cells were stimulated with IL-12 or anti-IL-12 mAb without the presence of $CD25^+CD4^+$ T cells. (E) $CD25^+CD4^+$ T cells were stained with CFSE, mixed with the same number of $CD25^-CD4^+$ T cells, and stimulated with or without IL-12. $CFSE^+CD4^+$ cells were gated. Data shown are representative of three independent experiments.

an important role in the functions of regulatory T cells (that is, suppression of cell proliferation)?

To answer this question, we added IL-12 to the above system and found that the decreased thymidine uptake and cell count of $CD25^-$ T cells cocultured with $CD25^+$ T cells for 3 days were partially rescued (Fig. 3). As expected, IL-12 addition did not increase the thymidine uptake and cell count of $CD25^-$ T cells cultured without $CD25^+$ T cells.

We also employed the CFSE-staining method to evaluate the number of cell divisions that either $CD25^-$ or $CD25^+$ T cells had undergone during the whole time course of the culture [23]. Most of the $CD25^-$ T cells divided in the presence of soluble anti-CD3 mAb and APCs, but they did not divide at all in the absence of the antibody (Fig. 4A). On the other hand, $CD25^+$ T cells hardly divided irrespective of the presence of the antibody (Fig. 4B). In the case of coculture of the two populations at an equal ratio, $CD25^-$ T-cell division was markedly inhibited. We found that the mean fluorescence intensity (MFI) of $CD25^-$ T cells cocultured with $CD25^+$ T cells was more than that of $CD25^-$ T cells cultured alone (144 vs 98; Figs. 4A and C). When IL-12 was added to this system, the MFI of $CD25^-$ T cells decreased (from 144 to 121). On the contrary, addition of anti-IL-12 mAb to this system made the number of cell divisions less (MFI: from 144 to 196). These data strongly suggest that IL-12 contributes, at least in part, to the suppressive effect of regulatory T cells. When $CD25^-$ T cells alone were stimulated with APCs and anti-CD3 mAb in the presence of IL-12, the added cytokine barely changed the MFI of $CD25^-$ T cells (Fig. 4D), suggesting that the $CD25^-$ T cells were already sufficiently stimulated by IL-12 released from APCs and no extra IL-12 was necessary (see Discussion). As expected, addition of anti-IL-12 mAb clearly made the cell division less (MFI: from 98 to 164). When $CD25^+$ T cells were stained with CFSE, cocultured with $CD25^-$ T cells, and then stimulated with APCs and anti-CD3 mAb, IL-12 addition did not decrease the MFI of these cells (305 vs 273, Fig. 4E).

During the experiments, it was noteworthy that although $CD25^+$ T cells did not divide at all in the

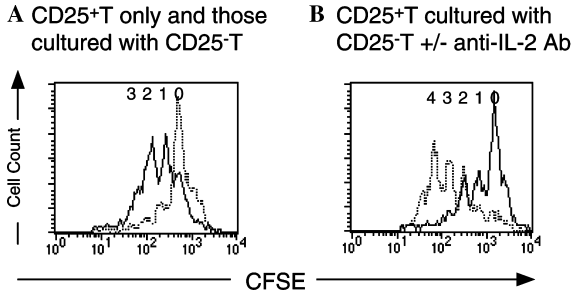

Fig. 5. Regulatory T cells proliferate in vitro in the presence of $CD25^{-}CD4^{+}$ T cells in an IL-2-dependent manner. (A) Flow cytometric analysis of stimulated $CD25^{+}CD4^{+}$ T cells cultured alone or with $CD25^{-}CD4^{+}$ T cells. The dotted line represents $CD25^{+}CD4^{+}$ T cells cultured alone and the solid lines represent $CD25^{+}CD4^{+}$ T cells stimulated in the presence of $CD25^{-}CD4^{+}$ T cells. (B) Similar experiments were performed with either 10 μg/ml of anti-IL-2 mAb or control antibody. Data shown are representative of three independent experiments.

absence of other T cells even when stimulated with APCs and soluble anti-CD3 mAb, they obviously divided when cocultured with $CD25^{-}$ T cells (Fig. 5A). Regulatory T cells have been considered to be anergic and the anergy was presumed to be overcome only by adding a high dose of IL-2 or anti-CD28 mAb [3,4]; however, in our experiment, neither reagent was added. Recently, activated APCs have been reported to help $CD25^{+}$ T cells proliferate in vitro [24], however, in our experiment, APCs were not activated before encountering T cells. It was evident from our experiment, too, that $CD25^{+}$ T cells barely proliferate without other T cells (Fig. 4B), therefore it may be that these regulatory T cells depend on other T cells for their aliment to proliferate. As for their aliment, IL-2 released from cocultured $CD25^{-}$ T cells was the most likely candidate. In accordance with this notion, the cell division of $CD25^{+}$ T cells cocultured with $CD25^{-}$ T cells and stimulated with APCs and soluble anti-CD3 mAb was strongly inhibited by the presence of anti-IL-2 mAb (Fig. 5B).

Finally, we analyzed the expression level of B7 molecules (CD80 and CD86) on APCs. These molecules bind to CD28 and CTLA-4 on T cells. To obtain sufficient APCs for flow cytometric analysis, we utilized DCs differentiated in vitro from bone marrow cells by GM-CSF stimulation. We confirmed that $CD25^{+}$ T cells also suppressed IL-12 production when cultured with $CD25^{-}$ T cells and DCs in the presence of anti-CD3 mAb (Fig. 6A). As for CD80 and CD86, the presence of $CD25^{+}$ T cells decreased the surface expression levels of these molecules (Fig. 6B).

Discussion

Two hypotheses have been proposed for the mechanism(s) of regulatory T cells [6]. One is that these cells directly suppress other T-cell function(s) and the other is that they suppress APC function(s). We have shown

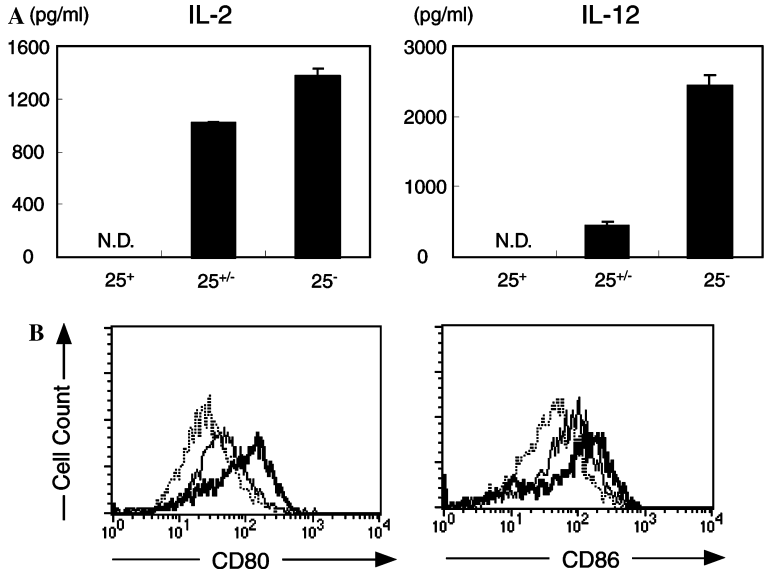

Fig. 6. CD80 and CD86 expressions on DCs are also downregulated in the presence of regulatory T cells. (A) Bone marrow-derived dendritic cells (1.5×10^{5}/ml) were used as APCs and cultured with $CD25^{+}CD4^{+}$ and/or $CD25^{-}CD4^{+}$ T cells (1.5×10^{6}/ml each) in the presence of 1 μg/ml soluble anti-CD3 mAb for 16 h. Culture supernatant was analyzed for IL-2 and IL-12 by ELISA. (B) After 16 h, CD80 and CD86 expressions on CD11c-positive cells were analyzed by flow cytometry. The dotted line represents APCs cultured with $CD25^{+}CD4^{+}$ T cells alone. The thin line represents APCs cultured with both $CD25^{+}CD4^{+}$ and $CD25^{-}CD4^{+}$ T cells (1.5×10^{6}/ml each). The bold line represents APCs cultured with $CD25^{-}CD4^{+}$ T cells alone. Data shown are representative of two independent experiments.

here that they regulate APC function(s) and that suppressed IL-12 production at least partially inhibits the proliferation of other $CD4^+$ T cells (Figs. 3 and 4C). Thus, our data favor the latter hypothesis, which are reminiscent of the results of Cederbom et al. [25]. On the other hand, regulatory T cells were reported to function in the presence of fixed APCs [4], indicating that mechanism(s) such as competition for costimulatory molecules on APCs may be involved. Seen in this light, the fact that regulatory T cells express CTLA-4 constitutively [8,9] may have significance because CTLA-4 was reported to bind to B7 molecules much more strongly than CD28 [26]. CTLA-4 may be able to tether regulatory T cells to APCs, inhibiting interaction between APCs and other T cells. In any case, the two hypotheses mentioned above do not necessarily contradict each other. During the first 16 h of APC and T-cell interaction, IL-12 production was more inhibited than IL-2 production by the presence of $CD25^+$ T cells. This may indicate that the degree of cell-to-cell interaction necessary for the release of the two cytokines is different (that is, IL-12 release may need more constant interaction between APCs and T cells). The observation that IL-2 release is not inhibited so much initially may reflect the number of cell divisions of $CD25^-$ T cells. The CFSE-staining method made it clear that even in the presence of $CD25^+$ T cells, $CD25^-$ T cells divide several times. This means that $CD25^+$ T cells do not inhibit proliferation of $CD25^-$ T cells suddenly, but only gradually. As the thymidine-uptake experiment evaluates only the last few hours' proliferation of cells, we can safely say that the total number of cell divisions during the entire culture period is more accurately estimated by the CFSE-staining method.

IL-12 was originally characterized as a growth factor and the upregulation of CD25 is one of the numerous functions of IL-12 [27,28]. CD25 is indispensable to the formation of high-affinity receptors for IL-2 and it is natural that cells that acquire high-affinity IL-2 receptors can respond readily to IL-2 and proliferate vigorously. $CD25^+$ T cells, on the other hand, have high-affinity IL-2 receptors from the beginning, so they may not be affected by the presence of IL-12 (Fig. 4E). As IL-12 is an essential cytokine in T helper type 1 (Th1)-specific response [29,30], it is no wonder $CD25^+$ T cells suppress Th1 response [31,32].

Another point we want to emphasize is that $CD25^+$ T cells clearly proliferate in vitro, depending on the presence of $CD25^-$ T cells, although their anergic characteristic has been emphasized previously (Fig. 5). Recently, it has been shown that $CD25^+$ T cells can proliferate well in the presence of activated APCs [24]. Our data differ in that, however, we used freshly prepared splenocytes from mice raised in a specific pathogen-free environment as APCs. For regulatory cells, this may be a rational strategy considering the relative scarcity of the subpopulation (about 5–10% of $CD4^+$ T cells in a normal mouse [2]) in a noninflammatory state.

As for the aliment given to $CD25^+$ T cells from activated $CD25^-$ T cells, the most likely candidate would be IL-2, because $CD25^+$ T cells do not release the cytokine, and nevertheless, they depend on it for their suppressive function [33]. When infectious agents invade, other T cells attempt to eliminate them from the body and release a large amount of IL-2 in the process. Regulatory T cells may proliferate only in this setting and thus eventually acquire a strong suppressive capacity (Fig. 7B). Thus, IL-2 seems to be necessary not only for the differentiation of $CD25^+$ T cells but also for the function of these cells. This mechanism may facilitate the termination of immune responses and prevent the occurrence of excessive inflammation. We presume this constitutes another novel feedback system, which is among several mechanisms for avoiding horror autotoxicus.

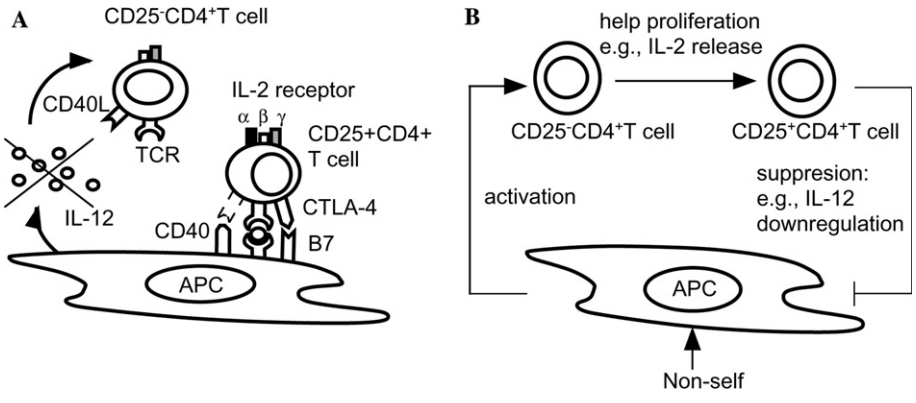

Fig. 7. Schematics of suppressive mechanisms of regulatory T cells. (A) CD40L-negative regulatory T cells block IL-12 production by APCs, suppressing proliferation of other T cells. CTLA-4 constitutively expressed may work as a strong binder between regulatory T cells and APCs. (B) $CD25^-$ T cells provide regulatory T cells with means (e.g., IL-2) to proliferate. Thus, regulatory T cells increase in number and eventually acquire a strong suppressive capacity.

Acknowledgments

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