De Novo-Developed T Cells Have Compromised Response to Existing Alloantigens: Using Ld-Specific Transgenic 2C T Cells as Tracers in a Mouse Heart Transplantation Model

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De Novo-Developed T Cells Have Compromised Response to Existing Alloantigens: Using L<sup>d</sup>-Specific Transgenic 2C T Cells as Tracers in a Mouse Heart Transplantation Model

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In this study, the phenotype, TCR signaling events, and function of T cells developed de novo during adulthood in the presence of extrathymic alloantigen were investigated. C57BL/6 mice (H-2<sup>b</sup>) were first transplanted heterotopically with BALB/c hearts (H-2<sup>d</sup>) and treated with rapamycin for 2 wk to create a tolerant status. Three weeks postoperation, the mice were whole body irradiated and transplanted with bone marrow cells from 2C mice, which are transgenic for TCR, and most of their T cells are L<sup>d</sup>-specific CD8 cells. The 2C T cells developed de novo in the C57BL/6 mice were not able to reject the heart allograft. No clonal deletion, TCR down-regulation, or CD8 down-regulation was found in the tolerated 2C T cells. There was no characteristic phenotype of these cells in terms of CD25, ICAM-1, CD44, and MEL-14 expression. Early TCR signaling events such as intracellular calcium concentration flux, tyrosine phosphorylation, Lck and Fyn kinase activities, and Lck and Fyn protein levels in the tolerated 2C T cells were comparable to their normal counterparts, but the tolerated T cells were defective in IL-2 production and proliferation upon H-2<sup>d</sup> alloantigen stimulation in vitro. Exogenous IL-2 could not reverse the compromised proliferation. The results of this study indicate that during adulthood, the de novo-developed T cells become tolerant to extrathymic Ag without clonal deletion. These newly minted T cells are functionally defective although they are indistinguishable from normal T cells in phenotypes and in some early signaling events.


Self-tolerance is achieved mainly by deletion of autoreactive T cells in the thymus by negative selection, which is mediated primarily by bone marrow-derived cells carrying self-Ags (1–4). However, there must also be a mechanism for T cells to tolerate extrathymic self-Ag. Soluble Ags might get into a thymus and be presented by APC there in the context of class II MHC via the exogenous pathway (5). For certain immune-privileged sites, such as an anterior chamber of the eye and testis, Fas ligands may play a role in curbing the self-reactive T cells from attacking peripheral Ags there (6, 7). Nevertheless, how class I MHC-associated self-Ags processed via the endogenous pathway in peripheral solid organs manage to avoid T cell recognition and reaction in general is still a challenging question.

In recent years, this question has been investigated using transgenic mice, which express foreign Ags under various tissue-specific promoters. Double transgenic mice expressing specific TCR as well as the corresponding Ag in the periphery have also been used for such investigations. Since most of the promoters used in the transgenic mouse models are functional in embryonic and neonatal stages, these models are useful to study the tolerance to peripheral Ags occurring early in development. There is evidence indicating that the neonatal thymic emigrants are more susceptible to tolerance induction than the adult ones (8). Therefore, these models are not the best to address the question of how during young adult life the newly produced T cells become tolerant to the peripheral Ags. Efforts have been made to use a C-reactive protein promoter to create LPS-inducible transgene expression in the liver, but in most cases, low level constitutive expressions still occur throughout life (9, 10).

In this study, L<sup>d</sup>-specific TCR transgenic T cells from 2C mice (11) were allowed to develop after 2C bone marrow transplantation (BMTx) into irradiated adult C57BL/6 mice, which carry an L<sup>d</sup> heart allograft. Since these alloantigen-specific T cells could be recognized by a clonotypic mAb 1B2, they could be identified and purified easily. The phenotypes, signal transduction, and function of these de novo-developed T cells in the presence of their specific Ag in the periphery have also been investigated with a view to understanding the behavior of the de novo-developed T cells in the presence of extrathymic Ags in adulthood.

Materials and Methods

Reagents

RPMI 1640, FCS, penicillin-streptomycin, and L-glutamine were purchased from Life Technologies (Burlington, Ontario, Canada). [γ-<sup>32</sup>P]ATP (3000 μCi/nmol) and 125 I-protein A (30 mCi/mg protein) were from Amersham (Oakville, Ontario, Canada). Rapamycin (RAPA)<sup>4</sup> was a

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<sup>4</sup> Abbreviations used in this paper: RAPA, rapamycin; HTx, heart transplantation; WBI, whole body irradiation; BMTx, bone marrow transplantation; MACS, magnetic cell sorting; PE, phycoerythrin; [Ca<sup>2+</sup>]<sup>i</sup>, intracellular calcium concentration; MLR, mixed lymphocyte reaction; MST, mean survival time.
Conjugated red-613 was from Life Technologies. The clonotypic mAb 1B2 was from Cedarlane and the PE-conjugated affinity-purified goat anti-C57BL/6 mice (H-2b) were used as recipients, and 16- to 20-g BALB/c mice (H-2d) were used as donors. Heterotopic mouse heart transplants were treated WBI/BMTx mice had a significantly higher graft survival rate than C57BL/6, and the RAPA-treated BALB/c mice as donors for HTx on day 1, as indicated by the short downward arrow. RAPA was given at 8 mg/kg/day i.p. for 14 days as indicated by a box. On day 21, the transplanted C57BL/6 mice were given 700 rad of WBI followed by BMTx using T cell-depleted 2C bone marrow. Twenty-three mice were sacrificed between days 54 and 74 for ex vivo experiments, while the grafts were still functional. The 2C mice rejected the BALB/c heart graft significantly faster than C57BL/6, and the RAPA-treated WBI/BMTx mice had a significantly higher graft survival rate than the C57BL/6 (p = 0.001 and p < 0.001, respectively, unpaired Student’s t test).

Flow cytometry

Two-color flow cytometry was performed as described in our previous publication (15, 16). Direct staining was used for most of the surface markers. The 2C clonotypic TCR determinant was stained by 1B2 mAb (mouse IgG1) followed by PE-conjugated goat anti-mouse IgG1. The cells were stained for Thy-1.2/1B2, CD8/1B2, CD44/1B2, ICAM-1/1B2, CD45RB/1B2, and CD25/1B2.

Cell culture, mixed lymphocyte reaction (MLR), and IL-2 assay

Total spleen cells were prepared from Lympholyte-mouse (Cedarlane) gradient, and were cultured at a density of 2 × 10^6/ml in 96-well flat-bottom plates in RPMI 1640 supplemented with 10% FCS, t-glutamine, and antibiotics. For MLR, 1 × 10^5 total responder spleen cells were stimulated with an equal number of mitomycin C-treated BALB/c spleen cells. The cell proliferation was monitored by a 16-h [3H]Tdr uptake on days 3, 4, and 5 as detailed previously (16, 17). The supernatants of MLR were collected on days 2, 3, and 4, and measured for IL-2 levels with a bioassay using an IL-2-dependent cell line (CTEV) as described previously (17).

Enrichment of T cells, CD8^+\textsuperscript{+}, or 1B2^+ T cells from spleen

The T cells were enriched by the anti-Ig columns from spleen cells according to the manufacturer’s instructions. The flowthrough contained 65 to 75% Thy-1.2^+ cells. The CD8^+ T cells were purified by MACS using anti-CD8-conjugated magnetic microbeads according to the manufacturer’s instructions. The positively selected population contained 85 to 95% CD8^+ cells. The 1B2^+ T cells were similarly purified by MACS using mAb 1B2 followed by anti-mouse IgG1-conjugated magnetic microbeads. The positively selected population contained more than 90% 1B2^+ cells.

Intracellular calcium concentration ([Ca^{2+}]\textsubscript{i}) flux assay

T cells (1B2^+) were loaded with Indo-1-AM (Molecular Probes, Eugene, OR), and were stimulated with Con A for the measurement of [Ca^{2+}]\textsubscript{i} as described before (15).

Treatment of T cells from the 2C mice for immunoblotting

For CD3 cross-linking experiments, T cells or CD8^+ T cells from 2C spleens were incubated in serum-free RPMI 1640 medium for 30 min on ice in the presence of anti-CD3 mAb 145-2C11. The cells were then washed in cold medium, and the cross-linking was terminated by adding an equal volume of 2× TNE buffer supplemented with protease inhibitors as described before (15).
The presence of peripheral alloantigens does not cause clonal deletion or TCR and CD8 down-regulation of de novo-developed alloantigen-specific 2C T cells

In our model, the mice with T cells de novo developed from 2C bone marrow in the presence of alloantigen did not reject the cardiac allografts. We first examined whether there was clonal deletion in these tolerated mice. The control animals experienced exactly the same manipulation of WBI and BMTx and received bone marrow cells from the same donor as the tolerated animals in each experiment. However, they were not transplanted with a BALB/c heart, or treated with RAPA before WBI and BMTx, i.e., they did not carry existing allografts at the time of BMTx. Our previous work clearly showed that RAPA disappears rapidly after termination of administration (15), and our pilot test showed that the surface markers to be examined in this study were not affected during or after RAPA administration at 8 mg/kg/day (data not shown).

According to two-color flow cytometry, the percentage of Thy-1.2+ T cells in total spleen cells of the tolerated and control mice were comparable (Fig. 2A). The percentages of 1B2+ 2C T cells within the Thy-1.2+ cells had no statistical significant difference between the tolerated and control mice according to eight independent paired experiments. This suggested that there was no apparent clonal deletion, nor was there apparent down-regulation of Ld-specific TCR recognized by mAb 1B2 in the tolerated mice.

We also employed another marker, CD8, to gate the Ld-specific 2C cells, and found that the ratio of 1B2+CD8+CD8+ cells was comparable between the tolerated and control mice in nine paired experiments with no statistically significant difference (Fig. 2B). This result substantiated our conclusion that there was no clonal deletion or TCR down-regulation. The CD8+1B2+ cells were derived from the C57BL/6 host. The interexperimental variations of 1B2+CD8+CD8+ ratios were likely due to differences of quality of bone marrow cells transferred in different experiments.

CD8 down-regulation has been described as a mechanism for tolerance (18, 19). This possibility was studied by evaluating CD8 expression in 1B2+ cells highly enriched by MACS as shown in Figure 2C. The ratio of CD8−1B2+ vs CD8+1B2+ cells in control and tolerated mice was similar in the paired experiments and no statistically significant difference was found in three paired experiments. This indicated that there was no CD8 down-regulation in the 1B2+ population and that this was not a cause for tolerance. The CD8−1B2+ population in both control and tolerated mice was actually CD4CD8 double negative 1B2+ cells (data not shown), and this population was observed not only in WBI/BMTx but also in naïve 2C mice (15). The cause and implication of such a phenomenon is beyond the scope of this study.

Surface activation markers of de novo-developed 2C T cells in the presence of Ld alloantigen

The 2C T cells in C57BL/6 mice were developed from the 2C bone marrow in the presence of periphery BALB/c heart graft. The newly developed 2C T cells had plenty of opportunities to encounter the Ld alloantigen in the graft. We wondered whether these 2C T cells expressed certain markers that could distinguish them from naïve 2C T cells. Spleen was the main draining lymphoid organ for the allograft. However, 40 days after WBI and BMTx, in spite of the existing allograft in the abdominal cavity, the spleen 2C T cells from the tolerated mice had no elevated early activation markers such as CD25 and ICAM-1, or a longer-lasting activation marker CD44 (Fig. 3, A-C). Another activation marker, MEL-14, on these cells was also expressed at a comparable level to that on control 2C
FIGURE 2. The 2C T cells de novo developed in the presence of peripheral alloantigen had no clonal deletion, or TCR or CD8 down-regulation. C57BL/6 mice were transplanted with BALB/c hearts and treated with RAPA for 14 days. On day 21, the mice underwent WBI and 2C BMTx as illustrated in Figure 1. These mice are referred to as “tolerized” in this and subsequent figures. The control mice experienced exactly the same WBI and 2C BMTx but without HTx and RAPA treatment before WBI. These mice are referred to as “controls” in this and subsequent figures. Representative data from three to nine similar experiments are shown.

A, Total spleen T cells from the tolerized and control mice were analyzed with Thy-1.2/1B2 two-color flow cytometry. There is no statistically significant difference between the tolerized and the control mice in the percentages of Thy-1.2 T cells among total spleen cells, or in the percentages of 1B2 T cells among Thy-1.2 T cells (p = 0.668, n = 8, and p = 0.130, n = 8, respectively, according to a paired Student’s t test after an arcsine square root conversion of the percentage).

B, Total spleen cells from two tolerized and two control C57BL/6 mice were analyzed by CD8/1B2 two-color flow cytometry. There is no statistically significant difference between the tolerized and the control mice in their percentage of 1B2-CD8 T cells among 1B2 T cells (p = 0.376, n = 9, according to the paired Student’s t test after an arcsine square root conversion of the percentage).

C, MACS-purified 1B2 T spleen T cells from the tolerized and control C57BL/6 mice were analyzed by CD8/1B2 two-color flow cytometry. There is no statistically significant difference between the tolerized and the control mice in the percentage of 1B2-CD8 T cells among 1B2 T cells (p = 0.25, n = 3, according to the paired Student’s t test after an arcsine square root conversion of the percentage).
FIGURE 3. Activation markers on 2C T cells de novo developed in the presence of peripheral alloantigen. The C57BL/6 mice were manipulated as depicted in Figure 1. The 1B2+ spleen cells from the tolerized or control mice were enriched by MACS columns and analyzed by two-color flow cytometry for activation markers expressed on 1B2+ cells. Percentages of cells expressing the markers among the total 1B2+ cells are indicated. The experiment was performed four to eight times and similar results were obtained. A representative experiment is shown. No statistically significant difference was found between the tolerized and the control mice in their percentage of CD25+1B2+/1B2+ cells, ICAM-1+1B2+/1B2+ cells, CD44+1B2+/1B2+ cells, or MEL-14+1B2+/1B2+ cells (p > 0.05, paired Student’s t test after an arcsine square root conversion of the percentage).
T cells (Fig. 3D). The results obtained from four to eight independent paired experiments were similar, and no statistical difference was found between tolerized and naive mice. Thus, the de novo-developed tolerized 2C T cells had no characteristic phenotype according to the markers examined.

**Signaling events of the de novo-developed 2C T cells**

The de novo-developed 2C T cells appeared to be nonresponsive to the existing alloantigens. We were interested to know whether TCR signal transduction was compromised in these cells. Defective [Ca\(^{2+}\)], flux has been described as a mechanism of anergy for T cells treated with immobilized anti-CD3 in vitro, or for CD4\(^+\) cells treated with Mls-1\(^+\) in vivo (20, 21). This prompted us to examine the [Ca\(^{2+}\)], flux in the de novo-developed tolerized 2C T cells. Our pilot study showed that naive 2C T cells were not responsive to anti-TCR mAb 1B2 or allogenic H-2\(^d\) cells in the [Ca\(^{2+}\)], flux assay, but were responsive to Con A (data not shown). Thus, Con A was used as a stimulant. 2C T cells were highly enriched from spleen cells by MACS (>90% 1B2\(^+\)). As shown in Figure 4A, the 2C T cells developed in the presence of absence of peripheral L\(^d\) alloantigen (tolerized and control, respectively) had similar [Ca\(^{2+}\)], flux upon Con A stimulation ex vivo. Similar results were obtained in three independent paired experiments. As an additional control, we showed in Figure 4B that there was no difference in terms of Con A-triggered calcium flux between naive 2C T cells and T cells from mice previously treated with RAPA (7 days after RAPA treatment at 8 mg/kg/day i.p. for 7 days), or T cells from mice that had undergone RAPA treatment (8 mg/kg/day for 2 days). This suggested that the 2-wk RAPA treatment in our test group had no effect on the calcium flux of their T cells several weeks later.

Protein tyrosine phosphorylation is another important signaling event following TCR ligation, and in some models of T cell anergy, this type of phosphorylation is compromised (20, 22). We examined the tyrosine phosphorylation in 2C T cells from the tolerized and control mice by immunoblotting. Highly enriched 1B2\(^+\) T cells were cross-linked with anti-CD3 mAb 2C11, since our pilot study showed that TCR-specific mAb 1B2 was not effective in triggering the phosphorylation. Our preliminary work also established that the phosphorylation was most prominent at 3 min after the 2C11 cross-linking, and defective phosphorylation, if any, will likely be manifested at this time point, as shown by us and others (15, 20, 22). This time point was thus chosen for evaluation as multiple time points were difficult to do due to a limited amount of purified 2C cells. A representative result of three similar experiments is shown in Figure 5A. Both tolerized and control 2C T cells up-regulated their tyrosine phosphorylation upon the TCR cross-link. Although there were some minor differences in the degree of phosphorylation between tolerized and naive 2C T cells, these differences did not consistently appear in all three experiments. We therefore concluded that there was no apparent defect in the tolerized 2C T cells in this regard.

We have also studied the integrity of the TCR signaling pathway in connection to Lck and Fyn. Lck can associate with CD4 and CD8, and Fyn can associate with TCR ζ-chain (23, 24). They are likely situated in the beginning of the TCR signaling pathway (25, 26). It has been suggested that Lck and Fyn initiate tyrosine phosphorylation of TCR ζ-chains, and the phosphorylation leads to the recruitment of ZAP-70 to the TCR (27, 28). One report shows that the ζ-chain phosphorylation in anergic cells is either compromised or altered (29). This implies a possible dysfunction of Lck and Fyn in the anergic cells. In several other reports, reduced levels Lck and Fyn protein are correlated with reduced T cell response in tumor-bearing animals (30, 31). We therefore evaluated both the kinase activities and the protein levels of these two molecules in the tolerized T cells.

This was first studied in MACS-purified CD8\(^+\) T cells, among which 70% were 2C T cells developed in the presence of alloantigen. Lck or Fyn of these CD8\(^+\) cells was precipitated, and their autophosphorylation was conducted in the presence of [γ-\(^32\)P]ATP. As shown in Figure 5B, the major phosphorylated bands were 56 kDa and 59 kDa, representing the autophosphorylated Lck and Fyn, respectively. There was no significant difference between CD8\(^+\) cells derived from tolerized or control mice in three independent experiments. It is to be noted that although TCR cross-link could augment Lck and Fyn activity in CD4 T cells, in our study it did not increase the Lck and Fyn activity in CD8 T cells and 2C T cells (data not shown). This is in agreement with a previous report by Veillette et al. (32). Therefore, the cross-link experiments were not conducted subsequently in the Lck and Fyn kinase assays. The kinase membranes were hybridized with anti-Lck and anti-Fyn Ab 5 mo later, and the Lck and Fyn protein levels
of tolerized and control CD8<sup>+</sup> cells were similar (the bottom panels of Fig. 5B). To make sure that the result from CD8<sup>+</sup> cells, which contained about 10 to 30% host-derived CD8<sup>+</sup>1B2<sup>+</sup> cells, truly reflected the property of the 1B2<sup>+</sup> T cells, we also purified 1B2<sup>+</sup> cells with MACS (<85% pure), and performed the above described experiments again. As shown in Figure 5B, the Lck kinase activity and protein levels were comparable in the tolerized and control 1B2<sup>+</sup> cells. Similar results were obtained in two other experiments.

An additional control was performed to show that the RAPA treatment would not alter the early signaling events examined in this section. For this purpose, 2C mice were injected with RAPA i.p. at 8 mg/kg/day for 3 days. Their spleen T cells were cross-linked with 2C11, and the cell lysates were immunoblotted for tyrosine phosphorylation as described in A. D. Lck and Fyn activity in naive and RAPA-treated 2C T cells. The nylon wool-purified T cells from the spleen of a naive 2C mouse (control) or from a mouse in the middle of RAPA treatment (8 mg/kg/day i.p. for 2 days) (Rapa) were lysed. The kinase activities of Lck and Fyn in the lysate were precipitated and measured according their autophosphorylation in an in vitro kinase assay as described in B.
2-wk RAPA treatment in our test model had no impact on the results of these parameters 40 days later.

The results in this section showed that 2C T cells de novo developed in the presence of peripheral alloantigen had no apparent defect in \([Ca^{2+}]_i\) flux, tyrosine phosphorylation, and Lck and Fyn kinase activities.

**De novo-developed alloantigen-specific T cells were functionally compromised in IL-2 production and in proliferation upon alloantigen stimulation in vitro**

We next examined these tolerized T cells for two important functions in a rejection response, i.e., IL-2 production and proliferation. The spleen cells from the tolerized and control mice were stimulated with mitomycin C-treated allogenic stimulation cells from the BALB/c spleen. The culture supernatants were harvested on days 2, 3, and 4 for IL-2 quantification. The cells were pulsed with \(^{3}H\)Tdr on days 3, 4, and 5 during the last 16 h of culture for proliferation assays. As shown in Figure 6A, the spleen cells from most of the control mice produced high levels of IL-2 on day 2. The levels declined on days 3 and 4. In contrast, the spleen cells from most of the tolerized mice only produced IL-2 at background levels during this period. The difference was statistically significant. Correlated to the compromised IL-2 production, the tolerant spleen cells did not proliferate vigorously upon H-2d alloantigen stimulation in MLR, as shown in Figure 6B. On the other hand, the control cells started to proliferate on day 3, and had peak \(^{3}H\)Tdr uptake on day 4. There was about a fourfold difference in \(^{3}H\)Tdr uptake between the control and tolerant cells on day 4.

We wondered whether the reduced level of IL-2 was the cause of compromised proliferation of the tolerized 2C cells. Exogenous IL-2 was added to the MLR, and as shown in Figure 6C, it could not reverse the diminished proliferation of the tolerized 2C cells upon H-2d stimulation. In the same experiment, we included mitomycin C-treated C3H spleen cells (H-2k) as control stimulator cells, and they failed to elicit vigorous MLR in the control 2C cells.

To relieve concerns that the initial 2-wk RAPA treatment might interfere with T cell proliferation more than 40 days later in our test, we took 2C spleen cells in the middle of in vivo RAPA treatment (8 mg/kg/day i.p. for 2 days), and tested their response to IL-2 levels in the culture supernatants on days 2, 3, and 4 were assayed.

The data were from eight independent experiments in which tolerized and control mice were paired and experienced exactly the same manipulation in WBi/2C BMTx. The levels of IL-2 on day 2 were 2.34 ± 1.93 U/ml for the controls and 0.35 ± 0.27 for the tolerized cells (p = 0.018, paired Student’s t test). On day 3, the levels were 1.16 ± 0.84 vs 0.19 ± 0.20 (p = 0.02) and on day 4, 0.41 ± 0.25 vs 0.12 ± 0.15 (p = 0.034). B, \(^{3}H\)Tdr uptake in MLR. The total spleen cells from the tolerized and control mice were stimulated with mitomycin C-treated BALB/c spleen cells and were pulsed with \(^{3}H\)Tdr for the last 16 h of the culture on days 3, 4, and 5. Data from two representative pairs of mice are shown. Similar results were obtained in three independent experiments. C, MLR in the presence of exogenous IL-2. MLR was performed as described above, except that exogenous recombinant human IL-2 (20 U/ml) was added to certain cultures as indicated. The cells were cultured for 3 days and were pulsed with \(^{3}H\)Tdr for the last 16 h of culture before being harvested. The mitomycin C-treated C3H spleen cells (H-2k) were also included as a control to verify the alloantigen specificity of the de novo-developed 2C cells. D, The effect of in vivo RAPA treatment on the proliferation of 2C cells in vitro. Spleen cells of a naive 2C mouse (control) or from a mouse in the middle of in vivo RAPA treatment (8 mg/kg/day i.p. for 2 days) (Rapa) were stimulated with mitomycin C-treated BALB/c spleen cells in vitro, and \(^{3}H\)Tdr uptake of the MLR was measured on days 3, 4, and 5.
H-2\textsuperscript{d} alloantigens in vitro. As shown in Figure 6D, the RAPA-treated and vehicle-treated 2C spleen cells respond equally well in the MLR. This is consistent with our observation that lymphocytes from patients under active cyclosporin A therapy could respond very well to mitogen or alloantigen stimulation in vitro. Likely explanations for such an observation are that 1) in spite of the nominal high dosage administered to the animals or patients, the actual biologically available drug concentration to the T cells is not very high, and 2) such limited bioavailable drug is rapidly metab-
olized or degraded during the process of cell preparation and/or in the early culture period. As a consequence, the in vitro activation of these cells is not affected by in vivo RAPA treatment. Our results indicated that the 2-wk RAPA treatment in our test group had a negligible effect on their in vitro MLR more than 40 days later.

The results in this section demonstrated that alloantigen-specific T cells developed de novo in the presence of alloantigen were functionally compromised in IL-2 production and in proliferation upon stimulation by the specific alloantigen in vitro. However, the compromised proliferation was unlikely due to the reduced IL-2 production by the tolerant 2C cells, since a large amount of exogen-
ous IL-2 could not drive the cells to proliferate either. Further-
more, we have shown that the de novo-developed 2C T cells were still H-2\textsuperscript{d}-specific, as expected, in that they could specifically re-

donstrate IL-2 production by the tolerant 2C cells, since a large amount of exogenous IL-2 could not drive the cells to proliferate either. Furthermore, we have shown that the de novo-developed 2C T cells were still H-2\textsuperscript{d}-specific, as expected, in that they could specifically re-
spond to the H-2\textsuperscript{d} but not H-2\textsuperscript{b} alloantigen.

Discussion
In this study, we examined how de novo-developed alloantigen-
specific T cells behaved in the presence of peripheral alloantigen. The study not only intended to explore fundamental mechanisms governing the tolerance to extrathymic tissue-specific Ags, but also to answer a clinically relevant question, i.e., do newly emigrated T cells from the thymus of young patients actively participate in graft rejection?

Our results indicated that there was no clonal deletion but, func-
tionally, the specific T cells were compromised. They could not reject the existing allograft in vivo, and failed to produce IL-2 and proliferate upon alloantigen stimulation in vitro. Moreover, their proliferation defect could not be reversed by exogenous IL-2. Phenotypically, the specific T cells had no characteristic markers. Some critical early TCR signaling events, such as [Ca\textsuperscript{2+}] flux, tyrosine phosphorylation, and Lck and Fyn activities, seemed to be normal.

It has been documented that the thymus-blood barrier is not as tight as we used to believe, and peripheral lymphocytes are able to enter the thymus (33). Although in theory some of the passenger leukocytes from the graft might enter the host thymus in our model, in reality the frequency should be very low. This is due to the fact that the heart graft carries a limited number of donor-type leukocytes, and that the subsequent WBI effectively eliminated most of the existing lymphocytes. Our study has established that even though there is such entry, it is inconsequential with respect to negative selection. There is no clonal deletion either centrally or peripherally for the T cells developed in the presence of peripheral alloantigens. This is consistent with findings in some models of double transgenic mice, which carry H-2\textsuperscript{K}\textsuperscript{b}-specific TCR and have H-2\textsuperscript{K}\textsuperscript{b}\textsuperscript{e} expression driven by the albumin promoter (Alb.K\textsuperscript{b} \times TCR mice) or by the keratin IV promoter (IV.K\textsuperscript{b} \times TCR mice) in the periphery (9), although in our case, the 2C T cells were developed during adulthood.

No characteristic phenotype indicative of T cell activation has been found in the de novo-developed tolerized 2C T cells in our model. We previously reported that mature 2C T cells could be tolerized by persistent alloantigen in a model where 2C mice were transplanted with a BALB/c heart and treated with a short duration of RAPA (15). In that model, the tolerized 2C T cells have no particular phenotype either. Thus, the lack of commonly known activation markers or memory markers of the tolerized cells seems to be a feature in both of these models.

We have observed similarities between our model of peripheral tolerance with the Alb.K\textsuperscript{b} \times TCR double transgenic mice (9), in that the specific T cells are incompetent to reject an allograft in vivo and fail to respond to the alloantigen in vitro. However, no TCR and CD8 down-regulation could be found in our model, as it could be in the Alb.K\textsuperscript{b} \times TCR mice (9). This has raised an intriguing possibility: that the moderate down-regulation of TCR and CD8 in those double transgenic mice are merely parallel but not causative events for the peripheral tolerance. In any case, a convincing mechanism to explain how a moderate down-regulation of CD8 or TCR could lead to tolerance is lacking.

Compared with the double transgenic mice mentioned above, the tolerance in our model is not complete in a sense that there was still some mononuclear cell infiltration in the graft (data not shown), and some 23% of the HTx/WBI/BMTx mice rejected their allografts. There are two possible reasons for this. First, this might be partly due to initial damage to the graft that occurred in the first 21-days postoperation during RAPA administration before WBI/BMTx. The damage was then manifested after the WBI/BMTx. Indeed, the protection of RAPA is not complete. In our previous study, about 27% of the 2C mice transplanted with BALB/c allografts under the cover of RAPA eventually reject the graft (15). The rate is quite similar to the total rejection rate observed in this study (four before WBI/BMTx + three after WBI/BMTx divided by a total of 30 HTx mice = 23%). Secondly, there might be a difference between perinatally developed T cells during and those developed in adulthood. The former were more easily tolerized to peripheral Ags, as reported by Adams, Alpert, and Hanahan (8). The above-described two possible reasons are not mutually exclusive.

Do the host-derived T cells have regulatory roles in our model? In some experiments not presented in this paper, we irradiated the recipient with 1000 rad. Under such conditions, all host-derived T cells were eliminated, yet similar tolerance was still observed. This suggests that the role of host-derived T cells, if there is any, is not critical in generating or maintaining the tolerant status.

We tried to identify defects in the signaling pathway of the de novo-developed tolerized T cells. Several parameters such as [Ca\textsuperscript{2+}] flux, tyrosine phosphorylation, and Lck and Fyn activities have been implicated in the signaling defect of the tolerized T cells (20–22, 29–31). However, we have found no apparent anomaly in these parameters in the tolerized T cells in our model. Migita et al. have reported that tolerized T cells are defective in TCR z-chain phosphorylation and z-chain/ZAP70 association upon Ag stimula-
tion (29). We also examined these parameters in the tolerized 2C T cells but they seemed to be comparable to those of naive 2C T cells (data not shown). Our data also showed that the tolerized 2C T cells had intrinsic defects in their capability to proliferate and the reduced IL-2 production was not the primary cause of their defec-
tive proliferation, since exogenous IL-2 could not rescue the compromised proliferation. It is likely that the signaling defect of the tolerized 2C T cells in our model lies downstream of the early events such as tyrosine phosphorylation and calcium flux, but before IL-2 production and proliferation. The exact nature of the defect is not clear, and we are left with the challenging task of discovering it.

In our previous model, mature 2C T cells were tolerized to H-2\textsuperscript{d} alloantigen by blocking their initial response with RAPA followed
by the persistent alloantigens. Those 2C T cells had compromised [Ca^{2+}], flux and tyrosine phosphorylation. In theory, the persistent alloantigen in this current model could also be a mechanism accountable for the observed tolerance. However, it is intriguing that the [Ca^{2+}], flux and tyrosine phosphorylation in this model seem to be normal. How do we reconcile the difference? It has been speculated, although not yet proven, that the newly emigrated T cells from the thymus have a short time window during which they could be rendered tolerant to peripheral Ags. If this is true, then the de novo-developed 2C T cells in this study might depend mainly on this window of opportunity to become tolerant to the alloantigens, while in our previous model, the mature 2C T cells depend mainly on constant desensitization of the alloantigen to establish tolerance. Thus, two completely different mechanisms might be involved; hence the difference in the signal transduction events.

In summary, the results of this study show that tolerance (defined as failing to reject an existing allograft) of de novo-developed 2C T cells to peripheral Ag is achievable during adulthood without clonal deletion. The functional defects of these tolerized T cells are more characteristic than changes of their phenotype or early signaling events. We believe that this is also the case in young transplantation recipients. The de novo-developed alloantigen-specific T cells from functional thymic in those patients are likely not deleted, but functionally compromised. Consequently, these cells are not a serious force to be reckoned with in acute rejection episodes. Whether these cells could contribute to a lesser extent to chronic rejection is still an open question.

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References
