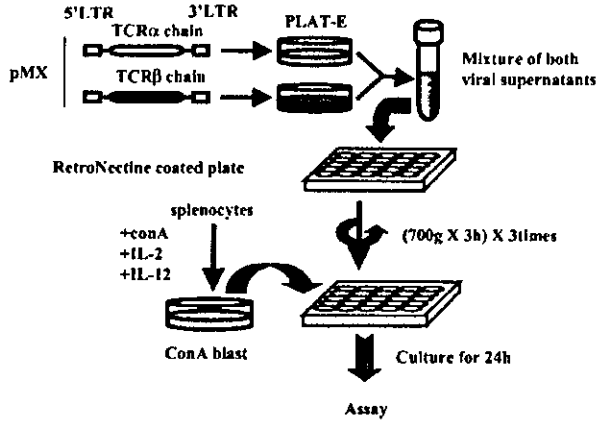

FIGURE 3. Schematic diagrams of the protocol for retrovirus infection to splenocytes. The mixture of both viral supernatants was placed on non-tissue culture 24-well plates coated with RetroNectin and centrifuged for 3 h at room temperature. This step was repeated three times. Then splenocytes were placed into the plates and cultured for 24 h.

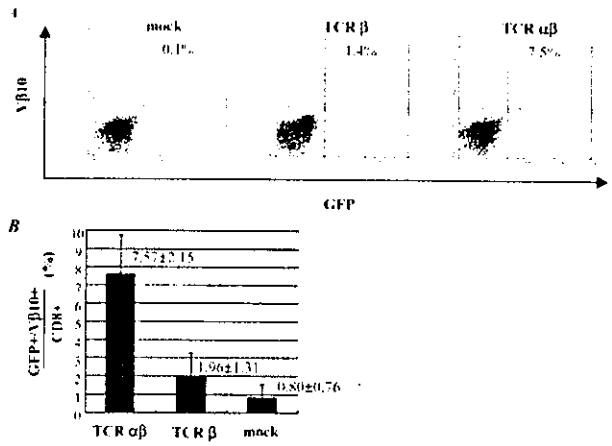

FIGURE 5. Analysis of the reconstituted T cell accumulation at the original tumor site in nude mice. The DBA/2 splenocytes were retrovirally transferred with IRES/GFP (mock), TCR β-chain gene plus IRES/GFP (β), or TCR αβ-chain genes (αβ), and CD8+ cells were isolated from the splenocytes. Nude mice ($n = 4$) were injected s.c. with 1×10^6 tumor cells and i.v. with 2×10^6 CD8+ gene-transduced T cells. After 12 days, CD8+ TIL were isolated, stained with PE anti-Vβ10, and subsequently analyzed by flow cytometry. *A*, Flow cytometric analysis of P815-infiltrating CD8+ T cells. The horizontal axis shows GFP intensity (TCRα-IRES/GFP or IRES/GFP for the controls), and the vertical axis shows Vβ10 expressions. Eight individual mice were examined, and representative data are shown. *B*, The ratio of GFP+/Vβ10+ TIL in CD8+ TIL from P815-bearing mice. Values represent the mean of two independent experiments.

β-chain plus IRES/GFP, the TCR αβ-chain-introduced CD8+ T cells were found to more efficiently accumulate at the P815 tumor site than the cells introduced with the TCR β-chain alone. The GFP+/Vβ10+ populations in the CD8+ TIL from each group are summarized in Fig. 5*B*. This observation suggests that, like the P09-2C clone, TCR αβ-chain-introduced T cells accumulate at the original tumor site, when reconstituted CD8+ T cells are adoptively transferred to tumor-bearing nude mice.

Discussion

In this study, we demonstrated that the TCR αβ-chains from clonally expanded TIL can be detected by the combination of the RT-PCR/SSCP method and the single-cell PCR method. Furthermore, when functional tumor-specific CTL were reconstituted by retroviral transfer of TCR αβ-chain genes from clonally expanded TIL, we proved that the TCR αβ-chain-introduced CD8+ T cells lyse the tumor cells in vitro and accumulate at the tumor site in vivo. This is the first approach for simultaneously determining the individual TCR α- and β-chains of tumor-infiltrating CD8+ T cells and for investigating the immune response of such TCR to the original tumor under both in vitro and in vivo conditions. Several studies have been made on the reconstitution of functional T cells

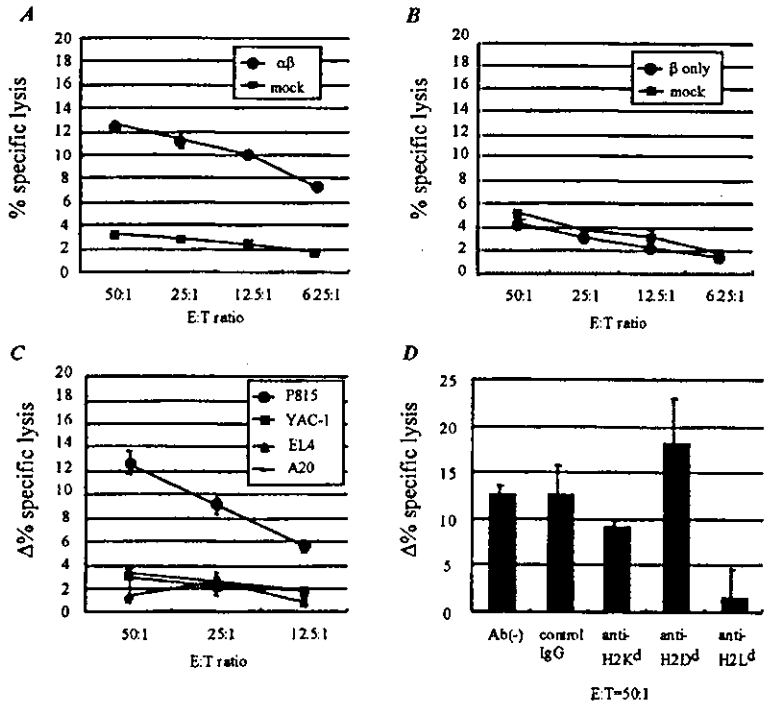

FIGURE 4. The cytotoxicity of T cells introduced with P09-2C TCR αβ-chain genes. Splenocytes from DBA/2 mice were retrovirally transduced with P09-2C TCR αβ-chain genes (*A*) or TCR β-chain gene alone (*B*), followed by a test of their cytotoxicity against P815 tumor cells. Mock-introduced T cells were used as a control. *C*, TCR αβ or mock-introduced splenocytes were tested for their cytotoxicity against A20, EL-4, and YAC-1 target cells. Δ% specific lysis = (% specific lysis of αβ-introduced T cells) − (% specific lysis of mock-introduced T cells). Each occurrence of lytic activity was evaluated in a 4-h ^{51}Cr release assay at different E:T ratios. *D*, P815 target cells were incubated with TCR αβ or mock-introduced splenocytes in the absence or presence of optimal concentrations of mAb at a 50:1 E:T ratio. Data points, means of triplicate determinants. Results are representative of three independent experiments.

by TCR $\alpha\beta$-chain gene transfer, but most of these studies have involved the reconstitution of TCRs into T cell hybridomas and T cell lines (20–24). Gene transfer into peripheral T lymphocytes has been achieved in the last few years (25, 26–29). Furthermore, these studies have used cloned TCR already proven to have a functional specificity to Ag, such as OVA and influenza nucleoprotein (25, 28, 29). In this report, we reconstituted the functional $CD8^+$ T cells by gene transfer of the functionally unknown TCR, which was supposed to recognize an unknown tumor Ag. This kind of approaches would enable us to reconstitute CTL specifically for an individual patient.

Several studies have shown that TIL are able to recognize tumor Ags and in some cases to lyse the malignant cells. These findings provided a basis for the use of in vitro cultured TIL in adoptive immunotherapy (15, 36). However, the efficacy of such cultured TIL is not sufficient for clinical use. For example, even when the freshly isolated TIL exhibited an activated phenotype and displayed strong cytotoxic activity in vitro, the TIL failed to prevent melanoma cell growth in vivo (37). Moreover, even in cases of clones capable of exerting strong antitumor cytotoxicity in vitro, these tumor-specific CTL were not sufficient to suppress tumor growth (38). The discrepancy between in vitro tumor lytic activity and in vivo tumor progression in the presence of such TIL remains to be clarified. Several studies suggest that tumors may escape from immune recognition by a variety of pathways, e.g., release of factors that inhibit the functioning of TIL.

In our previous experiments, we showed that the antigenic stimulation of a T cell population leads to clonal expansion, detected as a band by the RT-PCR/SSCP method (17, 39). Each band corresponds to an accumulated T cell clone in the heterogeneous T cell population (17). We have previously reported that, by this RT-PCR/SSCP method, distinct T cell clonotypes were detected in patients with solid tumors (18) as well as in samples from rheumatoid arthritis or systemic lupus erythematosus (35, 40). In murine P815 solid tumors, the clonal expansion of T cells with Vβ1, 2, 4, 7, 10, 13, and 14 was repeatedly detected by the SSCP method (Fig. 1). It is likely that these clones react to a tumor Ag. Previously, Levraud et al. (41) reported that T cells with Vβ1/Jβ1.2 or Vβ1/Jβ2.5 were found in P815-infiltrating lymphocytes, and the Vβ1/Jβ1.2 rearrangement was borne by CTL directed against an Ag derived from P1A, a self protein expressed in P815. Likewise, clonally expanded T cells using Vβ1 were reproducibly detected in our system. However, it was difficult to simultaneously determine the TCR α- and β-chains derived from a $CD8^+/V\beta1^+$ single TIL because no murine anti-Vβ1 Ab is available.

In this study, one of the principal problems involved the quantitative assessment of the RT-PCR/SSCP method. The dominant band on an SSCP gel may not reflect the dominant population in TIL because of differences in primer efficiencies among the Vβs and in clone numbers of each population, although it is reasonable to suppose that a dominant band in a distinct Vβ represents the dominant clone in the Vβ population. Therefore, we analyzed the Vβ usage of P815-infiltrating $CD8^+$ T cells by flow cytometry. Given that a clone using a certain Vβ displays clonal expansion on an SSCP gel and that the usage of the Vβ is predominant in TIL, it seems quite probable that the clone recognizes a P815 tumor Ag. In our system, the usage of Vβ10 was increased in TIL when compared with that in spleen (Table I). Recently, several investigators studied the Vβ usage of TIL (42, 43), but few attempts have been made to simultaneously determine the Vα and Vβ usage of such cells. In this study, we also tried to determine the Vα sequence of clonally expanded TIL by the single-cell PCR method. As shown in Fig. 2*A*, the Vα sequences were rendered by 5′ poly(A) addition and subsequent PCR amplification (31). It has been reported that the frequency of clonally expanded T cells could be analyzed by the single-cell PCR method (44–46). The efficiency for obtaining sequenceable PCR products from single cells is low (~10–30%) in our system compared with a previous report (46). The difference may be due to less abundant mRNA in TIL, because sequenceable PCR products were obtained from single spleen T cells in our system at the same efficiency as previously reported (46).

When the P09-2C TCR $\alpha\beta$-chain genes were transferred retrovirally to splenocytes, the ability of the transduced cells to secrete IFN-γ was not significant (data not shown). This finding may be due to the relatively weak activity of the CTL. However, the reconstituted $CD8^+$ T cells positively lysed P815 tumor cells and accumulated at the original tumor site. Hence, it is quite likely that the reconstituted $CD8^+$ T cells recognized a P815 tumor Ag. In fact, the MHC molecule presenting P815A and B Ags was shown to be $H\text{-}2L^d$ (8), so it is possible that the reconstituted CTL recognized the P815A or B Ag. Stronger activity against the tumor may be achieved by transferring another set of TCR $\alpha\beta$-chains, because $CD8^+$ T cells introduced with the $H2\text{-}K^b$-specific TCR $\alpha\beta$-chain genes strongly lysed the $H2\text{-}K^b$ gene-transfected P815 cells (unpublished data).

Although the TCR $\alpha\beta$-chain-introduced $CD8^+$ cells accumulated at the P815 tumor site, such T cells did not reject the original tumor in vivo (data not shown). This finding may be due to the character of P815 tumor cells. In fact, escape variants have been known to emerge from the original P815 tumor cells in vivo (47).

The P09-2C TCR $\alpha\beta$-chain-introduced $CD8^+$ T cells were definitely accumulated at the P815 tumor site. Recently, lymph node T cells transduced with IL-10 (48) and myelin basic protein-specific Th1 clone transduced with TGFβ1 (49) have been shown to ameliorate experimental autoimmune encephalomyelitis development. In addition, type II collagen-specific $CD4^+$ T cells or hybridomas with IL-12 antagonist (IL-12p40) significantly inhibited the development of collagen-induced arthritis, and these $CD4^+$ T cells accumulated in the inflamed joints in mice with collagen-induced arthritis (50). Moreover, when virus-specific TCR$\alpha\beta$ genes were introduced into peripheral T cells, such T cells expanded upon viral infection of mice and efficiently homed to effector sites (28). These observations suggest that the Ag-specific T cells would be useful as a therapeutic vehicle. Likewise, this approach may be effective in tumors.

The study presented in this work demonstrates that it is possible to reconstitute functional tumor-specific T cells accumulating at a tumor site. We expect that using the reconstituted tumor-specific T cells as a therapeutic vehicle will make possible the development of gene therapy for patients with malignant tumors.

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Peripheral Tolerance to a Nuclear Autoantigen: Dendritic Cells Expressing a Nuclear Autoantigen Lead to Persistent Anergic State of CD4$^+$ Autoreactive T Cells After Proliferation[1]

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It remains unknown why the T cell tolerance to nuclear autoantigens is impaired in systemic autoimmune diseases. To clarify this, we generated transgenic mice expressing OVA mainly in the nuclei (Ld-nOVA mice). When CD4$^+$ T cells from DO11.10 mice expressing a TCR specific for $OVA_{323-339}$ were transferred into Ld-nOVA mice, they were rendered anergic, but persisted in vivo for at least 3 mo. These cells expressed $CD44^{high}$, $CD45RB^{low}$, and were generated after multiple cell divisions, suggesting that anergy is not the result of insufficient proliferative stimuli. Whereas dendritic cells (DCs) from Ld-nOVA (DCs derived from transgenic mice (TgDCs)), which present rather low amount of the self-peptide, efficiently induced proliferation of DO11.10 T cells, divided T cells stimulated in vivo by TgDCs exhibited a lower memory response than T cells stimulated in vitro by peptide-pulsed DCs. Furthermore, we found that repeated transfer of either TgDCs or DCs derived from wild-type mice pulsed with a lower concentration of $OVA_{323-339}$ induced a lower response of DO11.10 T cells in Ag-free wild-type recipients than DCs derived from wild-type mice. These results suggest that peripheral tolerance to a nuclear autoantigen is achieved by continuous presentation of the self-peptide by DCs, and that the low expression level of the peptide might also be involved in the induction of hyporesponsiveness. ***The Journal of Immunology,*** **2002, 168: 1103–1112.**

One of the prominent features of systemic autoimmune diseases such as systemic lupus erythematosus is the presence of immune responses against ubiquitous nuclear autoantigens, such as spliceosomal components (U1 snRNP-A, 70K, C, B/B′, etc.) and nucleosomal components (dsDNA, histones). These systemic autoimmune responses are driven by autoantigen-specific CD4$^+$ T cells (1, 2). Although Ag compartmentation within cells could influence the processing and loading of antigenic determinants onto MHC molecules (3–6), only a few studies have focused on the mechanism of T cell tolerance to nuclear autoantigens (7, 8).

T cell tolerance is established and maintained by eliminating (9–12) and silencing (13–17) autoreactive T cells both in the thymus and in the periphery. In the thymus, TCR interactions with self-Ags presented on MHC molecules are known to be crucial for the selection of immature T cells. Recent studies also suggest that self-Ags presented in the periphery might play an important role in the maintenance of peripheral tolerance (13–17), the survival of naive mature T cells (18, 19), and the induction of autoimmunity (20, 21). It remains unclear what mechanism determines whether in vivo self-presentation leads to activation or to peripheral tolerance of autoreactive T cells.

To date, in vitro studies have demonstrated that TCR engagement of T cell clones in the absence of costimulation induces anergy (22–25). In this context, it has been suggested that the response of autoreactive T cells depends on either the activation status (resting or activated) or maturation stage of APCs (26), which influence the expression level of costimulatory molecules. As for dendritic cells (DCs),[3] it was demonstrated that immature DCs, which are able to efficiently phagocytose apoptotic (27) as well as necrotic cells (28), undergo maturation only when exposed to the latter (29) or to massive apoptotic cells (30). Thus, under inflammation-free conditions, in which few necrotic cells are generated, maturation of DCs that phagocytosed apoptotic cells derived from self-tissues might be impaired (31), resulting in avoidance of stimulatory self-presentation. Resting B cells are also suggested to be involved in T cell tolerance (32, 33), probably due to their low expression of costimulatory molecules.

It has been documented that this tolerizing stimulus induces proliferation of T cell clones either weakly (25) or not at all (22, 23), suggesting that generation of anergic T cells in vitro is not linked to cell cycle progression. However, previous studies using neo-self transgenic mice indicated that autoreactive T cells undergo transient clonal expansion followed by clonal elimination and anergy of the remaining population (10, 11, 14–16). Furthermore, self-Ags were shown to be highly expressed on DCs in vivo (34, 35), and were able to be presented to CD4$^+$ T cells in vivo by DCs which included DCs expressing B7 costimulatory molecules (36–38), compared with immature DCs generated in vitro by cytokines (39, 40). Thus, to understand in vivo CD4$^+$ T cell tolerance, it is

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[3] Abbreviations used in this paper: DC, dendritic cell; TgDC, DC derived from transgenic mice; WTDC, DC derived from wild-type mice; pepDC, $OVA_{323-339}$-pulsed WTDC.

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important to elucidate the association between cell cycle progression and differentiation into effector/memory T cells vs anergic T cells. In most studies using in vivo models, it was not clear whether anergy might be a transient state which precedes elimination, or if anergic T cells might be a distinct subpopulation that did not proliferate, probably due to the absence of costimulation or other ontogeny.

It has been demonstrated that altered peptide ligands induce T cell unresponsiveness (41, 42). In this context, a recent study reported that low numbers of agonist ligands induced T cell anergy (43). Regarding the case of Ag-presentation on MHC class II molecules to $CD4^+$ T cells, Ag localization (extracellular vs intracellular) was demonstrated to significantly influence the expression level (6) and processing (4–6) of Ags. Especially among intracellular Ags, compartmentalized Ags appeared to be expressed at lower levels, as shown in the model of hen egg white lysozyme (6). Thus, the findings of previous experiments, most of which used peptide-pulsed APCs or mice bearing soluble/membrane-bound neo-self Ags, might be difficult to apply directly to understanding $CD4^+$ T cell tolerance to intracellular autoantigens.

To investigate how a nuclear autoantigen leads to peripheral $CD4^+$ T cell tolerance, we generated transgenic mice (Ld-nOVA mice) expressing chicken egg OVA mainly in the nuclei. We used $CD4^+$ T cells obtained from DO11.10 TCR mice, which express a TCR specific for the $OVA_{323-339}$ dominant epitope bound to $I\text{-}A^d$ class II MHC molecules (44, 45), and performed adoptive transfer experiments. Our results show that proliferation stimulated by nuclear autoantigen-bearing DCs leads to a persistent anergic state of autoreactive $CD4^+$ T cells in vivo, suggesting that the property of nuclear autoantigens which controls the tolerance of CD4 T cells might be the low and continuous expression of a self-peptide on DCs.

Materials and Methods

Mice

BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). Mice were maintained in a temperature- and light-controlled environment with free access to food and water under specific pathogen-free conditions. Female age-matched mice were used in all experiments, and the mice were 7- to 10-wk old at the start of each experiment. DO11.10 transgenic mice, whose T cells express receptors specific for OVA, were kindly provided by Dr. T. Watanabe (Institute of Bioregulation, Kyushu University, Fukuoka, Japan), and were bred in our animal facility.

Generation of transgenic mice

Chicken egg OVA cDNA (kindly provided by Dr. P. Chambon, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Universite Louis Pasteur, Strasbourg, France) fused with the nuclear localization signal at the 3′-end was subcloned into pLG-Eμ, which had been produced by inserting a human Eμ enhancer into the 5′-end of the L^d class I promoter of pLG-2 plasmid. This OVA transgene construct was microinjected into the pronuclei of fertilized eggs from C57BL/6 mice. The microinjected eggs were transferred into the oviducts of pseudopregnant females. Mice carrying the transgene were identified by either Southern blot analysis or PCR analysis of tail DNA. Ld-nOVA BALB/c mice were produced by crossing Ld-nOVA C57BL/6 mice with normal BALB/c mice for less than eight generations.

Preparation of cell populations

A $CD4^+$ T cell population was prepared by negative selection with MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) using anti-CD19 mAb (BD PharMingen, San Diego, CA), anti-$I\text{-}A^d$ mAb (BD PharMingen), and anti-CD8 mAb (BD PharMingen). Naive $CD4^+$ T cells were positively selected from purified $CD4^+$ T cells with MACS using anti-CD45RB mAb (BD PharMingen). DCs were prepared as previously described (34, 36–38). Briefly, spleen cells or lymph node cells were digested with collagenase and DNase I at 37°C for 20 min, further dissociated in Ca^{2+}-free medium in the presence of EDTA at 4°C. Low density cells were selected by centrifugation in 14.5% metrizamide medium, followed by negative selection with MACS using anti-TCR β (BD PharMingen), anti-CD19 mAb, and overnight culture. Nonadherent cells were collected and used as a DC-enriched population. Adherent cells were collected as macrophages. Alternatively, DCs were freshly prepared by positive selection with MACS using N418 mAb (American Type Culture Collection, Manassas, VA) from low density cells. For peptide-pulsing, DCs were incubated in a tissue culture medium containing the indicated concentration of $OVA_{323-339}$ peptide for 2 h at 37°C, and then washed twice before injection.

Transfer experiments

For the transfer of $KJ1\text{-}26^+CD4^+$ cells into Ld-nOVA mice, naive $CD4^+$ T cells from the spleens of DO11.10 mice were prepared and resuspended in PBS. Then 5×10^6 cells were i.v. injected into Ld-nOVA mice and nontransgenic littermates. Cell viability was always <97%, as determined by trypan blue exclusion.

Proliferation assay

$CD4^+$ T cells from spleens were cultured at 4×10^5 cells/well with various concentrations of OVA and irradiated syngeneic spleen cells (5×10^5 cells/well) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FCS, and 5×10^{-5} M 2-ME for 3 days, followed by a final 16 h of culture in the presence of 1 μCi of [^{3}H]TdR per well. The incorporated radioactivity was counted with a gamma scintillation counter. The proliferative response was expressed as Δ cpm (mean cpm of the test cultures minus mean cpm of the control cultures without Ag) $\pm$ SD.

Assay for suppressive activity

Anergic $KJ1\text{-}26^+$ T cells positively selected from the Ld-nOVA recipients (2×10^4 cells/well) and $CD4^+CD25^-$ T cells from wild-type mice (2×10^4 cells/well) were cultured with irradiated syngeneic spleen cells (5×10^4 cells/well) in the presence of anti-CD3 mAb (10 μg/ml) for 3 days, followed by a final 6 h of culture in the presence of 1 μCi of [^{3}H]TdR per well. Three individual recipients were used. The proliferative response was expressed as Δ cpm (mean cpm of the test cultures minus mean cpm of the control cultures without anti-CD3 mAb) $\pm$ SD.

Flow cytometry

The following Abs were used for identification and phenotypic analysis of transferred T cells: FITC-conjugated or biotinylated KJ1-26; PE-conjugated anti-CD4, -CD25, -CD44, -CD62L, and -CD45RB (BD PharMingen); and streptavidin-Tricolor (Caltag Laboratories, Burlingame, CA). For CFSE-labeling (Molecular Probes, Eugene, OR), cells were resuspended in PBS at 10^7/ml and incubated with CFSE at a final concentration of 5 μM for 30 min at 37°C, followed by two washes in PBS.

Immunoprecipitation

Thymus, spleen, liver, and kidney were homogenized and solubilized in RIPA lysis buffer (20 mM Tris, pH 8.0, 0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 10 μg/ml leupeptin, and 1 mM PMSF). Whole cell lysates were immunoprecipitated with either anti-OVA polyclonal Ab (Cappel, Aurora, OH) or anti-OVA mAb (gifts from Dr. H. Karasuyama (Department of Immune Regulation, Tokyo Medical and Dental University, Tokyo, Japan) and Dr. T. Azuma (Research Institute for Biological Sciences, Science University of Tokyo, Noda, Chiba, Japan)). The immunoprecipitates were resolved by 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were incubated with anti-OVA polyclonal Ab and visualized with HRP-conjugated secondary Ab and ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

ELISA

The serum OVA concentration was assayed by sandwich ELISA. Briefly, 96-well plates (Immulon4; Dynatech, Chantilly, VA) were coated with anti-OVA capture mAb (Sigma Aldrich, St. Louis, MO) in 0.03 M carbonate buffer at pH 9.6 by overnight incubation at 4°C. After blocking with 1% BSA for 2 h at 37°C, the plates were incubated with mouse serum samples for 1 h at 37°C. After washing five times with 0.05% Tween 20 in PBS, the plates were incubated with rabbit anti-OVA detection polyclonal Ab (Cappel). The bound OVA was visualized with anti-rabbit IgG Ab coupled to HRP (Zymed Laboratories, San Francisco, CA), followed by development with 3,5,3′,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The OD was read at 450 nm. All samples were tested in duplicate.

Intracellular cytokine staining

$CD4^+$ T cells (5×10^6 cells) were stimulated with plate-bound anti-CD3 mAb for 20 h. Brefeldin A (10 μg/ml) and monensin (1 μM) were added 10 h before harvesting. Cells were first stained with FITC-conjugated KJ1-26 and biotinylated anti-CD4, followed by streptavidin-Tricolor. Stained cells were fixed in 4% paraformaldehyde, lysed in 0.5% saponin/1% BSA/0.1% NaN_3, and incubated with PE-conjugated anti-IL-2 (BD PharMingen).

Results

Generation of Ld-nOVA mice systemically expressing a nuclear neo-autoantigen

To generate transgenic mice showing systemic expression of a nuclear neo-autoantigen, we fused a nuclear localization signal to the 3′-end of chicken egg OVA cDNA and then subcloned into the cDNA downstream of the human Eμ enhancer and L^d class I promoter. Mice carrying this OVA transgene construct were generated, backcrossed to BALB/c background, and termed Ld-nOVA mice. We examined the subcellular localization of OVA in Ld-nOVA mice by immunofluorescence microscopy (Fig. 1*A*). Nuclear expression of OVA was confirmed in most spleen cells, although OVA was expressed not only in the nuclei but also in the cytoplasm to a lesser extent. When whole cell lysates of spleen, thymus, liver, and kidney from Ld-nOVA mice were immunoprecipitated and probed with anti-OVA Ab, OVA expression was detected in multiple organs, probably due to the L^d class I promoter (Fig. 1*B*). To exclude the possibility that OVA is secreted into the peripheral blood at a significant level, we examined whether OVA could be detected in the peripheral blood by sandwich ELISA. OVA was not detected in the sera of Ld-nOVA mice (>2 ng/ml) (data not shown). These results indicated that Ld-nOVA mice are a novel transgenic model which expresses a systemic intracellular, predominantly nuclear, neo-autoantigen.

Ld-nOVA mice are tolerant to OVA

To investigate whether the nuclear neo-autoantigen was able to induce tolerance, Ld-nOVA mice and wild-type mice were s.c. immunized with 100 μg of OVA in CFA at the base of the tail, and the draining lymph node cells were stimulated with various doses of OVA or $OVA_{323\text{-}339}$ 10 days after immunization. The proliferative responses were greatly reduced in Ld-nOVA mice in comparison with wild-type mice (Fig. 1*C*). This result indicates that Ld-nOVA mice are tolerant to OVA as well as to $OVA_{323\text{-}339}$, which is a major antigenic determinant of OVA immunized exogenously.

Transferred $KJ1\text{-}26^+CD4^+$ cells persist in Ld-nOVA mice after transient proliferation

To investigate peripheral tolerance to a nuclear autoantigen, we performed adoptive transfer experiments using Ld-nOVA mice as recipients. Naive $CD4^+$ T cells (5×10^6 cells) from DO11.10 TCR mice, which express a TCR specific for $OVA_{323\text{-}339}$ bound to $I\text{-}A^d$ class II MHC molecules, were i.v. transferred to Ld-nOVA mice and nontransgenic littermates. The fate of the transferred cells was followed by staining $CD4^+$ T cells of the recipients with a clonotype-specific mAb, KJ1-26. The percentage of $KJ1\text{-}26^+CD4^+$ cells in the total $CD4^+$ T cells of spleens from the recipients was determined by flow cytometry at several time points. As shown in Fig. 2, in the Ld-nOVA recipients, the percentage of $KJ1\text{-}26^+CD4^+$ cells started to increase 3 days after transfer and peaked on days 10–14. Evaluation of the total number of $KJ1\text{-}26^+CD4^+$ cells revealed that the kinetics of the total cell number was the same as that of the percentage (data not shown). In contrast, in the nontransgenic recipents, $KJ1\text{-}26^+CD4^+$ cells were always <0.5%. The number and percentage of $KJ1\text{-}26^+CD4^+$ cells in Ld-nOVA recipients increased 20-fold by day 14. Although the majority of $KJ1\text{-}26^+CD4^+$ cells disappeared after day 14, a substantially larger number of $KJ1\text{-}26^+CD4^+$ cells remained in the periphery of the Ld-nOVA recipients than the nontransgenic recipients. This population was able to be detected at least 3 mo later in the Ld-nOVA recipients, but not in the nontransgenic littermates. This result shows that a population of $KJ1\text{-}26^+$ $CD4^+$cells can persist in Ld-nOVA mice after transient proliferation.

Persisting transferred $KJ1\text{-}26^+CD4^+$ cells are hyporesponsive in Ld-nOVA mice

To address the question of whether the persisting transferred $KJ1\text{-}26^+CD4^+$ cells are anergic or not, the following experiments were performed. First, we examined the proliferative responses of

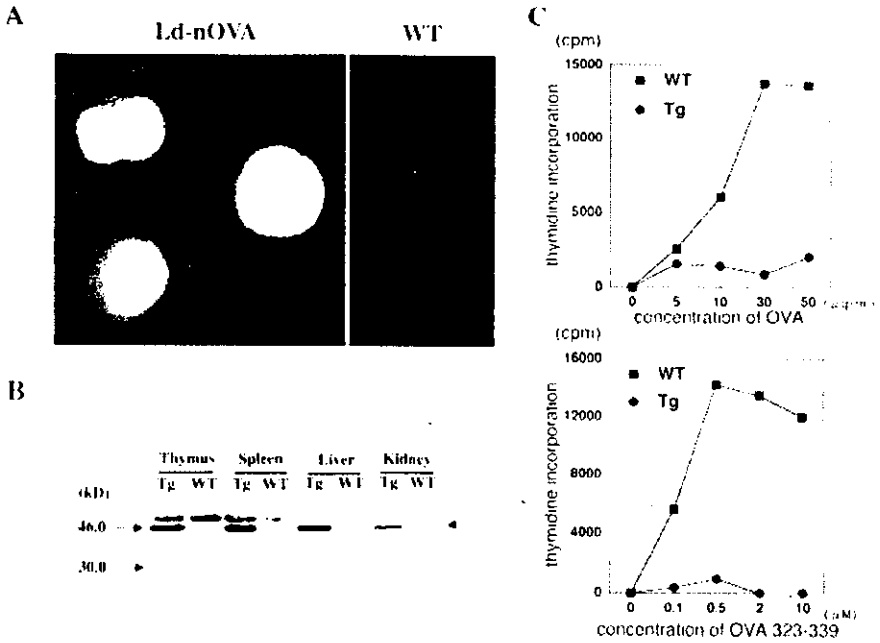

FIGURE 1. Ld-nOVA mice express OVA systemically in nuclei and are tolerant to OVA. *A*, Nuclear localization of OVA in the spleen cells of Ld-nOVA mice. OVA expressed in the spleen cells from Ld-nOVA mice was detected by immunofluorescence microscopy. The band corresponding to OVA is indicated by an arrow. *B*, OVA expression in multiple organs of Ld-nOVA mice. Total cell lysates of spleen, thymus, liver, and kidney from Ld-nOVA mice were immunoprecipitated and detected with anti-OVA polyclonal Ab. *C*, Tolerance to OVA in Ld-nOVA mice. Ld-nOVA mice (●) and wild-type mice (■) were immunized with 100 μg of OVA emulsified 1:1 (v/v) in CFA at the base of the tail. Proliferative responses of draining lymph node cells stimulated with OVA or $OVA_{323\text{-}339}$ were measured by thymidine incorporation at 10 days after the immunization.

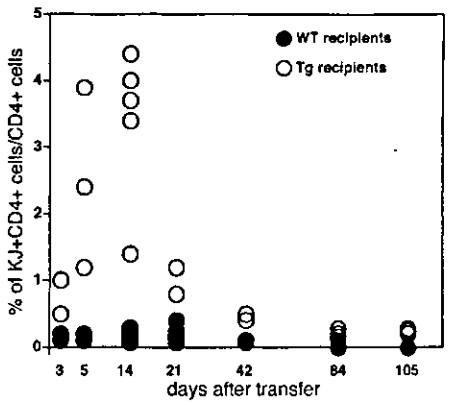

FIGURE 2. KJ1-26$^+$CD4$^+$ cells persist in Ld-nOVA mice after transient proliferation. Naive CD4$^+$ T cells (5×10^6 cells) from spleens of DO11.10 mice were i.v. injected into Ld-nOVA mice (○) and nontransgenic littermates (●). The percentage of KJ1-26$^+$CD4$^+$ cells in CD4$^+$ spleen cells of the recipients was determined at the time points indicated on the abscissa.

CD4$^+$ T cells from the spleens of recipients to $OVA_{323-339}$ in vitro. Because the number of KJ1-26$^+$CD4$^+$ cells remaining in the transgenic mice was larger than that of KJ1-26$^+$ cells in the control mice, we calculated the values of incorporated thymidine corresponding to the response of 10^3 KJ1-26$^+$CD4$^+$ cells. As shown in Fig. 3*A*, the remaining KJ1-26$^+$CD4$^+$ cells recovered from Ld-nOVA mice gave a lower response (~20 times lower on day 14) than those from nontransgenic mice. These proliferative responses were not recovered by the addition of IL-2 (data not shown). Second, we performed intracellular IL-2 staining of remaining KJ1-26$^+$CD4$^+$ T cells stimulated by anti-CD3 mAb in vitro. The IL-2 production gated for KJ1-26$^+$ cells recovered from the Ld-nOVA recipients was impaired in contrast to KJ1-26$^+$ cells recovered from the nontransgenic recipients, as shown in Fig. 3*B*. We were unable to detect IFN-γ, IL-4, or IL-10 in the culture supernatants, or by intracellular cytokine staining of KJ1-26$^+$ cells (data not shown), indicating that the remaining cells in Ld-nOVA mice did not show immune deviation. Third, we examined the ability of transferred KJ1-26$^+$CD4$^+$ T cells to respond to OVA in vivo. To prevent OVA expression in Ld-nOVA mice from influencing the outcome of in vivo antigenic stimulation, readoptive transfer experiments were performed. On day 14 after the initial transfer, 10^7 CD4$^+$ T cells were collected from Ld-nOVA recipients as well as from the control, and adoptively transferred into wild-type mice. The wild-type recipients were simultaneously injected with OVA/IFA. Five days after the re-adoptive transfer, draining lymph node cells were collected from the wild-type recipients and stained with

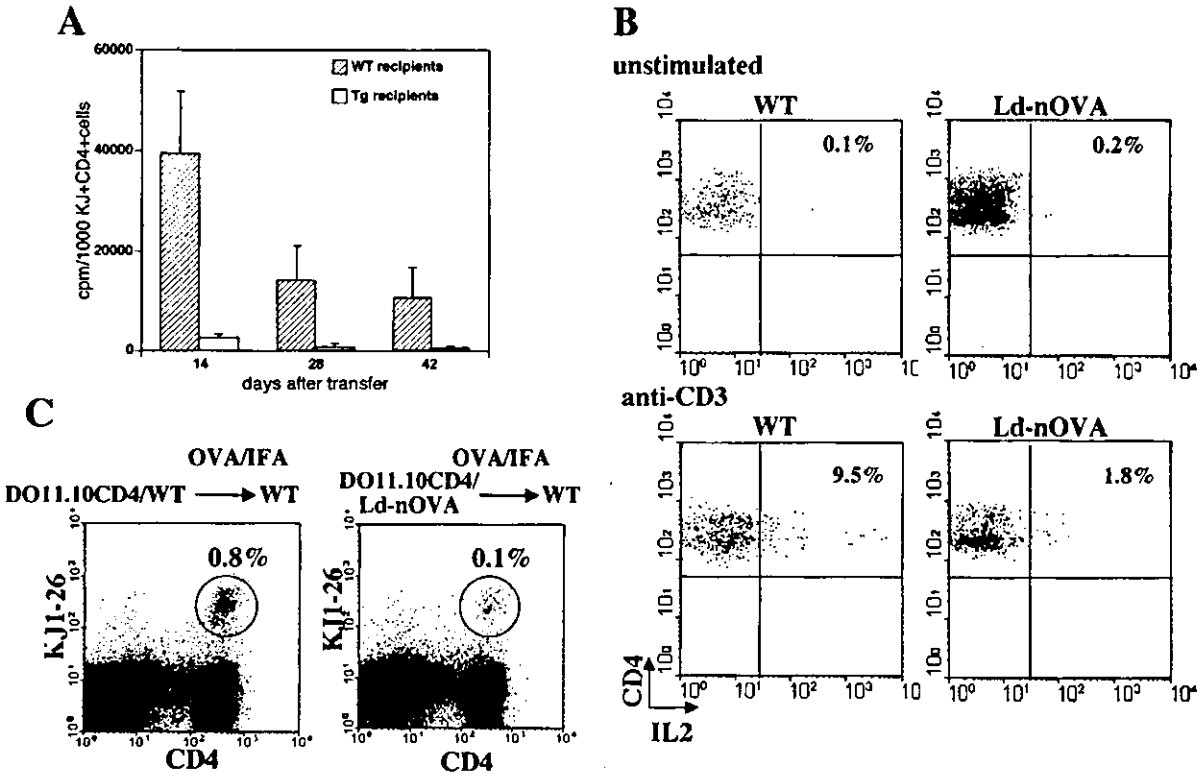

FIGURE 3. Transferred KJ1-26$^+$CD4$^+$ T cells are rendered hyporesponsive in Ld-nOVA recipients. *A*, Impaired proliferative responses of KJ1-26$^+$CD4$^+$ T cells recovered from Ld-nOVA recipients. At different time points after the transfer, CD4$^+$ T cells from the spleens of the recipients were cultured with $OVA_{323\ 339}$ and irradiated syngenic splenocytes for 3 days. Proliferative responses were measured by thymidine incorporation. All values were calculated to correspond to the response of 10^3 KJ1-26$^+$CD4$^+$ cells. *B*, Impaired IL-2 production of KJ1-26$^+$CD4$^+$ cells recovered from Ld-nOVA recipients. CD4$^+$ T cells (5×10^6 cells) recovered from the recipients were stimulated with anti-CD3 mAb for 20 h. Cells were first stained with FITC-conjugated KJ1-26 and biotinylated anti-CD4, followed by streptavidin-Tricolor. Stained cells were fixed, lysed, and incubated with PE-conjugated anti-IL-2. *C*, Impaired proliferation of KJ1-26$^+$CD4$^+$ cells recovered from Ld-nOVA recipients in readoptive transfer experiments into wild-type mice. On day 14 after the transfer, CD4$^+$ T cells were collected from the spleens of the recipients and adoptively transferred into wild-type mice. Wild-type recipients were simultaneously injected with OVA/IFA. Five days after readoptive transfer, draining lymph node cells were collected from the recipients and stained with anti-CD4 mAb and KJ1-26.

anti-CD4 mAb and KJ1-26. Fig. 3*C* shows that in vivo accumulation of KJ1-26$^+$CD4$^+$ T cells recovered from the Ld-nOVA recipients (0.1%) was impaired in comparison with KJ1-26$^+$CD4$^+$ T cells from the nontransgenic recipients (0.8%). Since the number of KJ1-26$^+$CD4$^+$ T cells within the retransferred CD4$^+$ T cells recovered from the Ld-nOVA recipients was 20-fold larger than that of KJ1-26$^+$CD4$^+$ T cells from the nontransgenic recipients, as shown in Fig. 2, the ability of KJ1-26$^+$CD4$^+$ T cells recovered from Ld-nOVA recipients to accumulate and proliferate in vivo by antigenic stimulation must be greatly impaired in comparison with the control. These lines of evidence indicate that autoreactive T cells specific for a nuclear autoantigen are rendered anergic in the periphery and persist in vivo.

Persistent anergic KJ1-26$^+$CD4$^+$ T cells are Ag-experienced and effectively divided cells

Next, we addressed the question of whether the persisting anergic KJ1-26$^+$CD4$^+$ T cells underwent antigenic stimulation enough to induce cell division or whether they were rendered anergic due to ineffective proliferative stimuli. First, we examined the expression of CD44, CD62L, and CD45RB, indicative of TCR engagement, on KJ1-26$^+$CD4$^+$ T cells of recipients 28 days after the transfer. Fig. 4*A* shows that KJ1-26$^+$CD4$^+$ T cells from the Ld-nOVA recipients expressed lower levels of CD62L and CD45RB and a higher level of CD44 than KJ1-26$^+$CD4$^+$ T cells from the nontransgenic recipients. These results indicate that persistent anergic KJ1-26$^+$CD4$^+$ T cells are Ag-experienced cells. In addition, we labeled CD4$^+$ T cells from DO11.10 mice with CFSE before transfer into Ld-nOVA mice and nontransgenic littermates. Since the progeny of a CFSE-labeled cell retains half of the initial fluorescence, the fluorescence intensity provides a quantitative measurement of the strength of the proliferative response. On day 28 after transfer, histograms of the CFSE fluorescence intensity were gated for KJ1-26$^+$CD4$^+$ cells from the spleens of recipients. As shown in Fig. 4*B*, the fluorescence intensity of persisting KJ1-26$^+$CD4$^+$ T cells in Ld-nOVA mice decreased almost equally to that of the nontransgenic recipients with OVA/CFA immunization as a positive control. This suggests that all of the persisting KJ1-26$^+$CD4$^+$ T cells underwent multiple cell divisions. These results show that the persisting KJ1-26$^+$CD4$^+$ T cells in the Ld-nOVA recipients underwent antigenic stimulation which was sufficient to induce multiple cell divisions. We next investigated whether anergic KJ1-26$^+$CD4$^+$ T cells had the ability to suppress the activation of T cells as demonstrated in CD4$^+$CD25$^+$ regulatory T cells, because a recent study showed that repeated stimulation by immature DCs induced CD4$^+$CD25$^+$ regulatory T cells (40). The persisting KJ1-26$^+$CD4$^+$ T cells in the Ld-nOVA recipients did not express CD25 as shown in Fig. 4*C*. Positively selected KJ1-26$^+$ T cells from the Ld-nOVA recipients and CD4$^+$CD25$^-$ T cells from wild-type mice were cultured with irradiated syngeneic spleen cells in the presence of anti-CD3 mAb for 3 days. Fig. 4*D* showed that persisting anergic KJ1-26$^+$CD4$^+$ T cells did not suppress the activation of CD4$^+$CD25$^-$ T cells. These results indicated that anergic KJ1-26$^+$CD4$^+$ T cells generated in the the Ld-nOVA recipients were not suppressive.

DCs present the nuclear autoantigen most efficiently

Next, we investigated which cell population can present a nuclear Ag to KJ1-26$^+$CD4$^+$ T cells, leading to multiple cell divisions. To compare the ability of Ag-presentation of the nuclear autoantigen in vitro among various populations of APCs, CD4$^+$ spleen cells from DO11.10 mice were cultured with irradiated splenic DCs, lymph node DCs, splenic macrophages, peritoneal macrophages, and B cells from Ld-nOVA mice, and the proliferative responses

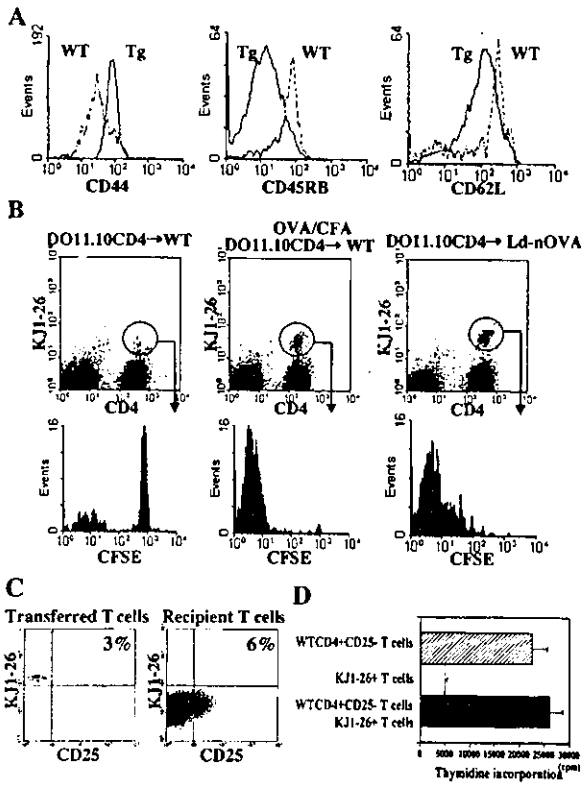

FIGURE 4. Persistent anergic KJ1-26$^+$CD4$^+$ T cells were Ag-experienced and effectively divided cells. *A*, Persistent anergic KJ1-26$^+$CD4$^+$ T cells at day 28 after the transfer show memory/effector phenotype. Expression of CD44, CD62L, and CD45RB on KJ1-26$^+$CD4$^+$ spleen cells from wild-type recipients (dashed line) overlaid with the corresponding stain from Ld-nOVA recipients. *B*, KJ1-26$^+$CD4$^+$ T cells persist in the Ld-nOVA recipients after multiple cell divisions. CFSE-labeled CD4$^+$ spleen cells from DO11.10 mice were adoptively transferred into Ld-nOVA mice and nontransgenic littermates. On day 28 after transfer, CD4$^+$ T cells from the spleens of the recipients were stained with KJ1-26, and histograms of the CFSE fluorescence intensity were gated for KJ1-26$^+$CD4$^+$ cells. *C*, Persistent KJ1-26$^+$CD4$^+$ T cells do not express CD25. Expression of CD25 in transferred KJ1-26$^+$CD4$^+$ spleen T cells and recipient KJ1-26$^-$CD4$^+$ spleen T cells were analyzed by flow cytometry. *D*, Persisting anergic KJ1-26$^+$CD4$^+$ T cells did not suppress the activation of CD4$^+$CD25$^-$ T cells from wild-type mice. Positively selected KJ1-26$^+$ T cells from the Ld-nOVA recipients (2×10^4 cells/well) and CD4$^+$CD25$^-$ T cells from wild-type mice (2×10^4 cells/well) were cultured with irradiated syngeneic spleen cells (5×10^4 cells/well) in the presence of anti-CD3 mAb (10 μg/ml) for 3 days, followed by a final 6 h of culture in the presence of 1 μCi of [^{3}H]-TdR per well. Three individual recipients were used. The proliferative response was expressed as Δ cpm (mean cpm of the test cultures minus mean cpm of the control cultures without anti-CD3 mAb) $\pm$ SD.

were measured. B cells (Fig. 5*A*) and peritoneal macrophages (data not shown) failed to induce proliferation. Although splenic macrophages induced proliferation to some extent, splenic and lymph node DCs showed the most efficient induction of proliferation in vitro, as seen in Fig. 5*A*. The presented data are derived from experiments using DCs purified after overnight culture. However, we performed the same experiments using freshly isolated splenic DCs. Even these DCs could induce proliferation more efficiently than macrophages (data not shown). Both types of DCs expressed IA^d, CD80, CD86, and CD40, indicating that DCs used in this paper were not immature DCs but maturing DCs, although DCs purified after overnight culture showed higher expression levels of

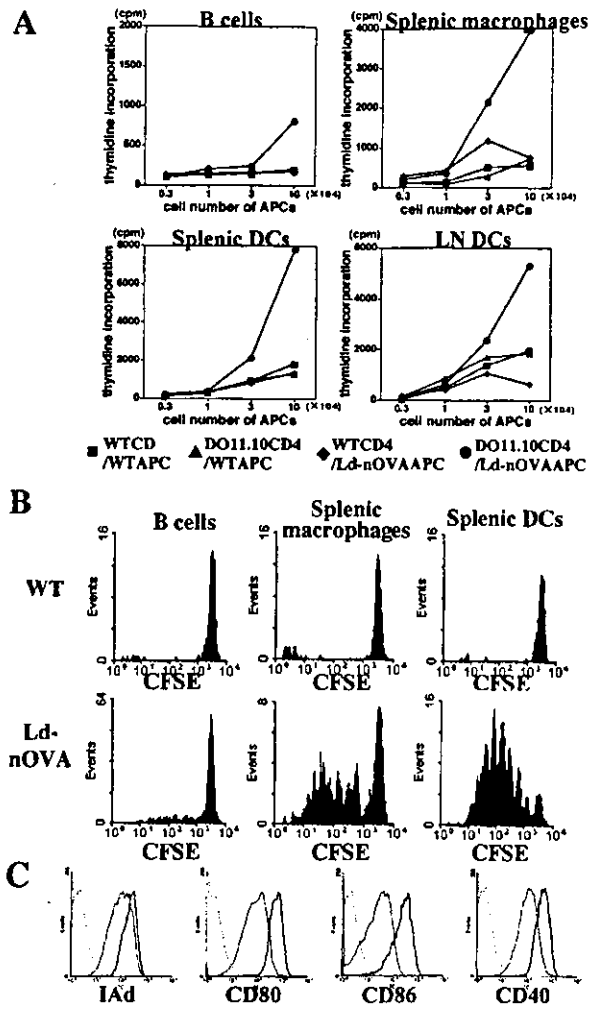

FIGURE 5. DCs most efficiently present the nuclear autoantigen. *A*, In vitro presentation of nuclear Ag by DCs from Ld-nOVA mice. $CD4^+$ spleen cells (5×10^4 cells/well) from DO11.10 mice were cultured with irradiated (1500 rad) B cells, splenic DCs, lymph node DCs, and splenic macrophages (5×10^4 cells/well) from Ld-nOVA mice and nontransgenic littermates (■, WTCD4/WTAPC, ▲, DO11.10CD4/WTAPC, ◆, WTCD4/Ld-nOVAAPC, and ●, DO11.10CD4/Ld-nOVAAPC). Proliferative responses were measured by thymidine incorporation. *B*, In vivo presentation of nuclear Ag by DCs from Ld-nOVA mice. Splenic DCs (10^6 cells), macrophages, B cells, and LPS-activated B cells from Ld-nOVA mice and nontransgenic littermates were i.v. transferred into wild-type mice that had already been injected with CFSE-labeled $CD4^+$ T cells (5×10^6 cells) from the spleens of DO11.10 mice. On day 5 after the transfer, $CD4^+$ T cells from the spleens of the recipients were stained with KJ1-26, and histograms of the CFSE fluorescence intensity were gated for $KJ1\text{-}26^+CD4^+$ cells. *C*, Expression of IA^d, CD80, CD86, and CD40 in DCs purified after overnight culture and freshly isolated DCs. DCs were double stained for N418 and the indicated markers. Analysis gates were set within $N418^+$ population. Freshly isolated DCs (solid line) were stained for the indicated markers and with an isotype Ab (dashed line). The expression of the indicated markers in DCs purified after overnight culture (bold line) overlaid.

IA^d, CD80, and CD86 than freshly isolated DCs as shown in Fig. 5*C*. This was consistent with the finding that DCs purified after overnight culture had a higher ability for Ag presentation than freshly isolated DCs (data not shown). To confirm the ability of DCs to present a nuclear autoantigen in vivo, we performed Ag-presenting cell transfer into wild-type mice which had already been injected with CFSE-labeled $CD4^+$ T cells from DO11.10 mice. On day 5 after APC transfer, $CD4^+$ T cells from the spleens of the recipients were stained with KJ1-26, and histograms of the CFSE fluorescence intensity were gated for $KJ1\text{-}26^+CD4^+$ cells. Fig. 5*B* shows that DCs from Ld-nOVA mice presented the nuclear Ag most efficiently in vivo, as demonstrated in vitro. Freshly isolated DCs from Ld-nOVA mice could also induce proliferation (data not shown). By flow cytometry analyses we confirmed that there is no difference in the expression level of IA^d, CD80, and CD86 between DCs from transgenic mice and from their littermates. These in vitro and in vivo data indicate that DCs are at least one population of the cells able to present the nuclear Ag efficiently, leading to multiple cell divisions of Ag-specific T cells.

T cells stimulated by DCs from Ld-nOVA failed to elicit an enhanced secondary in vitro response, whereas T cells stimulated by peptide-pulsed DCs did elicit an enhanced secondary in vitro response

Since DO11.10 $CD4^+$ T cells efficiently proliferated by stimulation of DCs from Ld-nOVA, we addressed the ability of these divided T cells to respond to secondary stimulation. We prepared three sorts of DCs: DCs from Ld-nOVA mice (TgDCs), DCs from wild-type mice (WTDCs) as an autoantigen-negative control, and $OVA_{323-339}$-pulsed WTDCs (pepDCs) as an autoantigen-positive control. These DCs were transferred into wild-type mice that had already been injected with $CD4^+$ T cells from DO11.10 mice. On day 21 after transfer, $CD4^+$ T cells from the spleens of the recipients were analyzed. The numbers of remaining $KJ1\text{-}26^+CD4^+$ cells in the TgDC and pepDC recipients were the same and were significantly higher than in the WTDC recipients (Fig. 6*A*). The $KJ1\text{-}26^+CD4^+$ cells in the TgDC and pepDC recipients showed the same memory/effector phenotype and >80% of them experienced cell division, suggesting that the majority of the transferred $KJ1\text{-}26^+CD4^+$ cells encountered the transferred DCs at the almost same efficiency (data not shown). However, the proliferative response of the $KJ1\text{-}26^+CD4^+$ cells in the TgDC recipients was lower than that in the pepDC recipients, and did not differ from that in the WTDC recipients (Fig. 6*B*). These results suggest that despite TgDCs and pepDCs having the same ability to generate remaining T cells after proliferative stimuli, they had different influences on the secondary responses of the remaining T cells.

Low expression of $OVA_{323-339}$ on the surface of DCs from Ld-nOVA mice

Since the preparation of DCs and their MHC class II and CD80/CD86 expression levels were almost the same, the expression level of $OVA_{323-339}$ on the surface of DCs is thought to be one of the most likely candidates for explaining their different abilities. Therefore, we investigated the expression level of $OVA_{323-339}$ on the surface of DCs by comparing the proliferative responses of $KJ1\text{-}26^+CD4^+$ clone stimulated by TgDCs with those stimulated by pepDCs. Fig. 7 shows that the expression of $OVA_{323-339}$ on the surface of splenic DCs from Ld-nOVA is functionally equivalent to 50 pM of $OVA_{323-339}$ in culture medium. This result confirms that even low expression of agonistic ligand is sufficient to induce proliferation of Ag-specific T cells, but not to generate T cells having the ability to respond effectively to secondary stimulation.

Repeated encounters with DCs induce tolerance to a nuclear autoantigen

Principally systemic autoantigens are constitutively expressed in vivo. However, previous experiments showed the disapearance of DCs after Ag-specific interaction with T cells in vitro (46) and in vivo (47). Even if DCs failed to encounter Ag-specific T cells, DCs

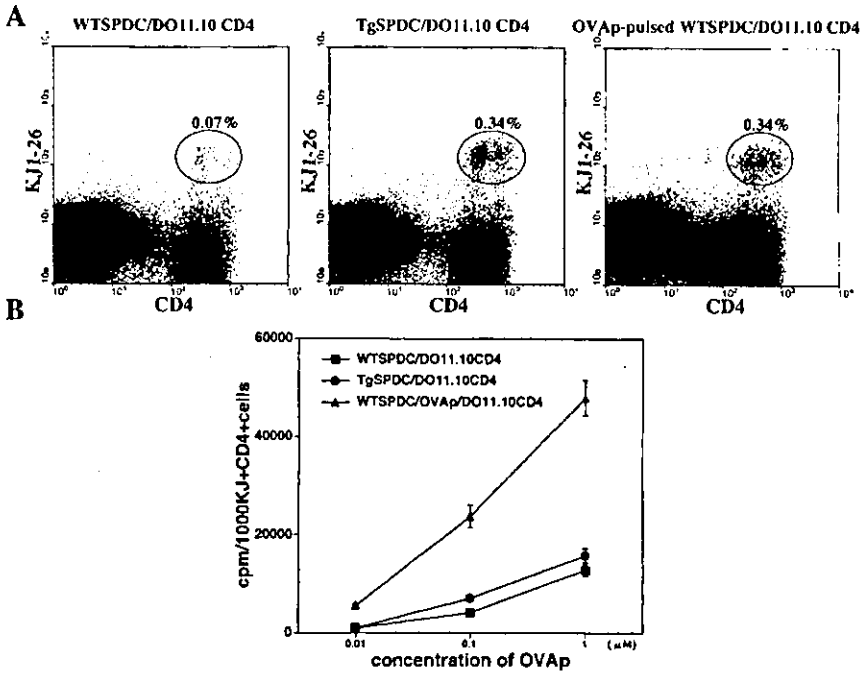

FIGURE 6. T cells stimulated by DCs from Ld-nOVA mice proliferate, but exhibit no enhanced secondary in vitro response compared with T cells stimulated by peptide-pulsed DCs. Wild-type mice which had already been injected with $CD4^+$ T cells from DO11.10 mice were injected with DCs from Ld-nOVA mice (●), WTDCs (■), or pepDCs (▲) which had been incubated in tissue culture medium containing 1 μM $OVA_{323-339}$ peptide for 2 h at 37°C, then washed twice before injection. On day 21 after transfer, $CD4^+$ T cells from the spleens of recipients were stained with KJ1-26 (*A*), or stimulated with $OVA_{323-339}$ and irradiated syngeneic splenocytes for 3 days. Proliferative responses were measured by thymidine incorporation. All values were calculated to correspond to the response of 10^3 KJ1-26^+CD4^+ cells (*B*).

are shown to have a rapid turnover with a $t_{1/2}$ of >1 wk (48–50), except for some DCs such as Langerhans cells. Therefore, to mimic the physiological conditions in our adoptive transfer model, DCs were repeatedly transferred four times at 4-day intervals into wild-type mice which had already been injected with CFSE-labeled $CD4^+$ T cells from DO11.10 mice. To reveal the role of antigenic peptide concencentration on DCs for the tolerance induction, we prepared WTDCs pulsed with various concentrations of $OVA_{323-339}$ (0, 0.04, and 0.2 μM) and TgDCs. Five days after the final transfer, KJ1-26^+CD4^+ T cells from the spleen of recipients were stimulated with $OVA_{323-339}$ and proliferative responses were measured. Fig. 8*A* shows that $CD4^+$ T cells from recipients injected with TgDCs gave a lower response than $CD4^+$ T cells from recipients injected with WTDCs. Moreover, we observed that WTDCs pulsed with a lower concentration of $OVA_{323-339}$ induced a lower response of $CD4^+$ T cells. These results show that continuous expression of antigenic peptides on DCs plays an important role for tolerance induction of nuclear autoantigens, and suggests that a lower concentration of a peptide on DCs might lead autoreactive T cells to a lower state of tolerance. To exclude the possibility that DCs failed to encounter $CD4^+$ T cells from DO11.10 mice, $CD4^+$ T cells from the recipients that were injected with TgDCs and WTDCs were stained with KJ1-26, and histograms of the CFSE fluorescence intensity were gated for KJ1-26^+CD4^+ cells (Fig. 8*B*). KJ1-26^+CD4^+ cells proliferated markedly in recipients injected with DCs from Ld-nOVA mice, and most of the KJ1-26^+CD4^+ cells (92.6%) divided. Therefore, these results indicate that $CD4^+$ T cells which proliferated by repeated encounter with DCs which express a nuclear autoantigen become hyporesponsive. This does not appear to be attributed to the encounter with immature DCs, as shown in Fig. 5*C*. In addition, we again confirmed that tolerized $CD4^+$ T cells did not express $CD25^+$ (<3%), which is a marker of anergic $CD4^+CD25^+$ regulatory T cells (data not shown). This is consistent with the results shown in Fig. 4*C*. Taken together, these results suggest that the property of nuclear autoantigens which controls the tolerance of $CD4^+$ T cells might be the low and continuous expression of a self-peptide on DCs.

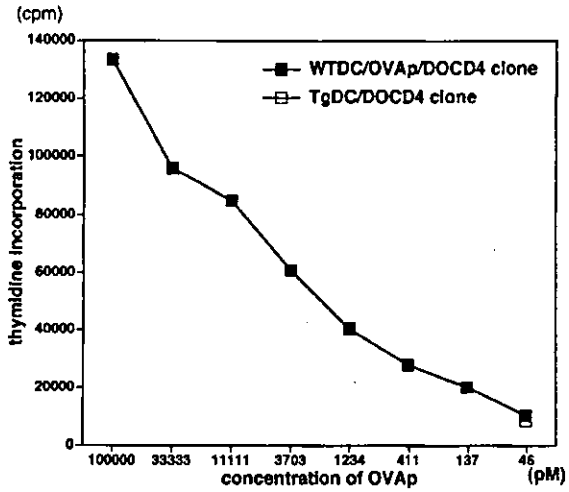

FIGURE 7. Low expression level of $OVA_{323-339}$ on the surface of DCs from Ld-nOVA mice. A KJ1-26^+CD4^+ clone (5×10^4 cells/well) was cultured with DCs (5×10^4 cells/well) from Ld-nOVA mice (□) or pepDCs (5×10^4 cells/well; ■). Proliferative responses were measured by thymidine incorporation.

Discussion

Our transgenic mice are unique in that the neo-self Ag is expressed predominantly in the nuclei. Since Ag localization exerts a significant influence on the expression level (6) and processing (4–6) of Ags, this transgenic model might help us understand systemic autoimmune diseases, which are characterized by the presence of immune responses to nuclear autoantigens. Using these mice, we demonstrated that peripheral tolerance to a nuclear autoantigen was achieved by anergy of the remaining population after transient clonal expansion. Previous studies using neo-self transgenic mice did not clarify whether proliferation and anergy are confined to

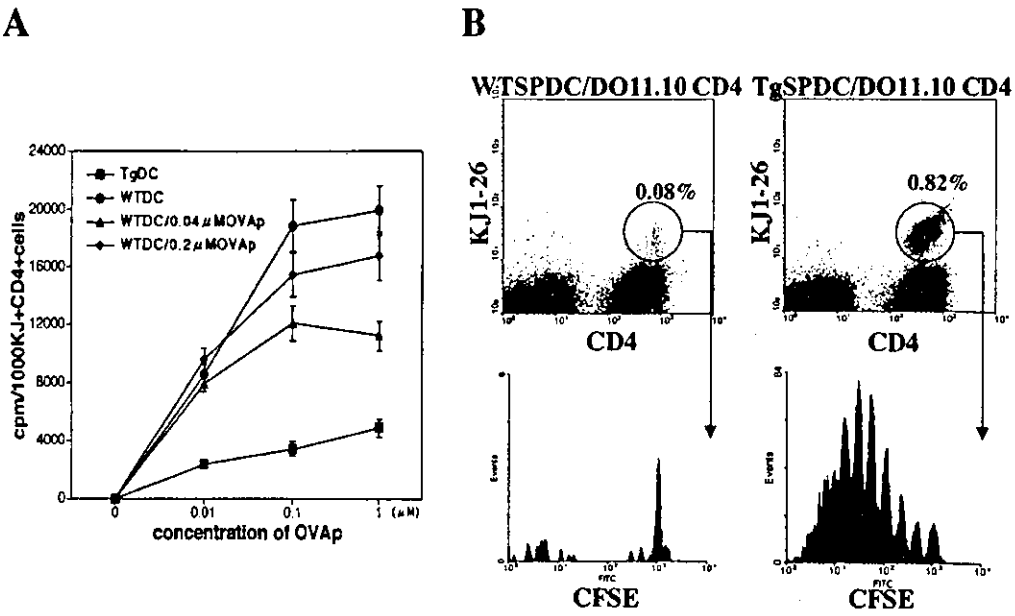

FIGURE 8. Repeated infusions of DCs induce tolerance to a nuclear autoantigen. TgDCs (10^6 cells; ■) and WTDCs pulsed with various concentrations of $OVA_{323-339}$ (0 μM (●), 0.04 μM (▲), and 0.2 μM (◆)) were i.v. transferred four times at 4 day intervals into wild-type mice which had already been injected with CFSE-labeled $CD4^+$ T cells (5×10^6 cells) from the spleens of DO11.10 mice. *A*, On day 5 after the last DC transfer, $CD4^+$ T cells from the spleens of recipients were prepared and stimulated with $OVA_{323-339}$ and irradiated syngeneic splenocytes for 3 days. Proliferative responses were measured by thymidine incorporation. All values were calculated to correspond to the response of 10^3 KJ1-26^+CD4^+ cells. *B*, $CD4^+$ T cells from the recipients which were injected with TgDCs and WTDCs were stained with KJ1-26, and histograms of the CFSE fluorescence intensity were gated for KJ1-26^+CD4^+ cells.

distinct subpopulations or whether the same cell becomes unresponsive after proliferation. However, our present study was able to shed light on the ontogeny of anergic T cells in vivo by analyses of cell division using CFSE-labeling and found that all of the anergic cells underwent cell division.

To date, anergy induced in the absence of costimulation has not been linked to proliferation (28–30, 52). In contrast to the in vitro-generated anergic T cells, $CD4^+$ T cell anergy to a nuclear autoantigen in vivo in our study was not the result of an insufficient proliferative response. It emerged after multiple cell divisions as a generation of Ag-specific memory T cells and persisted for a long period. This is consistent with recent studies using either a soluble OVA peptide injected i.v. (51), or hemagglutinin as a self-Ag expressed on parenchymal cells by the transgenic mice (52). These findings suggest that in addition to mitotic stimuli, the presence or absence of other factors on APC might be involved in induction of anergic $CD4^+$ T cells in vivo. It was demonstrated that DCs present dominant self-peptides of cell surface molecules on MHC class II products (53) as well as foreign-peptides (54), suggesting that DCs have a regulatory or tolerizing role for self-tolerance in the periphery, in addition to induction of responses to foreign Ags. In this context, DCs play an important role in the induction of peripheral tolerance to a nuclear autoantigen. This is because DCs are Ag-presenting cells which can most efficiently induce mitosis of autoreactive T cells responding to a nuclear autoantigen, compared with splenic macrophages and B cells, as indicated in Fig. 5*B*.

How can the self-Ag presentation by DCs be associated with peripheral tolerance? It has been demonstrated that the maturation stage of DCs significantly influences priming of Ag-specific T cells. This finding is thought to be critical for the induction of peripheral tolerance under physiological conditions without proinflammatory stimuli. However, Fig. 5*C* showed that DCs used in our experiments expressed IA^d, CD80, CD86, and CD40, indicating that these DCs were not immature DCs but maturing DCs, although DCs purified after overnight culture showed higher expression levels of these surface molecules than freshly isolated DCs. Futhermore, the results in Fig. 6 cannot to be explained only by this concept. Fig. 6 indicates that TgDCs induced impaired responses to secondary TCR engagement in comparison with peptide-pulsed WTDCs. This might be explained by the difference in peptides presented on TgDCs from peptide-pulsed WTDC. Since it has been demonstrated that processing of an endogenous Ag is different from that of the exogenous form of the same Ag (4–6), antagonistic ligands might be generated in TgDCs and inhibit the immune responses to $OVA_{323-339}$. However, because pepDCs have the same ability as peptide-coated WTDC to induce primary and secondary proliferative responses of DO11.10 $CD4^+$ clones in vitro (data not shown), other explanations are needed.

In contrast to foreign immunogenic Ags, autoantigens, especially nuclear autoantigens, share the following properties: persistent and low-level expression on resting APCs, as suggested in other anergic models (55–58, 43). Fig. 6 suggests that the expression level of an Ag on resting APCs might be important for tolerance induction. A recent study (43) which investigated in vitro anergy induced by a low number of agonistic ligands supports this idea. Furthermore, since repeated transfusions of TgDCs into wild-type mice are able to induce proliferation following tolerization of KJ1-26^+CD4^+ T cells, repeated encounters with proliferative stimuli provided by DCs might be important. We propose that the repeated encounters with DCs mimic the persistence of self-Ag and continuous stimulation by resting DCs. This explanation is supported by several other models (55–58) as follows. It was demonstrated that $CD4^+$ T cell clones which were repeatedly stimulated by agonistic ligands were rendered anergic in vitro (58), and that anergy of $CD8^+$ T cells was induced and maintained by Ag

persistence in vivo (56). Alternatively, repeated transfusion could eventually increase the amount of the self-peptide in vivo to a level which is sufficient to induce tolerance. Further studies are required to elucidate whether this anergy induction mechanism is associated with other mechanisms such as B7/CTLA-4 (59). There are at least two possible pathways for DCs to present a nuclear autoantigen to $CD4^+$ T cells. One is an exogenous Ag presentation pathway via uptake of autoantigen-bearing apoptotic or necrotic cells. The other is an endogenous Ag presentation pathway, in which DCs present an endogenous self-Ag on their own MHC class II products. Although in our transgenic model it is hard to distinguish the two presentation pathways, we suspect the latter based on the following findings. Significant proliferation of CFSE-labeled KJ1-26^+CD4^+ T cells was not observed in recipients to which various numbers of apoptotic cells from Ld-nOVA mice were adoptively transferred, as previously described (Ref. 60 and data not shown). Furthermore, $CD4^+$ T cells from DO11.10 mice did not proliferate when they were cultured with WTDCs which had captured apoptotic cells from Ld-nOVA mice (data not shown).

Since we have not generated other transgenic models which systemically express OVA in extracellular or membrane-bound form, it remains unclear whether the present findings in Ld-nOVA mice are specific for nuclear autoantigens. However, anergy after transient expansion of autoreactive T cells is also observed in other transgenic models, as described previously (10, 11, 14–16). Therefore, this raises the possibility that our findings in the Ld-nOVA mouse model might be applicable to autoantigens expressed in other cell compartments. In systemic autoimmune disease, peripheral tolerance to nuclear autoantigens is disturbed. Thus, we are now investigating the underlying fine mechanisms of $CD4^+$ T cell central and peripheral tolerance to nuclear autoantigens by using this Ld-nOVA transgenic model.

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Generation of $CD4^+CD25^+$ Regulatory T Cells from Autoreactive T Cells Simultaneously with Their Negative Selection in the Thymus and from Nonautoreactive T Cells by Endogenous TCR Expression[1]

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Normal T cell repertoire contains regulatory T cells that control autoimmune responses in the periphery. One recent study demonstrated that $CD4^+CD25^+$ T cells were generated from autoreactive T cells without negative selection. However, it is unclear whether, in general, positive selection and negative selection of autoreactive T cells are mutually exclusive processes in the thymus. To investigate the ontogeny of $CD4^+CD25^+$ regulatory T cells, neo-autoantigen-bearing transgenic mice expressing chicken egg OVA systemically in the nuclei (Ld-nOVA) were crossed with transgenic mice expressing an OVA-specific TCR (DO11.10). Ld-nOVA × DO11.10 mice had increased numbers of $CD4^+CD25^+$ regulatory T cells in the thymus and the periphery despite clonal deletion. In Ld-nOVA × DO11.10 mice, T cells expressing endogenous TCR $\alpha\beta$ chains were $CD4^+CD25^-$ T cells, whereas T cells expressing autoreactive TCR were selected as $CD4^+CD25^+$ T cells, which were exclusively dominant in recombination-activating gene 2-deficient Ld-nOVA × DO11.10 mice. In contrast, in DO11.10 mice, $CD4^+CD25^+$ T cells expressed endogenous TCR $\alpha\beta$ chains, which disappeared in recombination-activating gene 2-deficient DO11.10 mice. These results indicate that part of autoreactive T cells that have a high affinity TCR enough to cause clonal deletion could be positively selected as $CD4^+CD25^+$ T cells in the thymus. Furthermore, it is suggested that endogenous TCR gene rearrangement might critically contribute to the generation of $CD4^+CD25^+$ T cells from nonautoreactive T cell repertoire, at least under the limited conditions such as TCR-transgenic models, as well as the generation of $CD4^+CD25^-$ T cells from autoreactive T cell repertoire. ***The Journal of Immunology,*** **2002, 168: 4399–4405.**

Normal T cell repertoire contains $CD4^+CD25^+$ regulatory T cells that control autoreactive immune responses in the periphery (1–4). Impairment of the generation of $CD4^+CD25^+$ regulatory T cells results in various organ-specific autoimmune diseases, as demonstrated in mice thymectomized on day 3 of life (5). It has been demonstrated that development of regulatory T cells requires the thymus (6–8) and the presence of the relevant autoantigen in the periphery (9). However, development of $CD4^+CD25^+$ regulatory T cells remains poorly understood.

Although $CD4^+CD25^+$ T cells are anergic to TCR stimulation and suppress the activation of $CD4^+CD25^-$ T cells in an Ag-independent manner, it has been demonstrated that regulatory function of $CD4^+CD25^+$ T cells requires their activation via TCR in vitro (10–12). Seddon and Mason (9) observed that peripheral autoantigen is responsible for the survival of specific regulatory T cells in vivo. These findings suggest the critical role of TCR specificity of $CD4^+CD25^+$ regulatory T cells in their generation, survival, and ability to prevent autoimmunity. Recently, Jordan et al. (13) demonstrated that, in the thymus, self-reactive T cells were positively selected as $CD4^+CD25^+$ regulatory T cells and were not deleted. These results suggest that positive selection of $CD4^+CD25^+$ regulatory T cells requires higher avidity interactions of their TCRs with self ligands, but that the required avidity must not exceed the threshold of the deletion (14). However, it is unclear whether avidity of autoreactive TCRs that induce positive selection as $CD4^+CD25^+$ regulatory T cells is different from avidity of autoreactive TCRs that induce negative selection.

The immune system controls autoreactivity by several mechanisms such as clonal deletion and inactivation. Accumulating evidences suggest that receptor editing or revision, which is induced by autoreactive stimuli and involves endogenous TCR gene rearrangement, is also involved in the generation of nonautoreactive T cell repertoire from autoreactive T cell repertoire (15–17). Moreover, it is suggested that secondary TCR gene rearrangement occurs to escape not only from clonal deletion, but also from death by neglect during thymic selection (18, 19). Interestingly, disturbance of endogenous TCR gene rearrangement seems to be associated with the impaired development of regulatory T cells (20–25). Genetic manipulation of TCR α gene, as in TCR α-chain-deficient (20, 23) and 2B4 TCR α-chain transgenic mice (22), sometimes spontaneously induces organ-specific autoimmune diseases, such as inflammatory bowel disease, autoimmune gastritis, and thyroiditis. Itoh et al. (7) found that $CD4^+CD25^+$ T cells in a

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TCR-transgenic model expressed endogenous TCR chains and disappeared in recombination-activating gene 2 (RAG2)[3]-deficient TCR-transgenic mice. These findings have led us to consider how endogenous TCR gene rearrangement controls the generation of $CD4^+CD25^+$ T cells or $CD4^+CD25^-$ T cells, at least, under certain conditions that $CD4^+$ T cells lack the $CD4^+CD25^+$ T cells or $CD4^+CD25^-$ T cells, respectively. However, there are no studies that clearly demonstrate dual roles of endogenous TCR gene rearrangement for the generation of $CD4^+$ T cell repertoire in one model.

To examine the ontogeny of $CD4^+CD25^+$ T cells, we used neo-autoantigen-bearing transgenic mice expressing chicken egg OVA systemically in the nuclei (Ld-nOVA) and transgenic mice expressing an OVA-specific TCR (DO11.10). We found that part of autoreactive T cells could be positively selected as $CD4^+CD25^+$ T cells in parallel with their deletion in the thymus. We also found that endogenous TCR gene rearrangement generates autoreactive $CD4^+CD25^+$ regulatory T cells from nonautoreactive T cells and nonautoreactive $CD4^+CD25^-$ T cells from autoreactive T cells.

Materials and Methods

Mice

BALB/c mice were obtained from SLC (Shizuoka, Japan). They were maintained in a temperature- and light-controlled environment with free access to food and water under specific pathogen-free conditions. Female age-matched mice were used in all experiments, and the mice were 7–10 wk old at the start of each experiment. DO11.10 transgenic mice whose T cells express a receptor specific for OVA were kindly provided by T. Watanabe (Medical Institute of Bioregulation, Kyashu University, Fukuoka, Japan). DO11.10 TCR α single transgenic mice were kindly provided by S. Koyasu (Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan). RAG2-deficient BALB/c mice and TCR α-chain-deficient C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). Generation of Ld-nOVA transgenic mice has been described in another study (26). Briefly, chicken egg OVA cDNA (kindly provided by P. Chambon, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Universite Louis Pasteur, Strasbourg, France) fused with the nuclear localization signal at the 3′ end was subcloned into pLG-Eμ, which had been produced by inserting a human Eμ enhancer into the 5′ end of the L^d class I promoter of pLG-2 plasmid (27). This OVA transgene construct was microinjected into the pronuclei of fertilized eggs from C57BL/6 mice. Ld-nOVA BALB/c mice were produced by crossing Ld-nOVA C57BL/6 mice with normal BALB/c mice for more than eight generations. TCR α-chain-deficient BALB/c mice were produced by crossing TCR α-chain-deficient C57BL/6 mice with normal BALB/c mice for more than six generations.

Preparation of cell populations

Spleen cells were first enriched in T cells by using mouse $CD3^+$ T cell enrichment columns (R&D Systems, Minneapolis, MN). T cells were then stained with FITC anti-CD4 mAb (GK1.5; BD PharMingen, San Diego, CA) and biotin anti-CD25 mAb (7D4; BD PharMingen), followed by staining with anti-FITC microbeads. $CD4^+$ T cells were purified with MACS using a positive selection column (Miltenyi Biotec, Bergisch Gladbach, Germany). For the purification of $CD4^+CD25^+$ T cells, microbeads of purified $CD4^+$ T cells were released by FITC MultiSort kit. $CD4^+$ T cells were stained with streptavidin microbeads, followed by separation with MACS using a positive selection column. The purity of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells was ~88%.

In vitro proliferation assay

$CD4^+$ T cells, $CD4^+CD25^+$ T cells, or $CD4^+CD25^-$ T cells (2×10^4 cells/well) were cultured with irradiated (20 Gy) syngeneic spleen cells (5×10^4 cells/well) in the presence of $OVA_{323-339}$ at 0.5 μM for 3 days, followed by a final 16 h of culture in the presence of 1 μCi [^{3}H]TdR per well. Suppressor cell activity was assessed by coculturing $CD4^+CD25^+$ T cells (2×10^4 cells/well) with $CD4^+CD25^-$ T cells (2×10^4 cells/well) and with irradiated (20 Gy) syngeneic spleen cells (5×10^4 cells/well) in the presence of anti-CD3 mAb (145-2C11) at 10 μg/ml or Con A (Sigma-Aldrich, St. Louis, MO) at 1 μg/ml for 3 days, followed by a final 6 h of culture in the presence of 1 μCi [^{3}H]TdR per well. In some experiments, anti-CTLA-4 mAb (UC10-4F10-11) (100 μg/ml) was added to the culture. Cells were cultured in 96-well round-bottom plates in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FCS, and 5×10^{-5} M 2-ME at 37°C, 5% CO_2. The incorporated radioactivity was counted with a gamma scintillation counter. The proliferative response was expressed as Δcpm (mean cpm of the test cultures minus the mean cpm of the control cultures without Ag).

Flow cytometry

The following Abs were used for identification and phenotypic analysis of T cell populations: FITC-conjugated or biotinylated KJ1-26; FITC-conjugated or PE-conjugated anti-TCRVβ2, anti-TCRVβ6, anti-TCRVβ8, anti-TCRVβ14, anti-TCRVα2; FITC-conjugated or biotinylated anti-TCRβ (H57-597); FITC-conjugated anti-CD25; PE-conjugated anti-CD4; PE-conjugated CD8 (all from BD PharMingen); and streptavidin-Tricolor (Caltag Laboratories, Burlingame, CA).

Results

The numbers of $CD4^+CD25^+$ regulatory T cells increased in the thymus and the periphery of Ld-nOVA × DO11.10 mice

To investigate immunological tolerance to a systemic nuclear autoantigen, which is perturbed in systemic autoimmune diseases, we generated Ld-nOVA transgenic mice expressing OVA systemically in nuclei. We s.c. immunized Ld-nOVA mice and nontransgenic littermates with 100 μg OVA in CFA at the base of the tail and then cultured the draining lymph node cells with various doses of OVA or $OVA_{323-339}$ dominant epitope (OVAp) 10 days after the immunization. The proliferative responses were greatly reduced in the Ld-nOVA mice in comparison with nontransgenic littermates, indicating that Ld-nOVA mice are tolerant to OVA (26).

To address the question as to how autoreactive T cells specific for a nuclear autoantigen were rendered tolerized, we mated Ld-nOVA mice with DO11.10 transgenic mice that express a TCR (Vα13.1, Vβ8.2) specific for the OVAp bound to $I\text{-}A^d$ class II MHC molecules and monitored DO11.10 TCR-bearing cells using an anti-clonotypic Ab, KJ1-26.

Thymocyte numbers in Ld-nOVA × DO11.10 mice were significantly reduced to ~55% of those in DO11.10 mice (1×10^8 cells vs 1.8×10^8 cells, $p < 0.01$) (Table I). In comparison with DO11.10 mice, Ld-nOVA × DO11.10 mice exhibited a reduction in the percentage of CD4 single-positive (SP) (6.7 ± 0.4% vs 9.7 ± 0.9%, $p < 0.01$) (Fig. 1*A*) and CD4 CD8 double-positive thymocytes (61 ± 5.9% vs 69 ± 5.8%, $p < 0.05$), and an increase in the percentage of CD8 SP (2.8 ± 0.4% vs 1.5 ± 0.3%, $p < 0.01$) and CD4 CD8 double-negative thymocytes (29.5 ± 5.3% vs 19.8 ± 3.5, $p < 0.01$). These results indicated autoreactive T cells were negatively selected in the thymus. Splenic $CD4^+$ T cell numbers in Ld-nOVA × DO11.10 mice were reduced to ~45% of those in DO11.10 mice (1.3×10^7 cells vs 2.9×10^7 cells, $p < 0.01$) (Table I) (Fig. 1*A*).

$CD4^+$ T cells from the thymus and the spleen of Ld-nOVA × DO11.10 mice expressed a lower level of clonotypic TCR and Vβ8 than those of DO11.10 mice (clonotypic TCR in CD4 SP thymocytes, 37 vs 75%; Vβ8 in CD4 SP thymocytes, 81 vs 94%; clonotypic TCR in $CD4^+$ splenocytes, 26 vs 67%; Vβ8 in $CD4^+$ splenocytes, 70 vs 88%), despite the same expression level of TCR Cβ (Fig. 1*A*). Moreover, mean fluorescence intensity (MFI) of clonotypic TCR and Vβ8 was markedly reduced in Ld-nOVA × DO11.10 mice compared with DO11.10 mice (MFI of clonotypic TCR in CD4 SP thymocytes = 9.5 vs 32.5, MFI of Vβ8 in CD4

[3] Abbreviations used in this paper: RAG2, recombination-activating gene 2; MFI, mean fluorescence intensity; SP, single-positive.

Table I. *Ld-nOVA × DO11.10 mice have increased numbers of CD4⁺CD25⁺ regulatory T cells in the thymus and the periphery*

Organ	Cell Subset	DO11.10 (n = 7)	Ld-nOVA × DO11.10 (n = 7)
Thymus	Total ($\times 10^8$)	1.8 ± 0.3	1.0 ± 0.2
	$CD4^+$ SP ($\times 10^7$)[a]	1.7 ± 0.8 (9.7)	0.7 ± 0.3 (6.7)
	$CD4^+CD25^+$ ($\times 10^5$)[b]**	2.0 ± 0.9 (1.2)	2.7 ± 0.9 (4.0)
Spleen	Total ($\times 10^8$)	1.2 ± 0.3	1.0 ± 0.3
	$CD4^+$ ($\times 10^7$)[a]	2.9 ± 0.7 (24)	1.3 ± 0.3 (13)
	$CD4^+CD25^+$ ($\times 10^6$)[b]**	1.0 ± 0.3 (3.6)	1.6 ± 0.3 (12.3)
Lymph nodes[c]	Total ($\times 10^6$)	5.4 ± 0.9	4.3 ± 0.8
	$CD4^+$ ($\times 10^6$)[a]	3.3 ± 0.4 (62)	1.5 ± 0.2 (35)
	$CD4^+CD25^+$ ($\times 10^5$)[b]***	1.1 ± 0.2 (3.3)	2.7 ± 0.5 (18.2)

[a] The percentage of $CD4^+CD8^-$ T cells in total thymocytes, splenocytes, or lymph node cells is shown.
[b] The percentage of $CD4^+CD25^+$ T cells in $CD4^+CD8^-$ T cells is shown.
[c] Inguinal, axillary, and para-aorta lymph nodes were collected.
*, $0.2 > p \geq 0.05$; **, $0.05 > p \geq 0.01$; ***, $p < 0.01$. Statistical comparison is by Student's t test.

SP thymocytes = 19.2 vs 36.6, MFI of clonotypic TCR in $CD4^+$ splenocytes = 6.9 vs 20.8, MFI of Vβ8 in $CD4^+$ splenocytes = 13 vs 21.9). These data suggest the expression of endogenous Vαs and Vβs in Ld-nOVA × DO11.10 mice. This was confirmed by the increased expression of endogenous Vαs and Vβs in addition to Vα13.1 and Vβ8.2 in $CD4^+$ T cells of Ld-nOVA × DO11.10 mice (Fig. 1*B*).

We noticed that splenic $CD4^+$ T cells, lymph node $CD4^+$ T cells, and $CD4^+$ SP thymocytes in Ld-nOVA × DO11.10 mice contained a higher percentage of $CD4^+CD25^+$ T cells (12.3, 18.2, and 4%, respectively) than those in DO11.10 mice (3.6, 3.3, and 1.2%, respectively) (Table I). Absolute numbers of $CD4^+CD25^+$ T cells were also increased in Ld-nOVA × DO11.10 mice. Most of clonotypic T cells in DO11.10 mice were $CD4^+CD25^-$ T cells, whereas most of clonotypic T cells in Ld-nOVA × DO11.10 mice were $CD4^+CD25^+$ T cells (Fig. 2*A*). We next investigated whether these $CD4^+CD25^+$ T cells were regulatory T cells. $CD4^+CD25^+$ T cell-depleted $CD4^+$ T cells from Ld-nOVA × DO11.10 exhibited a more vigorous response to OVAp than $CD4^+$ T cells from Ld-nOVA × DO11.10, although they exhibited a lower response than $CD4^+CD25^+$ T cell-depleted DO11.10 $CD4^+$ T cells (Fig. 2*B*). $CD4^+CD25^+$ T cells from Ld-nOVA × DO11.10 mice had the ability to suppress the proliferative responses of $CD4^+CD25^-$ T cells not only from the Ld-nOVA × DO11.10 mice, but also from nontransgenic BALB/c mice. This inhibitory function was partially blocked by anti-CTLA-4 (Fig. 2*C*). Because we did not use anti-CTLA4 Fab, the abrogation of inhibition was not so distinguished as demonstrated in the previous reports (28–30). These results indicate that $CD4^+CD25^+$ T cells generated in Ld-nOVA × DO11.10 mice are regulatory T cells.

Clonotypic cells are positively selected into $CD4^+CD25^+$ regulatory T cells in Ld-nOVA × DO11.10 α-chain mice

To exclude the possibility that the generation of $CD4^+CD25^+$ regulatory T cells in Ld-nOVA × DO11.10 mice could be attributed to excessive production of autoreactive T cells beyond the capacity for clonal deletion, we crossed Ld-nOVA mice with DO11.10 α-chain single transgenic mice and examined CD25 expression of clonotypic T cells in Ld-nOVA × DO11.10 α-chain mice. Although lymph node $CD4^+$ T cells contained the small population of clonotypic T cells in DO11.10 α-chain and Ld-nOVA × DO11.10 α-chain mice, we could find clearly different results between these mice. Clonotypic T cells in DO11.10 α-chain mice were exclusively $CD4^+CD25^-$ T cells, whereas clonotypic T cells in Ld-nOVA × DO11.10 α-chain mice were exclusively $CD4^+CD25^+$ T cells (Fig. 3). These results are consistent with Fig. 2*A* and indicate that part of autoreactive T cells is positively selected as $CD4^+CD25^+$ regulatory T cells. Although $CD4^+CD25^+$

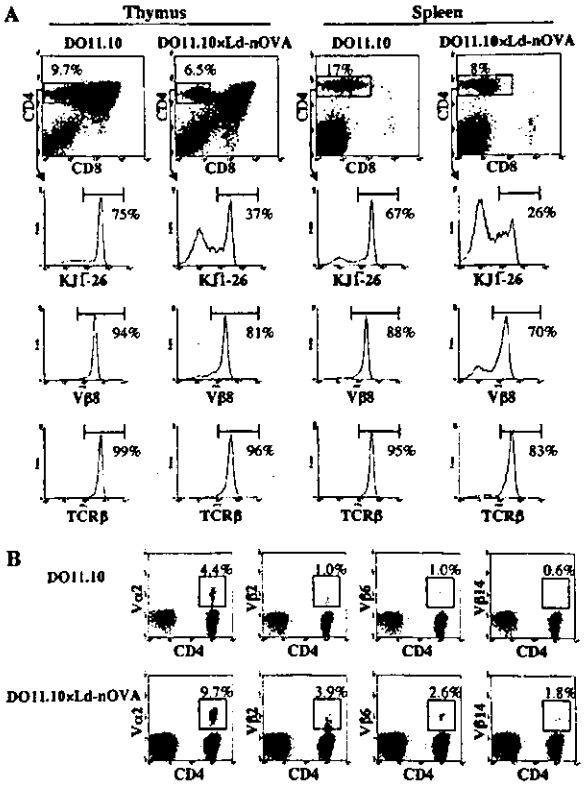

FIGURE 1. Expression of endogenous TCR α- and β-chains in DO11.10 mice and Ld-nOVA × DO11.10 mice. *A*, Thymocyte and splenocyte suspensions prepared from a 2-mo-old DO11.10 mouse and a 2-mo-old Ld-nOVA × DO11.10 mouse were stained with anti-CD4, anti-CD8, and KJ1-26, anti-Vβ8, or anti-TCRβ. Dot plots show staining of live gated cells with anti-CD4 and anti-CD8. The percentage of cells falling within the indicated region is shown in each dot plot. Histograms show staining of $CD4^+CD8^-$ cells with KJ1-26, anti-Vβ8, or anti-TCRβ. The percentage of cells falling within the indicated marker is shown in each histogram. *B*, T cell-enriched splenocytes from a 2-mo-old DO11.10 mouse and a 2-mo-old Ld-nOVA × DO11.10 mouse were stained with anti-CD4 and anti-Vα2, anti-Vβ2, anti-Vβ6, or anti-Vβ14. The percentage of $CD4^+$ T cells falling within the indicated region is shown in each dot plot. A representative result of seven independent similar experiments is shown.

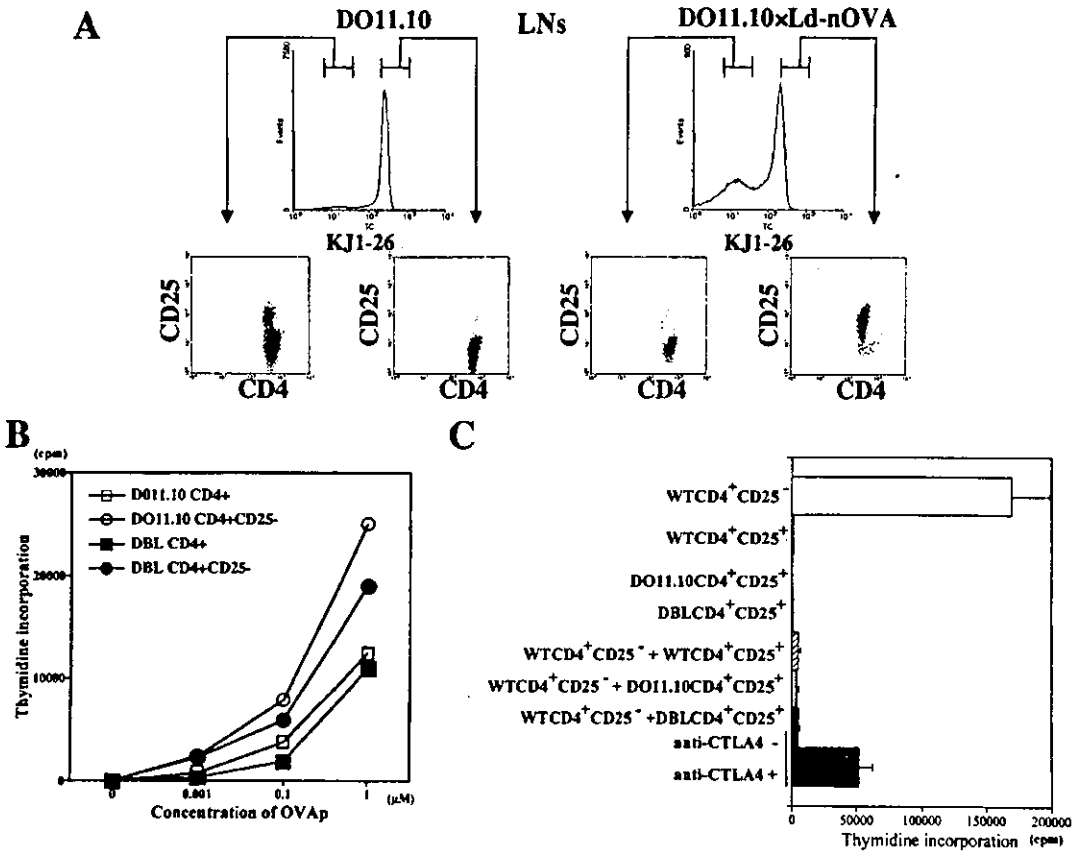

FIGURE 2. $CD4^+CD25^+$ T cells in Ld-nOVA × DO11.10 mice were regulatory T cells. *A*, $CD4^+$ T cells expressing endogenous TCR chains and $CD4^+$ T cells expressing a clonotypic TCR showed different CD25 expression levels in both DO11.10 mice and Ld-nOVA × DO11.10 mice. T cell-enriched splenocytes from a DO11.10 mouse and a Ld-nOVA × DO11.10 mouse were analyzed for the expression of CD25, CD4, and a clonotypic TCR. Histograms show staining of clonotypic and nonclonotypic $CD4^+$ T cell populations with anti-CD4 and anti-CD25. *B*, $CD4^+CD25^+$ T cell-depleted $CD4^+$ T cells from Ld-nOVA × DO11.10 mice exhibited a vigorous response to OVAp. $CD4^+$ T cells or $CD4^+CD25^+$ T cell-depleted $CD4^+$ T cells (2×10^4 cells/well) from three Ld-nOVA × DO11.10 mice and three DO11.10 mice were cultured in 96-well round-bottom plates with irradiated (20 Gy) syngeneic spleen cells (5×10^4 cells/well) in the presence of OVAp for 3 days, followed by a final 16 h of culture in the presence of 1 μCi [^{3}H]TdR per well. *C*, $CD4^+CD25^+$ T cells, $CD4^+CD25^-$ T cells, and a 1:1 mixture of the two populations were cultured in 96-well round-bottom plates with irradiated (20 Gy) syngeneic spleen cells (5×10^4 cells/well) in the presence of Con A at 1 μg/ml for 3 days, followed by a final 6 h of culture in the presence of 1 μCi [^{3}H]TdR per well. $CD4^+CD25^+$ T cells were prepared from four Ld-nOVA × DO11.10 mice, four DO11.10 mice, and four nontransgenic BALB/c mice. $CD4^+CD25^-$ T cells were prepared from four nontransgenic BALB/c mice. In some wells, an anti-CTLA-4 mAb (100 μg/ml) was added to the culture. A representative result of three independent similar experiments is shown. The proliferative response was expressed as Δcpm (the mean cpm of the test cultures minus the mean cpm of the control cultures without Ag). DBL, Ld-nOVA × DO11.10 double-transgenic mice.

T cells in Ld-nOVA × DO11.10 α-chain mice contained a higher percentage of clonotypic T cells than $CD4^+CD25^-$ T cells in DO11.10 α-chain mice, as shown in Fig. 3, cell numbers of clonotypic T cells in Ld-nOVA × DO11.10 α-chain mice were lower than those in DO11.10 α-chain mice.

T cells expressing endogenous TCR chains were differentially recruited into $CD4^+CD25^+$ regulatory T cells or $CD4^+CD25^-$ T cells between Ld-nOVA × DO11.10 mice and DO11.10 mice

We next investigated the role of endogenous TCR chain expression in the generation of $CD4^+CD25^+$ regulatory T cells and $CD4^+CD25^-$ T cells. In the thymus and spleen of Ld-nOVA × DO11.10 mice, $CD4^+CD25^+$ T cells were clonotype^{high} T cells, whereas $CD4^+CD25^-$ T cells were clonotype^{low} T cells (Fig. 4*A*). On the contrary, in the thymus and the spleen of DO11.10 mice, most $CD4^+CD25^-$ T cells were clonotype^{high} T cells, whereas $CD4^+CD25^+$ T cells were clonotype^{low} T cells (Fig. 4*A*). These clonotype^{low} T cells in lymph nodes (Fig. 4*B*) and the thymus (data not shown) expressed not only endogenous Vαs, but also endogenous Vβs. The percentages of $CD4^+$ T cells expressing endogenous Vβs in $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells from the thymus and lymph nodes of Ld-nOVA × DO11.10 mice and DO11.10 mice are summarized in Fig. 4*C*. The percentage of T cells expressing endogenous β-chains was defined as the percentage of TCR Cβ-positive cells in $CD4^+$ T cells minus the percentage of Vβ8-positive cells in $CD4^+$ T cells. These data and the comparison of MFI of Vβ8, as described above, indicate that $CD4^+CD25^+$ T cells of DO11.10 mice preferentially use endogenous Vβs like $CD4^+CD25^-$ T cells of Ld-nOVA × DO11.10 mice. These results imply that $CD4^+CD25^+$ regulatory T cells are derived from autoreactive T cells, which do not undergo clonal deletion, and that $CD4^+CD25^-$ T cells are derived from positively selected nonautoreactive T cells.

The perturbation of endogenous TCR gene rearrangement exclusively generates $CD4^+CD25^+$ T cells in Ld-nOVA × DO11.10 mice

The contribution of endogenous TCR gene rearrangement to the generation of $CD4^+CD25^-$ T cells in Ld-nOVA × DO11.10 mice

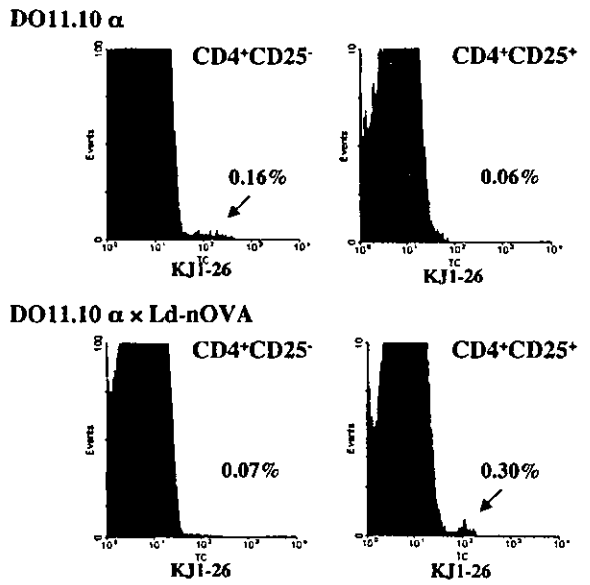

FIGURE 3. Clonotypic cells were positively selected into $CD4^+CD25^+$ T cells in Ld-nOVA × DO11.10 α-chain mice. Lymph node cells from two DO11.10 α-chain mice and two Ld-nOVA × DO11.10 α-chain mice were pooled and stained with anti-CD4, anti-CD25, and KJ1-26. Histograms of staining with KJ1-26 were gated for $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells. The percentage of clonotypic cells is shown in each histogram. The scale of histograms is different between $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells (10 vs 100) because cell numbers of $CD4^+CD25^+$ T cells were ~10% of those of $CD4^+CD25^-$ T cells. A representative result of three independent similar experiments is shown.

and to the generation of $CD4^+CD25^+$ T cells in DO11.10 mice suggests that endogenous TCR gene rearrangement is used not only for the avoidance of autoreactivity, but also for the generation of $CD4^+CD25^+$ regulatory T cells, probably by creating autoreactive TCRs. To confirm this possibility, we generated RAG2-deficient DO11.10 mice and RAG2-deficient Ld-nOVA × DO11.10 mice, in which endogenous TCR gene rearrangement was impaired. Although the numbers of $CD4^+$ T cells in spleens from RAG2-deficient Ld-nOVA × DO11.10 mice were reduced by clonal deletion, most of the $CD4^+$ T cells were $CD4^+CD25^+$ T cells (Fig. 5*A*), supporting the idea that autoreactive T cells were selected as $CD4^+CD25^+$ T cells. We confirmed that these $CD4^+CD25^+$ T cells had the ability to suppress the proliferative responses of $CD4^+CD25^-$ T cells from nontransgenic BALB/c mice in vitro (Fig. 6). On the contrary, most of $CD4^+$ T cells in spleens of RAG2-deficient DO11.10 mice were $CD4^+CD25^-$ T cells and lacked $CD4^+CD25^+$ T cells. Furthermore, we generated TCR α-chain-deficient DO11.10 mice and TCR α-chain-deficient Ld-nOVA × DO11.10 mice. These mice had almost the same phenotype as RAG2-deficient DO11.10 mice and RAG2-deficient Ld-nOVA × DO11.10 mice, respectively (Fig. 5*B*). We also confirmed the suppressive activity of $CD4^+CD25^+$ T cells in TCR α-chain-deficient Ld-nOVA × DO11.10 mice (data not shown). These results indicate that endogenous TCR expression, especially endogenous α-chain expression, plays an important role in the generation of regulatory T cells in DO11.10 mice.

Interestingly, although CD4 SP T cells in the thymus of RAG2-deficient Ld-nOVA × DO11.10 mice contained not only $CD4^+CD25^+$ T cells, but also a large population of $CD4^+CD25^-$ T cells, splenic $CD4^+$ T cells were exclusively $CD4^+CD25^+$ T cells. This finding suggests that there might be another selection mechanism in the periphery that differentially acts on $CD4^+CD25^+$ regulatory T cells.

Taken together, these findings indicate that autoreactive T cells are selected as $CD4^+CD25^+$ regulatory T cells and that endogenous TCR gene rearrangement plays a critical role in the generation of $CD4^+CD25^+$ regulatory T cells from nonautoreactive T cells and in the generation of nonautoreactive T cells from autoreactive T cells.

Discussion

We found that autoreactive T cells were positively selected as $CD4^+CD25^+$ regulatory T cells in parallel with clonal deletion in the thymus. These results indicate that part of autoreactive T cells that have a high affinity TCR enough to lead to clonal deletion could be positively selected as $CD4^+CD25^+$ T cells in the thymus. We also found that endogenous TCR expression generates $CD4^+CD25^+$ regulatory T cells from the nonautoreactive T cells and nonautoreactive $CD4^+CD25^-$ T cells from the autoreactive T cells.

In Sakaguchi and coworkers' papers (7, 22), the regulatory T cells in TCR-transgenic models were endogenous TCR α-chain-expressing cells, whereas, in our Ld-nOVA × DO11.10 mice, the regulatory T cells did not express endogenous TCRs. However, these findings do not contradict each other. In the case of $CD4^+$ T cells expressing high affinity TCRs with peripheral autoantigens or nonself ligands, these T cells could be positively selected as $CD4^+CD25^-$ T cells without clonal deletion or endogenous TCR chain expression. Because $CD4^+$ T cells expressing TCRs specific for autoantigens in the thymus might be selected as $CD4^+CD25^+$ regulatory T cells, in transgenic mice expressing a TCR specific for an exogenous Ag such as DO11.10 mice, $CD4^+CD25^+$ regulatory T cells are selected only from $CD4^+$ T cells expressing endogenous TCR chains. Therefore, the impairment of endogenous TCR chain expression led to the disappearance of regulatory T cells. On the contrary, in the case of transgenic mice expressing a TCR specific for a systemic autoantigen such as Ld-nOVA × DO11.10 mice, $CD4^+CD25^+$ regulatory T cells are selected from $CD4^+$ T cells expressing a transgenic TCR without endogenous TCR chain expression.

Because endogenous TCR gene rearrangement occurs to escape not only negative selection (15, 16), but also death by neglect during positive selection (18, 19), it is suggested that endogenous TCR gene rearrangement occurs to generate T cells expressing TCRs that have appropriate affinity for a self MHC/self peptide above the selection threshold. Thus, there is a possibility that endogenous TCR gene rearrangement might play an important role in the generation of $CD4^+CD25^+$ T cells from nonautoreactive T cells through creating autospecific TCRs and the generation of $CD4^+CD25^-$ T cells from autoreactive $CD4^+$ T cells. Therefore, it should be addressed whether second TCR gene rearrangement contributes to the generation of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells in nontransgenic mice.

Our experiment revealed that $CD4^+CD25^+$ T cells contain a certain T cell repertoire specific for a systemic nuclear autoantigen. Although $CD4^+CD25^+$ T cells are anergic to TCR stimulation and suppress the activation of $CD4^+CD25^-$ T cells in an Ag-independent manner, it has been demonstrated that regulatory function of $CD4^+CD25^+$ T cells requires their activation via TCR in vitro (10–12). Autoreactivity of regulatory T cells increases the chance that they encounter their stimulators in periphery. Thus, it is rational that regulatory T cells are specific for autoantigens in the thymus, which may be systemic autoantigens.

Jordan et al. (13) demonstrated that $CD4^+CD25^+$ regulatory T cells are positively selected by a self peptide in the thymus. In their study, autoreactive T cells are positively selected as $CD4^+CD25^+$

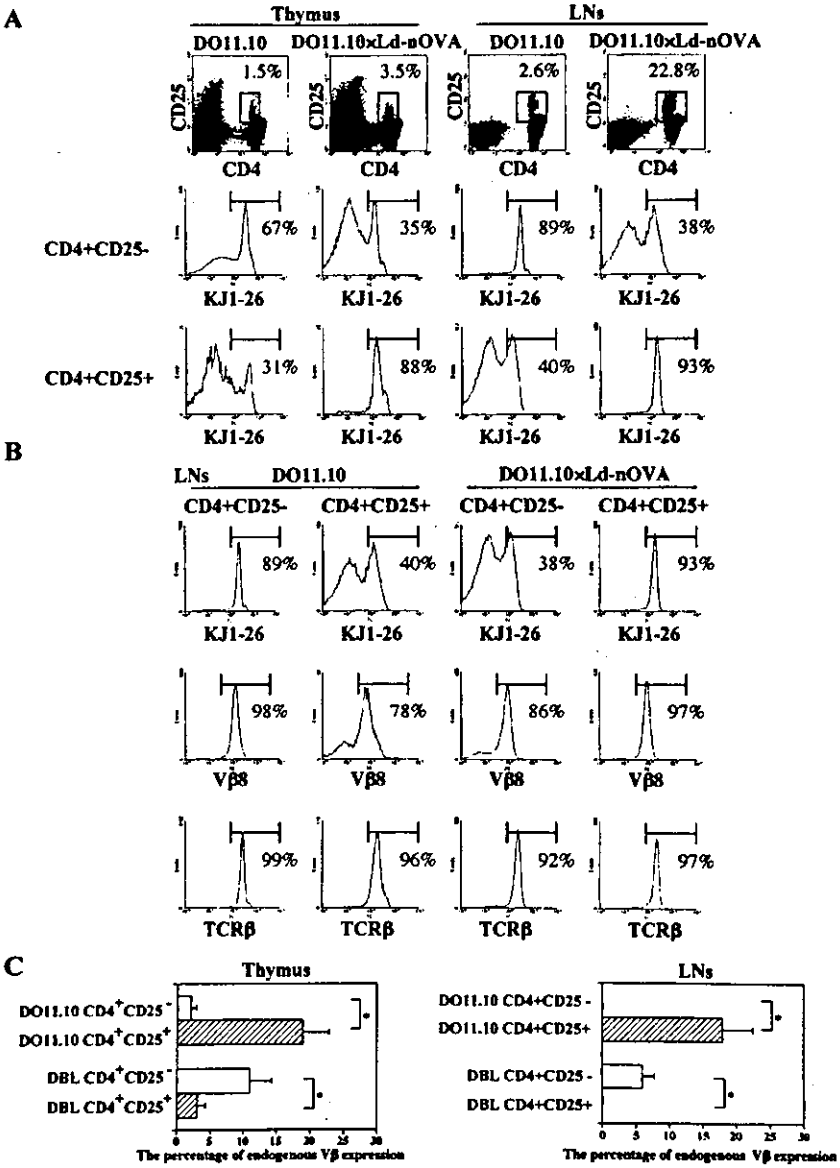

FIGURE 4. Endogenous TCR chain expression in $CD4^+CD25^+$ T cells from DO11.10 mice and $CD4^+CD25^-$ T cells from Ld-nOVA × DO11.10 mice. *A*, $CD4^+CD25^-$ T cells were clonotype^{low} T cells in Ld-nOVA × DO11.10 mice, whereas $CD4^+CD25^+$ T cells were clonotype^{low} T cells in DO11.10 mice. CD8-depleted thymocytes and lymph node cells from a DO11.10 mouse and a Ld-nOVA × DO11.10 mouse were stained with anti-CD4, anti-CD25, and KJ1-26. Dot plots show staining of these cells with anti-CD4 and anti-CD25. The percentage of $CD25^+$ cells in $CD4^+$ cells is shown in each dot plot (the mean percentages are shown in Table I). Histograms of staining with KJ1-26 were gated for $CD4^+CD25^-$ cells and $CD4^+CD25^+$ cells. The percentage of cells falling within the indicated maker is shown in each histogram. A representative result of seven independent similar experiments is shown. *B*, Clonotype^{low} T cells expressed endogenous Vβ chains. Lymph node cells from a DO11.10 mouse and a Ld-nOVA × DO11.10 mouse were stained with anti-CD4, anti-CD25, and KJ1-26, anti-Vβ8, or anti-TCRβ. Histograms of staining with KJ1-26, anti-Vβ8, or anti-TCRβ were gated for $CD4^+CD25^-$ cells and $CD4^+CD25^+$ cells. The percentage of cells falling within the indicated marker is shown in each histogram. A representative result of seven independent similar experiments is shown. *C*, The percentages of T cells expressing endogenous Vβ chains in $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells from the thymus and lymph nodes of DO11.10 mice and Ld-nOVA × DO11.10 mice are shown. Seven Ld-nOVA × DO11.10 mice and seven DO11.10 mice were analyzed. The mean percentage of T cells expressing endogenous Vβ chains was defined as the mean percentage of TCRβ-positive cells in $CD4^+$ T cells minus the mean percentage of Vβ8-positive cells in $CD4^+$ T cells. Statistical comparison is by Student's *t* test (*, $p < 0.01$). DBL, Ld-nOVA × DO11.10 double-transgenic mice.

regulatory T cells and did not undergo clonal deletion in contrast to our transgenic models. The lack of deletion can probably be attributed either to a lower affinity TCR compared with our transgenic model or to the expression level of the self ligand. These findings suggest that $CD4^+CD25^+$ regulatory T cells comprise a broad autoreactive T cell repertoire.

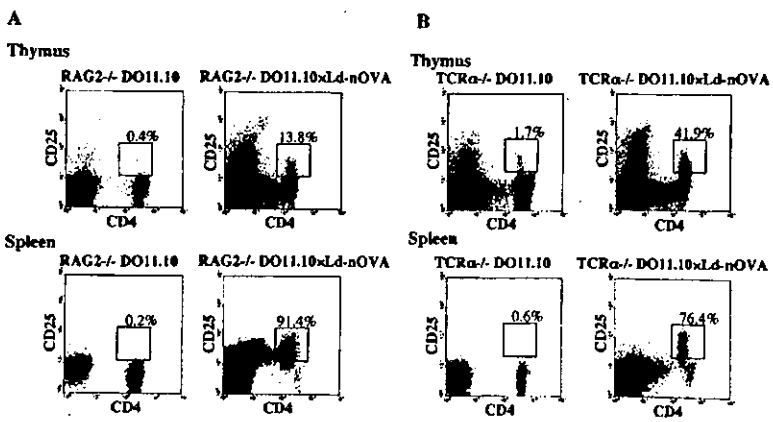

FIGURE 5. Perturbation of endogenous TCR gene rearrangement exclusively generated $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells in Ld-nOVA × DO11.10 mice and DO11.10 mice, respectively. Thymocytes and splenocytes of RAG2-deficient Ld-nOVA × DO11.10 mice and RAG2-deficient DO11.10 mice were analyzed for the expression of CD4 and CD25. *B*, Thymocytes and splenocytes of TCR α-chain-deficient Ld-nOVA × DO11.10 mice and TCR α-chain-deficient DO11.10 mice were analyzed for the expression of CD4 and CD25. A representative result of three independent similar experiments is shown.

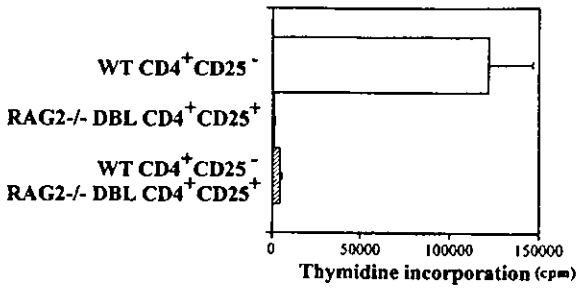

FIGURE 6. $CD4^+CD25^+$ T cells in RAG2-deficient Ld-nOVA × DO11.10 mice were regulatory T cells. $CD4^+CD25^+$ T cells (2×10^4 cells/well) from RAG2-deficient Ld-nOVA × DO11.10 mice were cultured with $CD4^+CD25^-$ T cells (2×10^4 cells/well) from nontransgenic BALB/c mice and irradiated (20 Gy) syngeneic spleen cells (5×10^4 cells/well) in the presence of Con A at 1 μg/ml for 3 days, followed by a final 6 h of culture in the presence of 1 μCi [^{3}H]TdR per well. These T cell subpopulations were purified from pooled splenocytes from six RAG2-deficient Ld-nOVA × DO11.10 mice or three nontransgenic BALB/c mice. Means and SDs of triplicate are shown. The data were representative of three independent similar experiments. DBL, Ld-nOVA × DO11.10 double-transgenic mice.

Our results provide new insight into the ontogeny of regulatory T cells. The thymus has the ability to generate regulatory T cells from autoreactive T cells simultaneously with negative selection, sometimes actively generating autoreactive T cells by endogenous TCR gene rearrangement. The critical contribution of endogenous TCR gene rearrangement to the control of autoreactivity is that it reduces self reactivity in effector precursor T cells by generating nonautoreactive $CD4^+CD25^-$ T cells from autoreactive T cells and generates autoreactive $CD4^+CD25^+$ regulatory T cells from the nonautoreactive T cells.

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