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_J Immunol_ 2006; 177:8400-8409; doi: 10.4049/jimmunol.177.12.8400
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Regulatory Roles of NKT Cells in the Induction and Maintenance of Cyclophosphamide-Induced Tolerance

Toshiro Iwai,* Yukihiro Tomita,† Shinji Okano,‡ Ichiro Shimizu,* Yohichi Yasunami,‡ Takashi Kajiwara,* Yasunobu Yoshikai,§ Masaru Taniguchi,¶ Kikuo Nomoto,‖ and Hisataka Yasui*†

We have previously reported the sequential mechanisms of cyclophosphamide (CP)-induced tolerance. Permanent acceptance of donor skin graft is readily induced in the MHC-matched and minor Ag-mismatched recipients after treatment with donor spleen cells and CP. In the present study, we have elucidated the roles of NKT cells in CP-induced skin allograft tolerance. BALB/c AnNCrj (H-2d, Lyt-1.2, and Mls-1b) wild-type (WT) mice or Vα14 NKT knockout (KO) (BALB/c) mice were used as recipients, and DBA/2 NCrj (H-2d, Lyt-1.1, and Mls-1a) mice were used as donors. Recipient mice were primed with 1 × 10^8 donor SC i.v. on day 0, followed by 200 mg/kg CP i.p. on day 2. Donor mixed chimerism and permanent acceptance of donor skin allografts were observed in the WT recipients. However, donor skin allografts were rejected in NKT KO recipient mice. In addition, the donor reactive Vβ6+ T cells were observed in the thymus of a NKT KO recipient. Reconstruction of NKT cells from WT mice restored the acceptance of donor skin allografts. In addition, donor grafts were partially accepted in the thymectomized NKT KO recipient mice. Furthermore, the tolerant-specific suppressor cell was observed in thymectomized NKT KO recipient mice, suggesting the generation of regulatory T cells in the absence of NKT cells. Our results suggest that NKT cells are essential for CP-induced tolerance and may have a role in the establishment of mixed chimerism, resulting in clonal deletion of donor-reactive T cells in the recipient thymus. The Journal of Immunology, 2006, 177: 8400–8409.

N atural killer T cells, which are characterized by coexpression of NK cell receptors and a single invariant T cell Ag receptor encoded by Vα14 and Jα281 gene segments, have been identified as a novel lymphoid lineage distinct from conventional T cells or NK cells. Although the physiological roles of NKT cells remain obscure, Vα14 NKT cells have been demonstrated to play important roles in tumor immunity (1), autoimmune disease (2), and infectious immunity (3, 4) via the dominant production of Th1 cytokine γ-IFN and Th2 cytokine IL-4. Regarding transplantation immunity, two reports have suggested a regulatory role of NKT cells in both allogeneic and xenogeneic tolerance systems induced by mAbs (5, 6).

Since 1982, we have investigated cyclophosphamide (CP)-induced tolerance that consists of an i.v. injection of 1 × 10^8 allogeneic spleen cells (SC) (day 0) followed by i.p. administration of 200 mg/kg CP on day 2 (7–18). By using this method, we were able to readily induce long-lasting skin allograft tolerance in most H-2-compatible combinations (10–12), but not in fully H-2-mismatched combinations (7, 13). Our previous studies have elucidated the three major mechanisms involved using H-2-compatible, Mls-1a-disparate combinations and Mls-1a Ag-reactive Vβ6+ T cells (11–14). The first is the destruction of Ag-stimulated and then proliferating T cells in the periphery by CP treatment. CD4+ Vβ6+ T cells proliferated and then disappeared in the periphery of the recipients tolerated to H-2-compatible, Mls-1a-disparate Ags. The second, at 4–6 wk after the treatments, is the establishment of intrathymic chimerism at both the thy-mocyte and dendritic cell levels, followed by the clonal deletion of Vβ6+ T cells that begins in the thymus. The third mechanism is the generation of regulatory cells in the late stage of tolerance.

The aim of the present study was to investigate the regulatory role of NKT cells in our CP-induced tolerance system by using Vα14 NKT knockout (KO) mice. Although an essential role for NKT cells in the induction of transplantation tolerance has been suggested in two previous reports (5, 6), the detailed mechanisms have not been clarified. Here, we evaluated the role of NKT cells in our three important mechanisms, i.e., clonal deletion, intrathymic clonal deletion, and generation of regulatory cells. The results clearly showed that NKT cells were essential for CP-induced tolerance through the establishment of intrathymic clonal deletion. Without NKT cell-mediated immunoregulation, however, our results demonstrated that the generation of regulatory cells for the maintenance of tolerance in the late stage of tolerance can occur, in addition to clonal destruction at the early stage.

Materials and Methods

Animals

Inbred mice of the BALB/c AnNCrj (H-2d, Lyt-1.2, and Mls-1b) and DBA/2 NCrj (H-2b, Lyt-1.1, and Mls-1a) strains were obtained from...
Charles River Laboratories. Inbred mice of the B10.D2.SnSlc (H-2b) strain were obtained from Japan SLC. Jo281 KO (Vα14 NKT KO) mice with a BALB/c background were also used as recipients (1). The recipients were used at 12–16 wk of age. All animals received humane care in compliance with the Guidelines for Animal Experiments of Kyushu University and Law no. 105 and Notification no. 6 of the Japanese government.

Cell preparation
Mice were sacrificed by decapitation. The spleens were collected and kept on ice in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). Spleens were disrupted in the medium by pressing spleen fragments between two glass slides. Cell suspensions were filtered through cotton gauze and washed three times with the RPMI 1640 medium. Viable nucleated cells were counted and usually adjusted to 20 × 10^6/ml.

Conditioning of CP-induced tolerance
A 0.5-ml aliquot containing 1 × 10^8 SC from DBA/2 mice was injected into the tail vein of recipient BALB/c mice. Two days later, CP (Endoxan; Shinonogi) dissolved in PBS at a concentration of 10 mg/ml was injected i.p.

Reconstitution of NKT cells in NKT KO mice
We set up two methods to reconstitute NKT cells in NKT KO mice. First, a 0.5-ml aliquot containing 1 × 10^8 SC from WT mice (containing ~1% NKT cells) was injected into the tail vein of recipient NKT KO mice on day −7. Second, recipient NKT KO mice were irradiated with three gray (Gy) on day −28 and then reconstituted with 1 × 10^8 SC and 5 × 10^7 BMC (containing ~0.1–0.4% NKT cells) from WT mice on the same day. The preparation of BMC was performed according to a previous method (19). Briefly, the bone marrow in the femoral and tibial bones was flushed out using a 5-ml syringe with a 26-gauge needle (Terumo).

Thymectomy
Recipients were anesthetized with phenobarbital (Nembutal) at 50 mg/kg and had thymectomy performed using anti-CD4 mAb (L3/T4), anti-CD8 mAb (Ly2.2) (Cedar-}

Flow cytometry
Phenotyping was performed at various times, beginning 2 wk after the injection of SC. Recipients were tail bled and white blood cells (WBC) were prepared by hypotonic shock (21). In some experiments, SC and thymocytes were used for chimeric assays. Staining with both donor-specific and T cell-specific mAbs was performed on each recipient and control mouse. Cells were incubated with an PE-conjugated anti-CD4 (BD Pharmingen) mAb, and allophycocyanin-conjugated anti-CD8 (BD Pharmingen) mAb for 30 min at 4°C. To determine the percentage of CD4 single-positive cells that were Vβ6+ or Vβ8.1/8.2+, 5,000 to 10,000 gated CD4+ and CD8− cells were collected. We investigated the effect of SC/C on the ratio of CD4+ Vβ6+ T cell or CD4+ Vβ8− T cell subsets to the total CD4+ T cell number in the spleen or WBC and on the ratio of CD4+ CD8− Vβ6+ T cell or CD4+ CD8+ Vβ8− T cell subsets to the total CD4+ CD8− T cell number in the thymus. We also investigated the effect of SC/C on the absolute number of CD4+ Vβ6+ T cells or CD4+ Vβ8− T cells in the spleen and thymus.

For the staining of NKT cells, SC or liver mononuclear cells (LMNC) were stained with PE-conjugated α-galactosyl ceramide (αGalCer)/CD1d tetramers and FITC-conjugated anti-CD3 mAb (BD Pharmingen). PE-conjugated αGalCer/CD1d tetramers were prepared as previously described (22). The liver was disrupted in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS by pressing liver fragments between two glass slides and then washed, resuspended in a 40% isotonic Percoll solution (Amersham Biosciences) and underlaid with a 67.5% isotonic Percoll solution. Centrifugation for 30 min at 3,000 rpm at room temperature isolated the LMNC at the interface. Cells were washed twice with HBSS containing 2% FCS and resuspended in the same solution.

Adoptive transfer experiment
To elucidate the existence of regulatory cells in the tolerant recipients, adoptive transfer experiments were performed as described previously (14). Briefly, 1 × 10^6 or 4 × 10^6 SC from the recipient mice accepting DBA/2 skin allografts for over 100 days were transferred into WT mice that had been irradiated with 3 Gy on the same day. The SC were harvested from WT or NKT mice that had been thymectomized and treated with DBA/2 SC and CP. Skin grafting was performed 1 day following the adoptive transfer. In one experiment, CD4+ CD8− Thy1.2− T cell depletion was performed using anti-CD4 mAb (L3/T4), anti-CD8 mAb (Ly2.2) (Cedarlane Laboratories), anti-Thy-1.2 mAb (Meijji), and complement (Low-Tox-M rabbit complement; Cedarlane Laboratories).
**Table I. Chimerism and clonal destruction in WBC of recipients treated with DBA/2 SC and CP**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>SC (day 0)</th>
<th>CP (day 2)</th>
<th>No. of Mice</th>
<th>2 wk</th>
<th>8 wk</th>
<th>3 wk</th>
<th>9 wk</th>
<th>3 wk</th>
<th>9 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 BALB/c WT</td>
<td>(−)</td>
<td>(−)</td>
<td>6</td>
<td>0</td>
<td></td>
<td>10.7 ± 1.2</td>
<td></td>
<td>16.6 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>2 BALB/c NKT KO</td>
<td>(−)</td>
<td>(−)</td>
<td>6</td>
<td>0</td>
<td></td>
<td>11.3 ± 1.4</td>
<td></td>
<td>12.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>3 DBA/2</td>
<td>(−)</td>
<td>(−)</td>
<td>6</td>
<td>96.3 ± 2.4</td>
<td></td>
<td>0</td>
<td>13.0 ± 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 BALB/c WT</td>
<td>DBA/2</td>
<td>200&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6</td>
<td>2.6 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.8 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.6 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>17.1 ± 1.9</td>
<td>16.6 ± 2.0</td>
</tr>
<tr>
<td>5 BALB/c NKT KO DBA/2</td>
<td>200&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>6</td>
<td>1.5 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>12.9 ± 1.2</td>
<td>2.6 ± 1.7</td>
</tr>
</tbody>
</table>

*The recipient mice were primed i.v. with 1 × 10<sup>8</sup> viable DBA/2 SC on day 0 and then given 200 mg/kg CP on day 2.

<sup>c</sup> Milligrams per kilogram (mg/kg).

<sup>p</sup> < 0.01 compared with group 5.

**Statistics**

The statistical significance of the data was determined by a Mann-Whitney U test when the data were nonparametric or a Student’s t test when the data were parametric. A value of p < 0.05 was considered to be statistically significant.

**Results**

**Skin allograft prolongation in H-2-matched DBA/2 (H-2<sup>d</sup>) → BALB/c WT (H-2<sup>e</sup>) or BALB/c background V<sub>ca14</sub> NKT KO (H-2<sup>d</sup>) combination mice by using 1 × 10<sup>8</sup> DBA/2 SC followed by 200 mg/kg CP**

When BALB/c WT (H-2<sup>e</sup>) or BALB/c background NKT KO mice were grafted with H-2-matched DBA/2 skin allografts (H-2<sup>d</sup>), the DBA/2 grafts were rejected within 14 days following grafting (Fig. 1a). Similarly, DBA/2 skin grafts were rejected within 14 days in BALB/c WT or NKT KO mice treated with DBA/2 SC alone or 200 mg/kg CP alone (data not shown). All of the DBA/2 skin allografts survived for >100 days in the recipient BALB/c WT mice treated with DBA/2 SC followed by CP (n = 6; MST, >100 days). When syngeneic (BALB/c) WT SC or PBS (0.5 ml) was used instead of DBA/2 SC or CP, respectively, the survival times of DBA/2 skin grafts were not prolonged (data not shown). In contrast, all DBA/2 skin grafts were rejected within 48 days in the recipient NKT KO mice treated with DBA/2 SC followed by CP (n = 6; MST, 38 days), although the survival of the grafts was moderately prolonged. The skin allograft prolongation in both BALB/c WT mice and NKT KO mice, which were treated with DBA/2 SC followed by CP, was tolerogen-specific, because the third party skin grafts of the B10.D2 strain (H-2<sup>d</sup>) were rejected in a normal fashion (Fig. 1b).

**Chimerism and reduction of Mls-1<sup>R</sup>-reactive CD4<sup>+</sup>V<sub>B6</sub><sup>+</sup> T cells of WBC in the recipient mice treated with DBA/2 SC plus CP**

As we previously reported (14), a minimal degree of mixed chimerism was detected in the BALB/c WT (Lyt-1.2) mice made tolerant of DBA/2 (Lyt-1.1) skin allografts. The mixed chimeric state induced with DBA/2 SC and CP was examined using...
PE-conjugated anti-Lyt-1 (Lyt-1.1 and Lyt-1.2) mAb and FITC-conjugated Lyt-1.1 mAb. WBC were obtained from the recipient mice at 2 and 8 wk after tolerance induction (Table I).

In the T (Lyt-1<sup>+</sup>) cells of BALB/c WT mice treated with DBA/2 SC and CP (Table I; group 4), 2–4% of Lyt-1.1 cells were clearly detected in the recipient WBC after tolerance induction. In contrast, a lower degree of chimerism was clearly detected at 2 wk (mean ± SD, 1.5 ± 0.1; p < 0.01 compared with group 4) and became <1% at 8 wk in the T (Lyt-1<sup>+</sup>) cells of NKT KO mice treated with DBA/2 SC followed by CP (Table I; group 5). A higher degree of chimerism was always observed in recipient BALB/c WT mice treated with DBA/2 SC and CP. These results were reproducible in five independent experiments (data not shown).

We examined the expression of the Mls-1<sup>a</sup>-reactive TCR V<sub>β</sub>6 in BALB/c WT or NKT KO (Mls-1<sup>b</sup>) mice treated with DBA/2<sup>a</sup> (Mls-1<sup>+</sup>) SC and CP. The WBC from the recipients were stained with FITC-conjugated anti-V<sub>β</sub>6 mAb and PE-conjugated anti-CD4 mAb (Table I).

In the WBC of untreated BALB/c WT or NKT KO mice, CD<sub>4</sub><sup>+</sup>/V<sub>β</sub>6<sup>+</sup> T cells were detected (Table I; group 1 or 2, respectively), whereas they were hardly detected in the WBC of untreated DBA/2<sup>b</sup> mice (Table I; group 3). In all of the BALB/c WT mice treated with DBA/2 SC and CP (Table I; group 4), CD<sub>4</sub><sup>+</sup>/V<sub>β</sub>6<sup>+</sup> T cells were significantly reduced by 3 wk. The same results were obtained in the WBC of NKT KO mice treated with DBA/2 SC and CP (Table I; group 5). There was no statistically significant difference in the results between groups 4 and 5. The disappearance of T cells from the WBC was specific for V<sub>β</sub>6<sup>+</sup> T cells, because the percentage of V<sub>β</sub>8.1/8.2<sup>+</sup> T cells was not significantly altered.

**Induction of DBA/2 skin graft prolongation in NKT KO mice reconstituted with NKT cells from BALB/c WT mice**

To clarify whether NKT cells were involved in the skin graft tolerance in CP-induced tolerance, NKT cells were reconstituted in NKT KO mice (Fig. 2). When SC and LMNC were stained with PE-conjugated αGalCer/CD1d tetramers and FITC-conjugated anti-CD3 mAb, αGalCer/CD1d tetramer<sup>CD<sub>3</sub></sup> cells accounted for ~1.0 ± 0.3 and 19.5 ± 5.4% of SC and LMNC in untreated BALB/c WT mice (n = 3), respectively, and 0.3 ± 0.1 and 1.2 ± 0.2% of SC and LMNC in untreated NKT KO mice (n = 3), respectively. A small percentage of αGalCer/CD1d tetramer<sup>CD<sub>3</sub></sup> cells were detected in NKT KO mice, because the NKT KO mice used in this study were generated by disruption of the Jae281 gene (1). In contrast, αGalCer/CD1d tetramer<sup>CD<sub>3</sub></sup> cells accounted for ~0.4 ± 0.1 and 4.3 ± 0.5% in SC and LMNC of NKT KO mice (n = 3) injected with BALB/c WT SC 7 days earlier, respectively. Therefore, we planned an additional experiment to further reconstitute NKT cells in NKT KO mice. For this purpose, recipient NKT KO mice were irradiated with 3 Gy on day −28 and then injected with 1 × 10<sup>7</sup> SC and 5 × 10<sup>5</sup> untreated BMC from WT mice on the same day. In NKT KO mice (n = 5) irradiated and injected with BALB/c WT SC and BMC 28 days earlier, αGalCer/CD1d tetramer<sup>CD<sub>3</sub></sup> cells accounted for ~0.7 ± 0.1 and 9.5 ± 2.6% of SC and LMNC, respectively. When NKT KO mice were injected with 1 × 10<sup>6</sup> SC from BALB/c WT mice on day −7 and treated with SC on day 0 and CP on day 2, the survival of DBA/2 skin grafts was significantly prolonged (n = 7; MST, >100 days), and four of seven recipients accepted donor DBA/2 skin grafts for >100 days (Fig. 2a). DBA/2 skin grafts were accepted for >100 days in all of the NKT KO mice irradiated with 3 Gy on day −28, reconstituted with 1 × 10<sup>7</sup> SC and 5 × 10<sup>5</sup> BMC from BALB/c WT mice on day −28, and then treated with DBA/2 SC on day 0 and CP on day 2 (Fig. 2a). Survival of donor skin grafts was not significantly prolonged in NKT KO mice reconstituted with SC and/or BMC from NKT KO mice treated with DBA/2 SC and CP as compared with that for NKT KO mice treated with DBA/2 SC and CP. In contrast, no skin graft prolongation was observed in NKT KO mice reconstituted with BALB/c WT SC or BMC, irradiated NKT KO mice reconstituted with BALB/c WT SC and BMC, NKT KO mice reconstituted with NKT KO SC or BMC, or irradiated NKT KO mice reconstituted with NKT SC and BMC if the recipient mice were not treated with donor SC and CP (Fig. 2a). This skin allograft prolongation was tolerogen-specific, because the third party skin of the B10.D2 strain (H-2<sup>d</sup>) was rejected in a normal fashion (Fig. 2b).

**Analysis of splenic clonal destruction and intrathymic clonal deletion and mixed chimerism in BALB/c WT or NKT KO mice treated with DBA/2 SC and CP**

As reported previously (12, 13), the induction mechanism of CP-induced tolerance is the clonal destruction of Ag-stimulated and proliferating T cells by the antimitotic drug CP. To further analyze the role of NKT cells in the tolerance induction, we examined the kinetics of Mls-1<sup>a</sup>-reactive CD4<sup>+</sup>/V<sub>β</sub>6<sup>+</sup> T cells in the CD4<sup>+</sup> T cells of SC in recipient BALB/c WT or NKT KO mice. When DBA/2 SC were injected into untreated BALB/c WT mice on day 0, CD4<sup>+</sup>/V<sub>β</sub>6<sup>+</sup> T cells significantly increased to ~35% on day 2 and then eventually declined to the normal range by days 15–21 (Fig. 3a). The same result was observed in NKT KO mice. In BALB/c WT mice treated with DBA/2 SC on day 0 and CP on day 2, CD4<sup>+</sup>/V<sub>β</sub>6<sup>+</sup> T cells significantly increased to ~35% on day 2, rapidly decreased to the normal range on day 5, and then gradually

![FIGURE 3. Clonal destruction in the periphery of recipient mice.](http://www.jimmunol.org/)

**FIGURE 3. Clonal destruction in the periphery of recipient mice.** The kinetics of CD4<sup>+</sup>/V<sub>β</sub>6<sup>+</sup> (a) or CD4<sup>+</sup>/V<sub>β</sub>8.1/8.2<sup>+</sup> (b) T cells in spleen cells harvested from the recipient mice are shown. SC were labeled with FITC-conjugated anti-V<sub>β</sub>6 or V<sub>β</sub>8.1/8.2 mAb and PE-conjugated anti-CD3 mAb. To determine the percentage of CD4<sup>+</sup> T cells that were V<sub>β</sub>6<sup>+</sup> or V<sub>β</sub>8.1/8.2<sup>+</sup>, 10,000–20,000 gated CD4<sup>+</sup> cells were collected. SC cells were obtained from WT (H-2<sup>d</sup>; Mls-1<sup>a</sup>) mice treated with DBA/2 (DBA/2; H-2<sup>d</sup>; Mls-1<sup>a</sup>) SC and CP (n = 4), NKT KO mice treated with DBA/2 SC and CP (n = 4), WT mice treated with DBA/2 SC alone (n = 4), and NKT KO mice treated with DBA/2 SC alone (Δn = 4). Vertical bars represent the SD. The statistical significance of the differences among groups was analyzed and the results are given in a.

**Analysis of splenic clonal destruction and intrathymic clonal deletion and mixed chimerism in BALB/c WT or NKT KO mice treated with DBA/2 SC and CP**

As reported previously (12, 13), the induction mechanism of CP-induced tolerance is the clonal destruction of Ag-stimulated and proliferating T cells by the antimitotic drug CP. To further analyze the role of NKT cells in the tolerance induction, we examined the kinetics of Mls-1<sup>a</sup>-reactive CD4<sup>+</sup>/V<sub>β</sub>6<sup>+</sup> T cells in the CD4<sup>+</sup> T cells of SC in recipient BALB/c WT or NKT KO mice. When DBA/2 SC were injected into untreated BALB/c WT mice on day 0, CD4<sup>+</sup>/V<sub>β</sub>6<sup>+</sup> T cells significantly increased to ~35% on day 2 and then eventually declined to the normal range by days 15–21 (Fig. 3a). The same result was observed in NKT KO mice. In BALB/c WT mice treated with DBA/2 SC on day 0 and CP on day 2, CD4<sup>+</sup>/V<sub>β</sub>6<sup>+</sup> T cells significantly increased to ~35% on day 2, rapidly decreased to the normal range on day 5, and then gradually...
decreased to ~3%. The percentage of CD4⁺Vβ6⁺ T cells was significantly reduced in BALB/c WT mice treated with DBA/2 SC compared with that for BALB/c WT mice treated with DBA/2 SC alone. The disappearance of T cells in WBC was specific for Vβ6⁺ T cells, because the percentage of Vβ8.1/8.2⁺ T cells was not significantly altered (Fig. 3b). Furthermore, the absolute number of CD4⁺Vβ6⁺ T cells in the spleen was analyzed, and similar results were obtained (Fig. 4). We have already reported this phenomenon, which we termed clonal destruction (12, 13), and similar results were obtained in NKT KO mice treated with DBA/2 SC on day 0 and CP on day 2 (Fig. 4). In contrast, when BALB/c WT or NKT KO mice were treated with CP alone (Fig. 4b), the numbers of CD4⁺Vβ8⁺ T cells in the spleens from WT mice treated with DBA/2 SC and CP (●; n = 4), WT mice treated with CP (●; n = 4), NKT KO mice treated with CP (■; n = 4), and NKT KO mice treated with DBA/2 SC (▲; n = 4), WT mice treated with CP (●; n = 4), NKT KO mice treated with CP (■; n = 4), WT mice treated with DBA/2 SC and CP (●; n = 4), NKT KO mice treated with DBA/2 SC and CP (▲; n = 4), WT mice treated with DBA/2 SC and CP (●; n = 4), and NKT KO mice treated with DBA/2 SC (▲; n = 4).

To further investigate the cellular events in the thymuses of BALB/c mice made tolerant of DBA/2 mice, the association of the clonal deletion with the mixed chimerism was examined (Fig. 5). Whole thymocytes were stained with FITC-conjugated anti-Vβ6 mAb, PE-conjugated anti-CD4 mAb, and allophycocyanin-conjugated anti-CD8 mAb. We previously reported that intrathymic clonal deletion occurs by 6 wk after SC and CP treatment (12, 13), but we did not investigate whether intrathymic CD4 single-positive T cells are depleted by clonal destruction or when intrathymic clonal deletion begins. The present analysis was performed by gating CD4⁺CD8⁻ single-positive thymocytes.

Among the CD4⁺CD8⁻ thymocytes of the BALB/c WT or NKT KO mice, CD4⁺Vβ6⁺ T cells represented ~9% (Fig. 5a),...
and the injection of DBA/2 SC did not significantly alter the percentage of CD4⁺CD8⁻Vβ6⁺ T cells during our observation. In the thy-
muses of BALB/c WT mice treated with DBA/2 SC and CP, the percentage of CD4⁺CD8⁻Vβ6⁺ T cells was not significantly changed by day 8 but then declined to ~3% by day 21 and reached <2% on day 35. The reduction in CD4⁺CD8⁻Vβ6⁺ T cells was strongly associated with the intrathy
mic mixed chimerism (Fig. 5c). After 28 days, mixed chimerism was detected in the thy-
muses of BALB/c WT mice treated with DBA/2 SC and CP. In contrast, in the thy-
musses of NKT KO mice treated with DBA/2 SC and CP, the percentage of CD4⁺CD8⁻Vβ6⁺ T cells was not significantly changed by day 8, then declined to ~5% on day 21, and returned to the normal range by day 70 (Fig. 5a). Mixed chimerism was not de-
tected in the thy-
muses of BALB/c NKT KO mice treated with DBA/2 SC and CP during our observation (Fig. 5c). The intrathy-
mic clonal deletion in the tolerant BALB/c mice was specific for Mls-1a-reactive T cells expressing TCR Vβ6, because Vβ8.1/8.2⁺ thymocytes were not deleted (Fig. 5b). Furthermore, the absolute number of CD4⁺CD8⁻Vβ6⁺ thymocytes was analyzed and similar results were obtained (Fig. 6). When BALB/c WT or NKT KO mice were treated with CP alone on day 2, a transient reduction of both CD4⁺Vβ6⁺ and CD4⁺Vβ8⁺ T cell subsets in the thymus was observed.

**FIGURE 6.** Absolute number of cells in the thy-
muses of recipients treated with DBA/2 (DBA) SC and CP. The kinetics of CD4⁺CD8⁻Vβ6⁺ (a) and CD4⁺CD8⁻Vβ8.1/8.2⁺ (b) T cells in thy-
mocytes harvested from the recipient mice are shown. a. The numbers of CD4⁺CD8⁻Vβ6⁺ cells in the thy-
muses from WT mice treated with DBA/2 SC and CP (□; n = 4), NKT KO mice treated with DBA/2 SC and CP (●; n = 4), WT mice treated with CP (▲; n = 4), NKT KO mice treated with CP (■; n = 4), WT mice treated with SC (○; n = 4), and NKT KO mice treated with SC (◆; n = 4). b. The numbers of CD4⁺CD8⁻Vβ8⁺ cells in the thy-
muses from WT mice treated with DBA/2 SC and CP (□; n = 4), NKT KO mice treated with DBA/2 SC and CP (●; n = 4), WT mice treated with CP (▲; n = 4), WT mice treated with CP (■; n = 4), NKT KO mice treated with CP (■; n = 4), WT mice treated with SC (○; n = 4), and NKT KO mice treated with SC (◆; n = 4).

**FIGURE 7.** Permanent DBA/2 (DBA) skin graft acceptance in the thy-
mectomized BALB/c NKT KO mice treated with DBA/2 SC and CP. Recipient mice were grafted with skin from donor DBA/2 (a) or third party B10.D2 (b) mice 4 wk after treatment. a. The groups and median skin graft survival times were as follows: •, thymectomized WT mice (n = 6; 10 days); ▲, thymectomized NKT KO mice (n = 6; 10 days); ●, thymectomized WT mice treated with DBA/2 SC and CP (n = 6; >100 days); ■, thymectomized NKT KO mice treated with DBA/2 SC and CP (n = 11; >100 days). b. B10.D2 skin grafts were rejected within 14 days after grafting in the following groups: •, thymectomized WT mice treated with DBA/2 SC and CP (n = 3); and ■, thymectomized NKT KO mice treated with DBA/2 SC and CP (n = 3).

**Induction of skin allograft prolongation in thymectomized NKT KO mice**

The previous results indicated that the effector T cells (CD4⁺CD8⁻Vβ6⁺) in the thy-
muses of WT mice were not depleted until intrathy-
mic clonal deletion occurred and that intrathy-
mic clonal deletion was associated with the establishment of mixed chimerism. Thus, we sup-
posed that the effector T cells generated in the thymus at the early phase of tolerance induction were regulated by NKT cells. To confirm this hypothesis, recipients were thymectomized on day −14. As shown in Fig. 7a, DBA/2 skin graft survival was permanently pro-
longed in 9 of 11 recipient NKT KO mice thymectomized on day −14 and treated with SC on day 0 and CP on day 2 (MST, >100 days). Similar results were obtained in thymectomized WT mice (n = 6; MST, >100 days). This skin graft prolongation was tolerogen-
specific, because third party B10.D2 (H-2b) allografts were rejected in a normal fashion (Fig. 7b).

**Generation of tolerogen-specific regulatory T cells in both WT and NKT KO recipients at the late stage of tolerance**

Previous studies have demonstrated that the third mechanism of cyclophosphamide-induced tolerance is a regulatory mechanism at the late stage of tolerance (11, 14). To examine whether NKT cells were involved in the generation of regulatory T cells, adoptive transfer experiments were conducted (Fig. 8). BALB/c WT mice were irradiated with 3 Gy and then received an i.v. transfer of 1 × 10⁶ SC from thymectomized WT or NKT KO recipients that had accepted DBA/2 skin grafts for >100 days. With respect to the T
cell percentage of the SC, no significant difference was observed between thymectomized NKT KO mice and BALB/c WT donors (20–25%). Skin grafting was performed 1 day following the transfer of the SC. DBA/2 skin grafts were rejected within 30 days after grafting in all groups. The groups and median skin graft survival times were as follows: •, irradiated WT mice treated with 4 × 10^7 WT SC (n = 9; 58 days); ■, irradiated WT mice treated with 4 × 10^7 NKT KO SC (n = 9; 50 days); ○, irradiated WT mice treated with 4 × 10^7 WT SC (n = 6; 38.5 days); □, irradiated WT mice treated with 4 × 10^7 NKT KO SC (n = 6; 35 days); and •, irradiated WT mice (n = 6; 24 days). b. B10.129 skin grafts were rejected within 24 days after grafting in all groups.

using low-dose SC, there was no statistically significant difference in survival between the groups treated with 4 × 10^7 SC from DBA/2 skin graft-accepting thymectomized WT mice and those treated with an equivalent number of SC from DBA/2 skin graft-accepting thymectomized NKT KO mice. The graft survival times in the irradiated BALB/c WT mice treated with a low dose (4 × 10^7) of SC from DBA/2 skin graft-accepting thymectomized BALB/c WT or NKT KO mice were shorter than those in the irradiated BALB/c WT mice treated with a high dose (1 × 10^8) of SC. These skin allograft prolongations were tolerogen-specific, because third party skin B10.D2 (H-2d) allografts were rejected within 24 days after grafting (Fig. 8b).

Furthermore, we investigated which T cell subset was dominant in the regulatory function. SC from tolerant BALB/c WT mice were treated with anti-CD4, -CD8, or -Thy-1.2 mAb and complement ex vivo, and 1 × 10^6 mAb-treated SC were transferred to the irradiated WT mice. Recipient mice were grafted 1 day following the transfer of tolerant SC (Fig. 9a). The graft survival time of the recipient treated with CD4^+ T cell-depleted SC from tolerant BALB/c WT mice was moderately prolonged (n = 6; MST ±
Reduced tolerance are mainly CD8 T cell-depleted tolerant SC (n = 6; MST ± SD = 55.2 ± 9.7 days; median = 56 days). In contrast, the graft survival of the recipients treated with CD8 or Thy1.2 T cell-depleted tolerant SC was significantly shorter than that of the recipients treated with non-T cell-depleted tolerant SC (n = 6; MST ± SD = 29.7 ± 6.0 days; median = 29 days; and n = 6; MST ± SD = 30.0 ± 5.6 days; median = 31 days; respectively). These data indicated that the regulatory cells induced in CP-induced tolerance are mainly CD8 T cells rather than CD4 T cells. When SC from tolerant NKT KO mice were used, similar results were obtained (Fig. 9b).

Discussion

By using the H-2-matched murine combination of DBA/2 into BALB/c WT and mAbs against T cell markers (Ly-1.1 and Lyt-1.2) and TCR Vβ6, we have demonstrated the sequential mechanisms of CP-induced tolerance (11, 14). These mechanisms are as follows: 1) clonal destruction of Ag-stimulated and then proliferating T cells by CP at the early stage; 2) intrathymic clonal deletion at the intermediate stage; and 3) regulatory mechanisms at the late stage of tolerance. These three conditions are achieved by SC and 200 mg/kg CP alone without any other supportive treatment in most H-2-matched mouse combinations. In the present study, we have elucidated the roles of NKT cells in the induction of skin allograft tolerance in CP-induced tolerance.

The first mechanism essential to CP-induced tolerance is the selective destruction of Ag-stimulated and then proliferating T cells by CP treatment. This mechanism is considered to be responsible for destroying mature T cells but not immature T cells. As shown in Fig. 3, the CD4+ Vβ6+ T cells that are responsible for the MLR against Mls-1-encoded Ag (14) and probably the effector T cells that are responsible for the rejection of DBA/2 skin selectively proliferated on day 2 and were depleted by day 5 in the periphery of the WT mice given DBA/2 SC and CP, leaving most of the nonproliferative CD4+ Vβ8+ T cells. The same results were observed in NKT KO mice given DBA/2 SC and CP, suggesting that NKT-mediated immunoregulation was not required for the induction of clonal deletion in the periphery.

The second mechanism is the intrathymic clonal deletion, which is essential for maintaining the central tolerance in CP-induced tolerance and other chimerism-based tolerance systems (12, 13). By days 28–35 after the treatments with DBA/2 SC and CP, intrathymic chimerism was established due to regeneration of the stem cells of donor origin contained in the tolerogenic SC, and then clonal deletion of Vβ6+ T cells began in the thymuses of WT recipients (Fig. 4). In fact, intrathymic clonal deletion was well correlated with intrathymic mixed chimerism. Notably, in the thymuses of NKT KO recipient mice given DBA/2 SC and CP, the percentage of CD4+ Vβ6+ T cells decreased only transiently from day 21 through day 35 and returned to the normal level by day 70. Consistently, intrathymic chimerism was not established in NKT KO recipients given DBA/2 SC and CP. Because donor Ag-reactive effector T cells can break mixed chimerism in the periphery, it can be speculated that the effector T cells generated in the thymuses of recipient WT mice by DBA/2 SC administration must be suppressed or regulated by an unsolved mechanism to establish the intrathymic mixed chimerism, which is essential for clonal deletion of donor Ag-specific T cells in the thymus. We hypothesized that this unsolved mechanism could be mediated by the NKT cells.

To confirm this hypothesis, we performed a thymectomy and then conditioned the mice with DBA/2 SC and CP (Fig. 7). The results showed that skin graft tolerance was induced in 9 of 11 of the thymectomized NKT KO mice given DBA/2 SC and CP (Fig. 7).

It is important to consider why chimerism or clonal deletion was poorly observed in NKT recipient mice (group 5; Table I and Fig. 5a). Regarding the reduced level of chimerism, we conjectured that chimerism was established by the clonal destruction but was gradually rejected by effector T cells from the thymus. In fact, the level of chimerism was reduced from 2 to 8 wk (group 5; Table I). In BALB/c WT mice, as described above, effector T cells from the thymus were suggested as being regulated by NKT cells, chimerism was stably maintained, and donor skins were permanently accepted. By performing thymectomies in NKT KO mice, a higher level of chimerism could be induced compared with that in non-thymectomized NKT KO mice (group 6 vs 7; Table II). As a result, skin allograft tolerance could be induced in thymectomized NKT KO mice treated with DBA/2 SC and CP. However, the level of chimerism in thymectomized NKT KO mice treated with DBA/2 SC and CP tended to be lower than that in thymectomized BALB/c WT mice treated with DBA/2 SC and CP (group 6 vs group 4; Table II), although this difference did not reach the level of statistical significance. These results may be explained in the following ways. First, we detected T cell chimerism, which may not correlate with bone marrow chimerism. Second, NKT-mediated immunity may contribute to the homeostatic proliferation or self-renewal of T cells. Regarding the poor level of deletion of CD4+ CD8− Vβ6+ thymocytes in NKT mice (Fig. 5a), we can hypothesize that NKT cells may regulate negative selection in the thymus.

Table II. Chimerism and clonal destruction in recipients treated with thymectomy, DBA/2 SC and CP

<table>
<thead>
<tr>
<th>Group</th>
<th>Recipient</th>
<th>Treatment*</th>
<th>Chimeric Analysis (percent positive cells ± SD)</th>
<th>Analysis of TCR Expression (percent positive cells ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thymectomy (day -14)</td>
<td>SC (day 0)</td>
<td>CP (day 2)</td>
</tr>
<tr>
<td>1</td>
<td>BALB/c WT</td>
<td>(+)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>2</td>
<td>BALB/c NKT KO</td>
<td>(+)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>3</td>
<td>DBA/2</td>
<td>(+)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>4</td>
<td>BALB/c WT</td>
<td>(+)</td>
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<tr>
<td>5</td>
<td>BALB/c WT</td>
<td>Sham</td>
<td>DBA/2</td>
<td>200*</td>
</tr>
<tr>
<td>6</td>
<td>BALB/c NKT KO</td>
<td>(+)</td>
<td>DBA/2</td>
<td>200*</td>
</tr>
<tr>
<td>7</td>
<td>BALB/c NKT KO</td>
<td>Sham</td>
<td>DBA/2</td>
<td>200*</td>
</tr>
</tbody>
</table>

* The recipient mice were primed i.v. with 1 × 10^6 viable DBA/2 SC on day 0 and then given 200 mg/kg CP on day 2. Thymectomies were performed on some groups on day −14.

a Milligrams per kilogram (mg/kg).

b No statistical significance as compared with group 6.

c p < 0.01 compared with group 7.
roles of nkt cells in cyclophosphamide-induced tolerance

thymus. We intend to elucidate these unsolved mechanisms in a future study.

The third mechanism is the generation of regulatory cells in the late stage of tolerance (11, 14). Any significant contribution of suppressor factors, such as enhancing Abs or anti-idiotypic Abs, was excluded from the transfer experiments by using the serum from long-term tolerant mice (11). Recent reports have clarified that the regulatory mechanism is mediated by both CD25+ CD4+ and CD25+ CD4− T cells via CTLA-4 molecules and Th2 cytokines in mAb-induced tolerance systems (23–25). Furthermore, another study has reported that CP depleted CD25+ CD4+ T cells (26). We have reported that CD8+ T cells are generally involved in the suppressor activity in CP-induced tolerance, whereas CD4+ T cells are not (11, 14). The present study confirmed that CD8+ T cells exhibit the main suppressor activity, indicating that CD25+ CD4+ T cells are not involved in the regulatory mechanisms. One of the aims in the present study was to examine the role of NKT cells in the generation of regulatory cells. The results showed that regulatory cells could be generated without the contribution of NKT cells. However, regarding the suppressor activity, NKT may have some effects on the suppression of the alloreactivity in the recipients, because the survival of DBA/2 skin grafts was significantly longer in irradiated recipients receiving a high dose (1 × 108) of SC from tolerant WT mice than in those receiving the same amount of SC from tolerant NKT KO mice.

Two reports have described the critical role of NKT cells in inducing transplantation tolerance (5, 6). However, the precise mechanisms at the cellular and molecular levels have remained unclear. It has been well documented that NKT cells produce large amounts of both IL-4 and IFN-γ upon activation (27–29). Given that IL-4 and IFN-γ have opposite effects on the development of Th1 and Th2 cells, extensive analyses have been performed with various experimental systems, and conflicting results have been reported (30–32). By using IL-4 KO and IFN-γ KO mice, two groups analyzed the mechanisms of the NKT-mediated role in transplantation tolerance induction and produced conflicting results (5, 6). Ikehara et al. (6) suggested that there was little involvement of these two cytokines in C57BL/6 mice injected with anti-CD4 mAb and grafted with rat islets. In contrast, Seino et al. (5) suggested that IFN-γ partially contributes to tolerance induction in C57BL/6 mice injected with anti-LFA-1 and ICAM-1 mAbs and grafted with heart grafts from BALB/c (H-2b) mice. However, these results did not seem to be definitive, because they could not show clearly whether the IFN-γ produced by NKT cells was involved in one or more of the steps that induce and maintain transplantation tolerance, i.e., activation of effector T cells, apoptosis of effector T cells, reprogramming of effector T cells (anergy induction), and the generation of regulatory T cells. In the present study, we can strongly suggest two roles for NKT cells in CP-induced tolerance. One is to regulate the effector T cells generated in the thymuses of recipient WT mice by DBA/2 SC administration through the establishment of intrathymic clonal deletion. The other is to allow generation of regulatory cells without NKT cell-mediated immunoregulation.

As for the NKT reconstitution assay (Fig. 2), unfortunately we could not show how many NKT cells are needed to completely reconstitute NKT-mediated immunoregulation. In our laboratory, the Vα14 transgenic mice (RAG-1 KO background) needed for reconstituting NKT cells in NKT (Vα14) KO mice are unavailable. However, even in the experiments using the Vα14 transgenic mice, a previous attempt to perform adoptive transfer of Vα14+ cells from Vα14 transgenic mice in an allogeneic tolerance system was not successful, probably because the dose of Vα14+ cells was not sufficient to restore these cells to the normal level (Y. Yasunami, unpublished observation). We initially transferred 1 × 108 SC from WT mice to NKT KO mice but could not induce permanent acceptance donor skin grafts in three of seven recipients. NKT (αGalCer/CD1d tetramer ‘CD3+’) cells were restored to 0.4 and 4.3% in SC and LMNC of these mice, respectively, suggesting that the level of NKT reconstitution was not enough. In contrast, Seino et al. had reconstituted WT BMC (including NKT cells and progenitors) in irradiated NKT KO mice (5). To further reconstitute NKT cells, recipient NKT KO mice were irradiated with 3 Gy and reconstituted with SC and BMC from WT mice. Although NKT cells were not fully restored (0.7 and 9.5% in SC and LMNC, respectively), permanent skin graft acceptance was induced in all of the irradiated and reconstituted NKT KO mice.

Acknowledgments

We thank Dr. Hisanori Mayumi, General Manager, Watanabe Hospital (Kagoshima, Japan) for reviewing this manuscript and giving helpful comments. We also thank the Edanz Editing Co. Ltd. (Fukuoka, Japan) and KN International, Inc. (Iowa City, IA) for the English editing of this manuscript.

Disclosures

The authors have no financial conflict of interest.

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