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

Kimito Kawahata, Yoshikata Misaki, Michiko Yamauchi, Shinji Tsunekawa, Keigo Setoguchi, Jun-ichi Miyazaki and Kazuhiko Yamamoto

*J Immunol* 2002; 168:1103-1112; doi: 10.4049/jimmunol.168.3.1103
http://www.jimmunol.org/content/168/3/1103

References
This article cites 60 articles, 43 of which you can access for free at:
http://www.jimmunol.org/content/168/3/1103.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Peripheral Tolerance to a Nuclear Autoantigen: Dendritic Cells Expressing a Nuclear Autoantigen Lead to Persistent Anergic State of CD4⁺ Autoreactive T Cells After Proliferation

Kimito Kawahata,* Yoshikata Misaki,‡ Michiko Yamauchi,* Shinji Tsunekawa,† Keigo Setoguchi,* Jun-ichi Miyazaki,‡ and Kazuhiko Yamamoto*

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 OVA323-339 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 OVA323-339 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), 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

---

*Department of Allergy and Rheumatology, University of Tokyo Graduate School of Medicine, Tokyo, Japan; †Medical and Biological Laboratories, Ina, Japan; and ‡Department of Nutrition and Physiological Chemistry, Osaka University Medical School, Suita, Japan

Received for publication April 23, 2001. Accepted for publication November 19, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by grants from the Ministry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Address correspondence and reprint requests to Dr. Yoshikata Misaki, Department of Allergy and Rheumatology, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail address: misaki-tky@umin.ac.jp

---

© 2002 by The American Association of Immunologists
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 OVA323–339 dominant epitope bound to I-Aβ 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. Chambron, 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 L1 class I promoter of pLG-2 plasmid. This OVA transgene construct was microinjected into the pronu- clei 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-Aβ 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 Ca2+/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 OVA323–339 peptide for 2 h at 37°C, and then washed twice before injection.

Transfer experiments

For the transfer of KJ1-26+ 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 × 106 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 × 105 cells/well with various concentrations of OVA and irradiated syngeneic spleen cells (5 × 106 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 × 10−3 M 2-ME for 3 days, followed by a final 6 h of culture in the presence of 1 μCi of [3H]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) ± SD.

Assay for suppressive activity

Anergic KJ1-26+ T cells positively selected from the Ld-nOVA recipients (2 × 106 cells/well) and CD4+ CD25+ T cells from wild-type mice (2 × 106 cells/well) were cultured with irradiated syngeneic spleen cells (5 × 106 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 [3H]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) ± SD.

Flow cytometry

The following Abs were used for identification and phenotypic analysis of transferred T cells: FITC-conjugated or biotinylated KJ1-26; PE-conjug- ated 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 107/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 mouse IgG1 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 x 10⁶ 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% NaN₃, 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 Ld 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. 1A). 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 Ld class I promoter (Fig. 1B). 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 OVA323-339 10 days after immunization. The proliferative responses were greatly reduced in Ld-nOVA mice in comparison with wild-type mice (Fig. 1C). This result indicates that Ld-nOVA mice are tolerant to OVA as well as to OVA323-339, which is a major antigenic determinant of OVA immunized exogenously.

Transferred KJ1-26+ 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 x 10⁶ cells) from DO11.10 TCR mice, which express a TCR specific for OVA323-339 bound to I-Ab, were transferred to Ld-nOVA mice and non-transgenic 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 KJ1-26+ 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 KJ1-26+ CD4+ cells started to increase 3 days after transfer and peaked on days 10–14. Evaluation of the total number of KJ1-26+ 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 recipients, KJ1-26+ CD4+ cells were always <0.5%. The number and percentage of KJ1-26+ CD4+ cells in Ld-nOVA recipients increased 20-fold by day 14. Although the majority of KJ1-26+ CD4+ cells disappeared after day 14, a substantially larger number of KJ1-26+ 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 KJ1-26+ CD4+ cells can persist in Ld-nOVA mice after transient proliferation.

Persisting transferred KJ1-26+ CD4+ cells are hyporesponsive in Ld-nOVA mice

To address the question of whether the persisting transferred KJ1-26+ 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 OVA323-339 were measured by thymidine incorporation at 10 days after the immunization.

Downloaded from http://www.jimmunol.org/ by guest on April 11, 2017
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 10^6 KJ1-26 CD4^+ cells. As shown in Fig. 3A, 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. 3B. 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

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 10^6 KJ1-26^+ CD4^+ cells. As shown in Fig. 3A, 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. 3B. 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 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.

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 syngeneic splenocytes for 3 days. Proliferative responses were measured by thymidine incorporation. All values were calculated to correspond to the response of 10^6 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 re-adoptive 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. 3C 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. 4A 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. 4B, 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. 4C. 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. 4D 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 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 were measured. B cells (Fig. 5A) 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. 5A. 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 IAα, 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...
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 KJ1-26+CD4+ cells. Fig. 5B 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 IAα, 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 autoreactive-negative control, and OVA23–339-pulsed WTDCs (pepDCs) as an autoreactive-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 KJ1-26+CD4+ cells in the TgDC and pepDC recipients were the same and were significantly higher than in the WTDC recipients (Fig. 6A). The KJ1-26+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 KJ1-26+CD4+ cells encountered the transferred DCs at the almost same efficiency (data not shown). However, the proliferative response of the KJ1-26+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. 6B). 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 OVA23–339 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 OVA23–339 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 OVA23–339 on the surface of DCs by comparing the proliferative responses of KJ1-26+CD4+ clone stimulated by TgDCs with those stimulated by pepDCs. Fig. 7 shows that the expression of OVA23–339 on the surface of splenic DCs from Ld-nOVA is functionally equivalent to 50 pM of OVA23–339 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 disappearance 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...
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 concentration 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 spleens of recipients were stimulated with OVA$_{323-339}$ and proliferative responses were measured. Fig. 8A 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 suggest 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 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. 5C. 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. 4C. 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.

**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 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 $10^5$ KJ1-26$^+$ CD4$^+$ cells (B).

**FIGURE 7.** Low expression level of OVA$_{323-339}$ on the surface of DCs from Ld-nOVA mice. A KJ1-26$^+$ CD4$^+$ clone (5 x 10$^4$ cells/well) was cultured with DCs (5 x 10$^3$ cells/well) from Ld-nOVA mice (□) or pepDCs (5 x 10$^4$ cells/well; ■). Proliferative responses were measured by thymidine incorporation.
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 in CD4^+ T cells (5 × 10^6 cells) from the spleens of DO11.10 mice. 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 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. 5B.

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. 5C 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. Furthermore, the results in Fig. 6 cannot 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 induction 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 transcription 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.

Acknowledgments

We thank Drs. Hajime Karasuyama, Takachika Azuma, Takeshi Watanabe, and Pierre Chambon for kindly providing us with their valuable materials. We are also grateful to Naoko Sato, Kazumi Abe, and Atumi Ohkubo for their excellent technical assistance.

References


