$CD8^+$effector T cells and cytotoxicity increase appreciably, but in these patients the absolute numbers of these effector cells in whole blood remained profoundly depleted. Such excessive downregulation of CTLs may impair infection eradication in a patient and increase the patient's vulnerability to other invading pathogens. Lack of $CD4^+$ cell help may well be a crucial factor predisposing a patient to the induction of severe life-threatening infections that lead to multiple organ failure.

The main function of $CD4^+$EM T cells relates to functional maturation of the $CD8^+$ T-cell response to bacteria and viruses; this response is additional to the destruction of infected cells by antimicrobial lymphokines. With regard to the underlying mechanism, most activated $CD8^+$CTL-precursor cells require additional IL-2, which is produced by proliferating $CD4^+$EM T cells, to proliferate and differentiate into effector CTLs. IL-2 and costimulation are important in the transformation of naïve CTL-precursor cells into mature effector cells; $CD4^+$EM T cells may act as mediators that facilitate this cell differentiation process. Thus, helper cells may play a major role in providing help to responding $CD8^+$ T cells as a consequence of their capacity to activate antigen-presenting cells by interacting with CD40-CD40 Legands.[20,21]

Finally, in naïve-cell-dominant recipients, preexisting $CD8^+$ T cells have the lowest cytotoxic activity, but after LDLT there is prompt and substantial upregulation of effector T cells in response to severe infection. In contrast, in most EM- or effector-cell-dominant recipients, memory T cells induced by preexisting viral infection responded to infection after LDLT by triggering only limited further generation of CTLs. These findings suggest that recipients with markedly downregulated naïve T cells are strongly dependent on heterologous immunity, whereas naïve-cell-dominant recipients are not. In particular, the available T-cell repertoire of effector-cell-dominant recipients may be markedly skewed and limited.[22] Thus, it seems likely that the capacity to generate CTLs in response to newly invading pathogens after LDLT is lower in EM- or effector-cell-dominant recipients than in naïve-cell-dominant recipients.

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Antidonor Antibody in Patients Receiving ABO-Identical and HLA-Mismatched Living Donor Liver Transplants: Effect on Survival

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We retrospectively determined the correlation of results of lymphocyte crossmatch tests by direct complement-dependent cytotoxicity, to the outcomes of 585 consecutive ABO-identical and human leukocyte antigen (HLA)-mismatched living donor liver transplants (LDLTs) (male:female=276:309; median age, 18 years). Crossmatch test results were positive in 14 recipients (2.4%). Patient survival at eight years in the crossmatch-positive group was significantly lower than in the crossmatch-negative group (positive group, 56.3%; negative group, 77.6%; $P=0.014$). The survival at five years of the crossmatch-positive group was significantly lower than the negative group in both older recipients (≥18 years of age: positive group, 41.7%; negative group, 76.4%; $P=0.0065$), and female recipients (positive group, 37.5%; negative group, 81.9%; $P=3.3\times10^{-5}$). We conclude that antidonor antibodies have adverse effects on the clinical outcome of LDLTs, and that being female and/or older aged (≥18 years of age) are risk factors for LDLT.

Keywords: Direct lymphocyte crossmatch, Living donor liver transplantation, Human leukocyte antigen, Antidonor antibody.

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Solid organ grafts, especially renal or heart transplants, into a recipient with a positive lymphocyte crossmatch test against the donor is correlated with acute rejection (AR) (*1, 2*). However, the relevance of a positive crossmatch to the outcome of liver transplantation remains unclear, although there are several published studies of crossmatch test results and their relationship to the outcomes of living donor liver transplantations (LDLTs) (*3–8*). We retrospectively examined the relationship of lymphocyte crossmatch test results, using direct complement-dependent cytotoxicity (CDC), to the outcomes of ABO-identical and human leukocyte antigen (HLA)-mismatched LDLTs. In this study, we report that the presence of antidonor antibodies is detrimental to patient survival in ABO-identical and HLA-mismatched LDLTs.

From 1996 through 2005, 804 LDLTs were performed at Kyoto University Hospital, of which 585 (72.8%) were ABO identical and HLA mismatched. This study enrolled these 585 LDLTs, which included 43 retransplant cases. In accordance with the Declaration of Helsinki recommendations, approval was obtained from the institutional review board at Kyoto University Hospital, and informed consent was provided to all patients. There were 276 male and 309 female patients, with a median age of 18 years (range: 27 days to 69 years). The numbers of patients with blood types A, B, AB, and O were 248, 125, 27, and 185, respectively. The indications for transplantation are shown in Table 1. Hepatitis B virus (HBV) was positive in 68 recipients and hepatitis C virus (HCV) was positive in 63 recipients. Surgical procedures were performed as described previously (*9*). The immunosuppression protocol used tacrolimus and low-dose steroids (*10*). Briefly, tacrolimus was started on day one before transplantation at a dosage of 0.15 mg/kg/day divided into two doses, except in cases with hepatic encephalopathy. The posttransplantation whole blood trough target level for tacrolimus was 10–12 ng/mL during the first two weeks, and approximately 10 ng/mL thereafter. Methylprednisolone at 10 mg/kg body weight was given intravenously during the operation just after reperfusion. In the postoperative period, 1 mg/kg of methylprednisolone was given intravenously once a day for the first three postoperative days, followed by 0.5 mg/kg once a day for the next four days. It was switched to oral prednisolone at a dose of 0.5 mg/kg/day seven days after transplantation. Prednisolone was reduced to 0.3 mg/kg/day four weeks after transplantation, if there was no liver dysfunction during the postoperative course.

A lymphocyte crossmatch test using direct CDC was performed to detect antidonor antibodies (*11*). Briefly, 1 μL of donor lymphocyte suspension and 5 μL of recipient serum, which was obtained immediately before LDLT, were incubated in a Terasaki plate (Nunc, Roskilde, Denmark) at room

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TABLE 1. Indications for living donor liver transplantation in 585 transplant recipients

Diseases before transplantation	Number	Percent
Congenital bile duct disorders	242	41.4
Biliary atresia	231	
Alagille syndrome	8	
Congenital biliary dilatation	2	
Caroli's disease	1	
Hepatocellular carcinoma	80	13.7
Liver cirrhosis	57	9.7
Fulminant hepatic failure	36	6.2
Primary biliary cirrhosis	34	5.8
Metabolic liver diseases	33	5.6
Wilson's disease	16	
Citrullinemia type II	5	
Hypertyrosinemia	4	
Glycogen storage disease	2	
Oxalosis	2	
Ornithine transcarbamylase deficiency	2	
Propionic acidemia	1	
Unclassified	1	
Primary sclerosing cholangitis	24	4.1
Fulminant hepatitis	24	4.1
Hepatoblastoma	12	2.1
Progressive familial intrahepatic cholestasis	7	1.2
Budd-Chiari syndrome	7	1.2
Hepatic failure due to chronic rejection	6	1.0
Autoimmune hepatitis	5	0.85
Congenital hepatic fibrosis	4	0.68
Acute phase of HBV chronic hepatitis	4	0.68
Polycystic liver and kidney	3	0.51
Metastatic liver tumor	3	0.51
Idiopathic portal hypertension	1	0.17
Hepatic vein thrombosis	1	0.17
Acute hepatic failure due to heat shock	1	0.17
Hepatic failure due to graft-versus-host disease after allogeneic bone marrow transplantation	1	0.17

temperature for 30 min. Five microliters of rabbit complement was added to each well, and the mixture was incubated at room temperature for 60 min. Then 2 μL of 5% eosin solution was added, and the cells were then fixed with formalin. The plate was examined using phase contrast microscopy (IMT-2, Olympus; Tokyo, Japan). The results were considered positive when more than 20% of the donor lymphocytes were killed by the recipient's serum.

Survival after LDLT was determined in the patient groups testing positive or negative for donor lymphocyte antibodies by direct CDC. Chi-squared test, Student's *t* test, and the Mann-Whitney *U* test were used to evaluate sex, age, and blood types for independence, respectively. Survival rates were estimated by the Kaplan-Meier method and compared by a log-rank test. A *P* value of <0.05 was regarded as statistically significant.

There were 14 graft recipients (2.4%) positive by the donor-specific T-cell crossmatch test, including four males and 10 females. Their ages ranged from 1 to 67 years (median, 29 years). Recipient blood types were: four type A, three type B, two type AB, and five type O. There were 571 recipients negative by the crossmatch test, including 272 males and 299 females. Their ages ranged from 27 days to 69 years (median, 17.5 years) and their blood types were: 244 type A, 122 type B, 25 type AB, and 180 type O. There were no significant differences in the median age, sex, and blood types between the antibody positive and negative groups. The survival rates are presented in Table 2. The survival rate of the crossmatch-positive recipients was significantly lower compared with that of the crossmatch-negative recipients ($P=0.014$; Fig. 1A). Of the 14 crossmatch-positive recipients, eight remain alive. Six died within one year after transplantation, with five of six recipients dying less than three months after transplantation. The cause of death in 83% of recipients was multiorgan failure (MOF) due to sepsis or infection. In the crossmatch-negative patients, 41% of recipients died from MOF, 11% from recurrence of their original disease, 9% from chronic rejection, and 6% from hemorrhagic shock.

We compared the survival rates between the positive and negative groups subdivided by age or gender. There were 291 younger recipients (<18 years) and 294 older recipients (≥ 18 years). Among the younger recipients there was no significant

TABLE 2. Comparison of patient survival rates after living donor liver transplantation

	Crossmatch test	Patient survival (%) 1 year	5 years	8 years	*P* value
All (n=585)	Positive (n=14)	56.3	56.3	56.3	0.014
	Negative (n=571)	82.1	79.1	77.6	
Age <18 (n=291)	Positive (n=5)	80	80	80	0.784
	Negative (n=286)	85.8	83.4	83.4	
Age ≥18 (n=294)	Positive (n=9)	41.7	41.7	41.7	0.0065
	Negative (n=285)	78.6	74.6	70.1	
Male (n=276)	Positive (n=4)	100	100	NA	0.276
	Negative (n=272)	80.7	75.4	72.6	
Female (n=309)	Positive (n=10)	37.5	37.5	NA	3.3×10^{-5}
	Negative (n=299)	83.2	81.9	81.2	

NA, not applicable: the duration of follow-up of male and female crossmatch-negative recipients has not reached eight years.

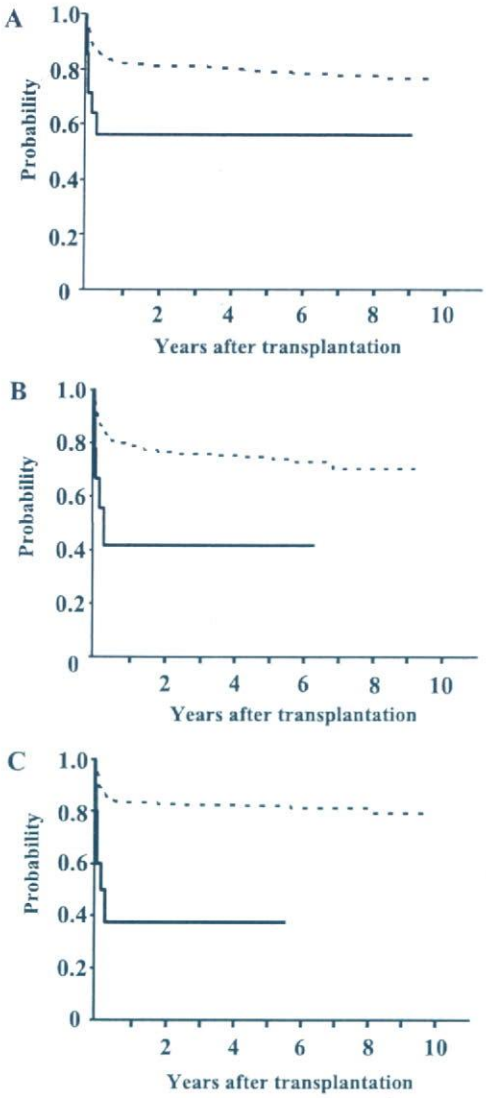

FIGURE 1. Kaplan-Meier analysis of survival rates in living donor liver transplantation (LDLT). (A) Kaplan-Meier patient survival curves of all LDLT recipients in the absence (n=571, dashed line) or presence (n=14, solid line) of a positive direct complement-dependent cytotoxic crossmatch. There is significant difference between them (P=0.014). (B) Kaplan-Meier patient survival curves of older (≥18 years old) LDLT recipients in the absence (n=571, dashed line) or presence (n=14, solid line) of a positive direct complement-dependent cytotoxic crossmatch. There is significant difference between them (P=0.0065). (C) Kaplan-Meier patient survival curves of female LDLT recipients in the absence (n=299, dashed line) or presence (n=10, solid line) of a positive direct complement-dependent cytotoxic crossmatch. There is significant difference between them ($P=3.3\times10^{-5}$).

difference in the survival rates between the crossmatch-positive (n=5) and negative groups (n=286). However, among the older recipients, the patient survival rate of the crossmatch-positive group (n=9) was lower than that of crossmatch-negative group (n=285, P=0.0065; Fig. 1B). Among the groups subdivided by sex, there was no significant difference between the survival rates of the crossmatch-positive and negative groups in 276 male recipients. However, in 309 female recipients, the patient survival rate was significantly lower in the crossmatch-positive group than in the crossmatch-negative group ($P=3.3\times10^{-5}$; Fig. 1C).

There are two published reports discussing the correlation of crossmatch test results and patient survival after liver transplantation (*5*, *12*). Sugawara et al. analyzed 113 adult LDLT cases, and demonstrated that there was a tendency towards decreased survival (but no statistical significance) in patients with positive crossmatch results (*5*). We analyzed 585 LDLT recipients, which are sufficiently larger than their sample number, and we were able to determine that there was a significant difference between crossmatch-positive and -negative patients. Moreover, our results are consistent with a larger study on cadaveric donor liver transplantations (*12*).

In this study, of the 14 crossmatch-positive recipients, six died within the first year after transplantation. AR occurred within one year after transplantation in crossmatch-positive recipients as previously demonstrated (*8*), and the dose of immunosuppressants was increased to prevent the worsening of AR. The recipients became compromised and infection or sepsis occurred. Death by MOF ultimately occurred in six recipients.

In a previous study at our institution, no correlation was found between the positive pretransplant flow cytometry crossmatches and the survival (*8*). That study analyzed 47 LDLT recipients with a mean age of 11.2 years. In this study, a larger number of recipients were enrolled, with a median age of 18 years, as well as an older mean age of 23.9 years. The patient survival rate of crossmatch-positive recipients in the older group of recipients in the present study was significantly lower than in the older crossmatch-negative recipients, although the survival rates in the younger crossmatch-positive and negative recipient groups were not statistically different.

In this study, we also demonstrated that female recipients are a high-risk group for LDLTs. Out of 306 female recipients, 146—including 8 out 10 crossmatch-positive female recipients—were older (≥18 years). We found that the sera of crossmatch-positive female recipients showed strong cytotoxicity, and postulate that older female recipients have high titers of anti-HLA antibodies because of sensitization during pregnancy.

In conclusion, the presence of antidonor antibody in liver graft recipients adversely affects the clinical outcome of LDLTs. Being female and/or older (≥18 years of age) are risk factors for LDLT. The crossmatch test against donor lymphocytes is useful in predicting clinical outcomes of recipients of LDLTs. It is important to monitor the crossmatch-positive recipients carefully, especially older and/or female recipients, and to treat them aggressively.

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T Cell Lineage Determination Precedes the Initiation of *TCRβ* Gene Rearrangement[1]

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Loss of dendritic cell potential is one of the major events in intrathymic T cell development, during which the progenitors become determined to the T cell lineage. However, it remains unclear whether this event occurs in synchrony with another important event, TCRβ chain gene rearrangement, which has been considered the definitive sign of irreversible T cell lineage commitment. To address this issue, we used transgenic mice in which GFP expression is controlled by the *lck* proximal promoter. We found that the double-negative (DN) 2 stage can be subdivided into GFP$^-$ and GFP$^+$ populations, representing functionally different developmental stages in that the GFP$^-$DN2, but not GFP$^+$DN2, cells retain dendritic cell potential. The GFP$^+$DN2 cells were found to undergo several rounds of proliferation before the initiation of TCRβ rearrangement as evidenced by the diversity of D-Jβ rearrangements seen in T cells derived from a single GFP$^+$DN2 progenitor. These results indicated that the determination step of progenitors to the T cell lineage is a separable event from TCRβ rearrangement. *The Journal of Immunology,* 2007, 179: 3699–3706.

T and B lymphocytes use a common gene-recombination machinery to form their diverse AgRs, the TCR in T cells and BCR in B cells. The precise timing of segregation of these two cell lineages and the occurrence of gene rearrangement of *TCR* and *BCR* genes have long been a matter of dispute. Because V to DJ rearrangement of IgH chain genes in B cells and of TCRβ chain genes in T cells is strictly regulated in a lineage-specific manner (1, 2), completion of this rearrangement step has been regarded as a sign of irreversible commitment to the B and T cell lineages, respectively. However, several studies indicate that T and B cell lineages are separated much earlier than the initiation of these gene rearrangements. In the case of B cell progenitors, Allman et al. (3) demonstrated that early B cell lineage committed progenitors contain *IgH* genes in germline configuration, and proposed that B cell lineage commitment takes place before D-J rearrangement at the H chain locus; however, the extent of proliferation of the B cell lineage committed progenitors before the IgH rearrangement remains unknown. In the case of T cell progenitors, we have shown that cells that have lost B cell and myeloid potential can expand in the thymus by >1000-fold before they initiate TCRβ rearrangement (4, 5). Thus, commitment to the T cell lineage, as defined by the shutoff of B cell potential, should occur much earlier than the initiation of *TCRβ* gene rearrangement.

Progenitors for T cells that have shutoff B cell and myeloid potential are not necessarily fully T cell lineage committed, because a large proportion of early thymocytes retain dendritic cell (DC)[3] potential (6–8). We have recently provided clonal evidence that early intrathymic T cell progenitors as well as prethymic T cell progenitors retain the potential to generate DCs and NK cells (9–12). DCs are primarily considered as members of the myeloid family, because their major functions, such as phagocytosis and Ag-presenting activity, are similar to those performed by macrophages. Indeed, some previous studies have also pointed out that early intrathymic progenitors can produce macrophages (13, 14). Because the frequency of DC-generating progenitors is much higher than that of macrophage-generating progenitors, it is probable that the DC potential is more stably retained by T cell progenitors. Thus, we reasoned that the shutoff of DC potential in T cell progenitors can be considered as one of the most important events for them to acquire their T cell lineage identity.

In developmental biology, the commitment process is divided into specified and determined phases (15). According to this characterization, T cell lineage "specified" progenitors are nearly committed to the T cell lineage, but still retain other potentials such as NK and DC potentials and possibly residual macrophage potential, whereas T cell lineage "determined" progenitors are irreversibly committed to T cell lineage. Thus, hereafter, we refer to the point where the progenitors lose their DC potential as "T cell lineage determination."

Some previous studies have focused on the DC shutoff point during intrathymic T cell development. Immature CD4$^-$CD8$^-$ (double-negative (DN)) thymocytes can be subdivided into subpopulations based on expression of CD44 and CD25; CD44$^+$ CD25$^-$ (DN1) cells represent the earliest population and they differentiate into CD44$^-$CD25$^+$ (DN3) stage through CD44$^+$CD25$^+$

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[3] Abbreviations used in this paper: DC, dendritic cell; DN, double negative; dpc, days post coitum; rm, recombinant murine; SCF, stem cell factor; Flt3L, Flt3 ligand; FT, fetal thymus; dGuo, deoxyguanosine; PI, propidium iodide; AT, adult thymus; DP, double positive; SP, single positive.

(DN2) stage. It has been shown that DN1 and DN2 cells but not DN3 cells are able to give rise to DC (6–8). Hence, the transition from DN2 to DN3 stage was assumed to be the point to shutoff the DC potential. It is also known that thymic progenitors undergo extensive proliferation at the DN2 stage (5, 16) and then enter a resting phase at the DN3 stage, where TCRβ rearrangement takes place. In general, differentiation of cells occurs when they are at the "resting" stage, as is the case here for TCRβ rearrangement. It is probable that the shutoff of DC potential additionally require a drastic change in the differentiation program by completely closing a broad spectrum of loci related to the myeloid lineage. The question addressed in the present study is whether the shutoff of DC potential also takes place at the DN3 stage in synchrony with the initiation of *TCRβ* gene rearrangement. We determined the developmental timing of these two events and found that DC potential is shutoff within the DN2 stage, while TCRβ rearrangement occurs at the DN3 stage, and that these two events are separated by several cell divisions.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from SLC. B6Ly5.1 mice were maintained in our animal facility. Transgenic mice of B6 background carrying enhanced GFP that is expressed under the control of the proximal lck promoter (plck-GFP mice) (17) were maintained in our animal facility. Embryos at 15 days postcoitum (dpc) were obtained from timed pregnancies of B6, B6Ly5.1, and plck-GFP mice. The day of finding the vaginal plug was designated as 0 dpc.

Antibodies

The following Abs were purchased from BD Pharmingen: anti-Ly5.1 (A20), anti-Ly5.2 (104), anti-c-*kit* (2B8), anti-erythroid lineage cells (TER119), anti-Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), anti-CD8 (53-6.7), anti-CD4 (H129.19), anti-NK1.1 (PK136), anti-CD3ε (145-2C11), anti-CD25 (PC61), anti-CD44 (IM7). CD4, CD8, CD3ε, TER, B220, Gr-1, and NK1.1 were used as lineage markers (Lin).

Growth factors

Recombinant murine (rm) stem cell factor (SCF), rmIL-2, rmIL-3, rmIL-7, rmGM-CSF, rmFlt3 ligand (Flt3L), rmIL-1α, and rmTNF-α were purchased from Genzyme Techne.

Culture conditions for DC generation

RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS (M.A. Bioproducts), L-glutamine (2 mM), sodium pyruvate (1 mM), sodium bicarbonate (2 mg/ml), nonessential amino acid solution (0.1 mM; Invitrogen Life Technologies), 2-ME (5×10^{-5} M), streptomycin (100 μg/ml), and penicillin (100 U/ml) were used. A mixture of the following cytokines—SCF (10 ng/ml), IL-3 (10 ng/ml), IL-7 (10 ng/ml), GM-CSF (10 ng/ml), Flt3L (10 ng/ml), IL-1α (10 ng/ml), and TNF-α (10 ng/ml)—was used as a cytokine mixture (7, 9). In the experiments shown in Fig. 1*C*, 100 cells were cultured in wells of a 96-well plate (Costar). In the experiments shown in Fig. 1*D*, single fetal thymus (FT) cells were individually cultured in the wells of a Terasaki plate (Nunc).

Coculture with a FT lobe

The procedures for the coculture with a deoxyguanosine (dGuo)-treated FT lobe under high oxygen submersion conditions have been described in detail previously (4, 5). Basically, single dGuo-treated FT lobes (B6Ly5.2) were placed into wells of a 96-well V-bottom plate, to which cells from B6Ly5.1 mice to be examined were added. Culture medium was supplemented with SCF (1 ng/ml) and IL-7 (3 ng/ml). The plates were centrifuged at $150 \times g$ for 5 min at room temperature, placed into a plastic bag (Ohmi Odor Air Service), the air inside was replaced by a gas mixture (70% O_2, 25% N_2, and 5% CO_2), and incubated at 37°C. After 10 days of cultivation, cells were harvested from each well. For the detection of T and NK cell potential, the culture system was the same as above except that IL-2 (1 ng/ml) was added to the culture medium (18).

Coculture with stromal cells

Murine stromal cell line TSt-4 cells were retrovirally transduced with the murine *DLL-1* gene (TSt-4/DLL-1) (12). Cells of FT subpopulations (200 or 500 cells/well) were cultured in a well of 24-well plate monolaid with TSt-4/DLL1. After cultivation, cells were harvested by trypsinization, stained in two colors with anti-CD44 and anti-CD25, and analyzed by a flow cytometer. Cells falling on lymphoid gate in scatter characteristics, gating out dead cells by propidium iodide (PI) staining, were flow cytometrically counted.

RT-PCR

RT-PCR was performed as described previously (4, 12). Primers used were: PU.1 sense: 5′-AGATGCACGTCCTCGATACT-3′, PU.1 antisense: 5′-TTGTGCTTGGACGAGAACTG-3′; Gata-3 sense: 5′-TCGGCCATTC GTACATGGAA-3′, Gata-3 antisense: 5′-GAGAGCCGTGGTGGATGG AC-3′; CD3ε sense: 5′-ATCACTCTGGGCTTGCTGAT-3′, CD3ε antisense: 5′-TAGTCTGGGTTGGGAACAGG-3′; pTα sense: 5′-AACAGGTAGCTC CTGGCTGCA-3′, pTα antisense: 5′-CAGGAAGAACAAAGCCC-3′; Tcf-1 sense: 5′-CCAGCTTTCTCCACTCTACG-3′, Tcf-1 antisense: 5′-TCAAGGATGGGTGGGTGAAC-3′; ikaros sense: 5′-GAGGCATGGC CAGTAATGTT-3′, ikaros antisense: 5′-AGGCCGTTCACCAGTA TGAC-3′; Rag2 sense: 5′-CCCAGAGAACCACAGAAAAAT-3′, Rag2 antisense: 5′-TAACCACCCACAATAACAAAT-3′; β-actin sense: 5′-TC CTGTGGCATCCATGAAACT-3′; β-actin antisense: 5′-GAAGCACTTG CGGTGCACGAT-3′.

Cycling times and temperatures were as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Amplification was performed for 25 cycles for *β-actin* and 35 cycles for all other genes. The PCR products were electrophoresed through a 1.2% agarose gel and stained with ethidium bromide.

Cell cycle analysis

The amount of nuclear DNA was determined by PI staining as follows. Cells were fixed in 50% ethanol, washed, and incubated in PBS containing 1 mg/ml RNase at 37°C for 20 min. The cells were washed in PBS, resuspended in PBS containing 100 μg/ml PI, and analyzed by a flow cytometer.

Assessment of the potential of individual progenitors to proliferate before the TCRβ chain gene rearrangement (pre-β proliferation)

In determining the pre-β proliferation of progenitors from normal mice, single cells were cultured with a dGuo-treated lobe for 12 days. Genomic DNA extracted from cells generated in each well (1000 cells equivalent) was PCR amplified using primers: Dβ1, 5′-TTATCTGGTGGTTTCTTC CAGC-3′; Jβ1.5, 5′-CAGAGTTCCATTTCAGAACCTAGC-3′; Dβ2, 5′-GCACCTGTGGGGAAGAAACT-3′; Jβ2.6, 5′-TGAGAGCTGTCTCCT ACTATCGATT-3′. The reaction volume was 20 μl containing 5 μl of the cell extract (equivalent to 1000 cells), 1.5 μl of 10× PCR buffer, 0.16 μl of 25 mM dNTPs, 0.4 μl of each primer (10 mM), and 0.6 U of *Taq* polymerase. Thermocycling conditions were as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C, and finally 10 min at 72°C. Resulting products were electrophoresed through an agarose gel and stained with ethidium bromide.

The extent of pre-β proliferation was calculated as described previously (5). The detected number of PCR bands does not necessarily represent the real number of rearranged *TCR* gene constructs actually present in T cells generated in a clonal culture, because overlap between bands is possible. Therefore, as the first step, the prediction of the actual number of D-J rearrangement constructs per clonal culture was statistically calculated. The next step is to estimate how many cells have initiated TCRβ rearrangement to generate the predicted number of D-J constructs. This step was done by simulation based on assumptions that 1) D-J rearrangement occurs in both alleles, 2) V-DJ rearrangement subsequently occurs in one allele and, if it is successful, V-DJ rearrangement does not occur in the other allele due to allelic exclusion, but if it fails, V-DJ rearrangement occurs in the second allele, and 3) all segments were used equally. It is important to understand that a D-J construct becomes undetectable by PCR when V-DJ rearrangement occurs in the locus. Because the probability for D1-J1, D2-J2, or D1-J2 constructs to remain detectable by PCR differs from each other, the equation for the mathematical calculation is separately given in each experiment based on D1-J1, D2-J2, and D1-J2 PCR data. In this study, PCR was performed for D1-J1.5 and D2-J2.6. When T cells derived from a single progenitor exhibit an average of m bands in the PCR analysis, the different equations predicting pre-β proliferation (N) are as follows: 1) from D1-J1.5 PCR data: $n = 25.8 \times (-\ln(5 - m)/5)$; 2) from D2-J2.6 PCR data: $n = 15.6 \times (-\ln(6 - m)/6)$. Theoretically, if PCR

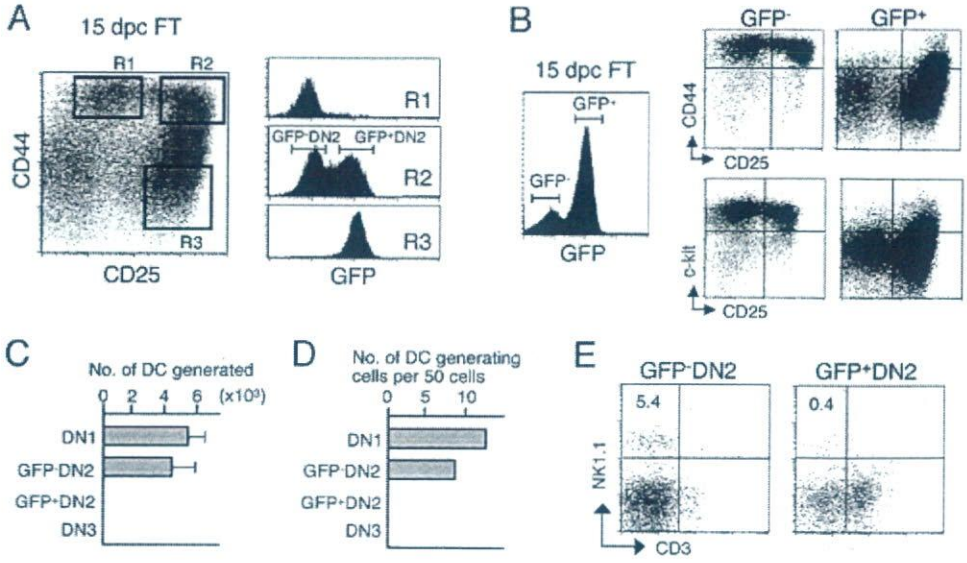

FIGURE 1. Thymic progenitors shutoff DC potential within the DN2 stage. *A*, FT cells from 15 dpc fetuses of plck-GFP mice were stained in three colors with anti-Lin, anti-CD25, and anti-CD44 mAb. *Left panel*, The CD44 vs CD25 profile of the Lin^- fraction. *Right panels*, GFP expression by cells in the gates denoted in the *left panel*. *B*, FT cells from 15 dpc fetuses of plck-GFP mice were similarly stained as in *A*, or separately stained in three colors with anti-Lin, anti-CD25, and anti-c-*kit* mAb. *Left panel*, A GFP expression profile of whole 15 dpc FT cells and gates to define GFP^- and GFP^+ fraction in Lin^- cells. *Right panels*, The CD44 vs CD25 profiles as well as c-*kit* vs CD25 profiles in GFP^- and GFP^+ gated fractions of the Lin^- cells denoted in the *left panel*. *C*, Cells in the indicated subpopulations from 15 dpc FT (100 cells/well) were cultured for 5 days in the presence of SCF, IL-7, Flt-3L, IL-3, GM-CSF, IL-1α, and TNF-α. The number of DC (mean $\pm$ SD) of triplicate cultures is shown. *D*, A total of 50 cells of the indicated subpopulations from 15 dpc FT were cultured at 1 cell/well in Terasaki plates in the presence of the same cytokine mixture as in *C*. The numbers of wells that showed DC generation on day 5 are scored. *E*, Cells of the indicated subpopulations from 15 dpc FT (100 cells) were cultured with a dGuo-treated FT lobe in the presence of SCF, IL-7, and IL-2 for 12 days. Representative flow cytometric profiles of generated cells are shown. The number of cells generated in a well of the *left* and *right panels* was 5.6×10^4 and 3.2×10^4, respectively.

analysis and calculation is done on the same sample, these two calculations for pre-β proliferation should result in a similar value.

Some PCR bands are very faint and therefore judgment for positive bands sometimes depends upon the individual. In our analysis, band counting was performed by four researchers and it was found that the numbers fluctuate about $\pm 5\%$ on an average among the four persons. Thus, we showed one representative data set for band number and value of pre-β expansion, together with the estimated range for each value of pre-β expansion, assuming that the band number count allows a $\pm 5\%$ margin.

Results

GFP^+ cells at the DN2 stage have lost the potential to generate DC in plck-GFP mice

Transgenic mice in which the expression of GFP is driven by the proximal *lck* promoter (plck) (17) were used to characterize the early stages of T cell development in the thymus. Flow cytometric profiles for expression of CD44, CD25, and GFP in Lin^- cells of 15 dpc FT from plck GFP mice are shown in Fig. 1*A*. GFP expression starts at DN2 stage, and DN2 cells can be subdivided into GFP^- and GFP^+ populations. Similar DN2 subpopulations are also seen in the adult thymus (AT) of the plck-GFP mice (data not shown). Because the DN2 stage is also characterized by c-*kit* expression, we examined whether GFP expression is initiated in c-kit^+ cells. Expression profiles of c-*kit* vs CD25 in GFP^- and GFP^+ fractions in 15 dpc FT are shown in comparison with that of CD44 vs CD25 in Fig. 1*B*. The results show that GFP expression starts at the c-kit^+CD25^+ stage.

DN1, GFP^-DN2, GFP^+DN2, and DN3 cells from 15 dpc FT were sorted and cultured at 100 cells/well in the presence of cytokine mixture that can induce DC generation. Several thousands of DC were generated in wells where DN1 cells or GFP^-DN2 cells were cultured, while neither GFP^+DN2 cells nor DN3 cells gave rise to DC (Fig. 1*C*). We then determined the frequency of DC-generating progenitors in FT subpopulations by culturing single cells in wells of a Terasaki plate. GFP^-DN2 cells contained progenitors with DC potential at a level comparable to that of DN1 cells (Fig. 1*D*). These data indicate that the DC potential is retained until the GFP^-DN2 stage while it is completely shut off during the transition from the GFP^-DN2 to GFP^+DN2 stages. Similar results were obtained with AT progenitors (data not shown).

We also compared the NK potential of cells in GFP^-DN2 and GFP^+DN2 subpopulations. One hundred cells were cultured with a dGuo lobe in the presence of IL-2. The GFP^-DN2 cells generated a distinct population of NK cells ($NK1.1^+CD3^-$ cells), whereas a very small number of NK cells were seen in the culture of GFP^+DN2 cells (Fig. 1*E*). Thus, the shutoff of NK potential may also occur at around the GFP^- to GFP^+ transition step.

In vitro recapitulation of the transition step from the GFP^-DN2 to GFP^+DN2 stages

To confirm the progenitor-progeny relationship of GFP^-DN2 and GFP^+DN2 cells, we cultured GFP^-DN2 cells on a monolayer of the stromal cells TSt-4/DLL1 (12). Results are shown in Fig. 2. On day 1, half of the cultured cells expressed GFP, and on day 2, almost all cells were found to express GFP. During these 2 days of culture, an ~4-fold cell increase was seen, suggesting that the transition from the GFP^-DN2 stage to the GFP^+DN2 stage is accompanied by cell proliferation. In the CD44 vs CD25 flow cytometric profile of cells on day 1, cells that have already down-regulated CD44 are seen. Therefore, the possibility that some GFP^-DN2 cells initiate down-regulation of CD44 without passing through the GFP^+DN2 stage cannot be ruled out.

Down-regulation of PU.1 at the transition step from the GFP^-DN2 to GFP^+DN2 stages

We next examined the expression of several developmentally regulated genes by RT-PCR in the FT subpopulations (Fig. 3). The

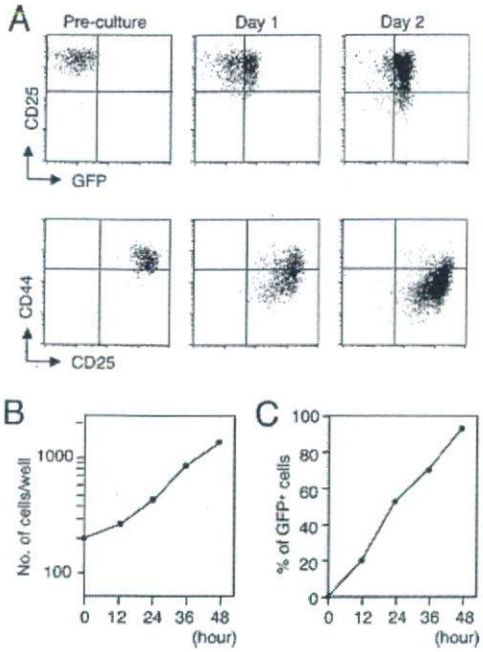

FIGURE 2. In vitro recapitulation of transition from the GFP$^-$DN2 stage to the GFP$^+$DN2 stage. GFP$^-$DN2 cells (200 cells/well) were cultured on a monolayer of stromal cells TSt-4/DLL1. *A–C*, Cells were harvested by trypsinization after indicated days of cultivation, counted, and analyzed for the expression of CD25 vs GFP, and CD44 vs CD25. Representative flow cytometric profiles of cells on indicated days of cultivation (*A*), number of cells per well (*B*; mean of triplicate cultures), and proportion of GFP cells among whole cells (*C*; mean of triplicate cultures) at the indicated time of cultivation are shown.

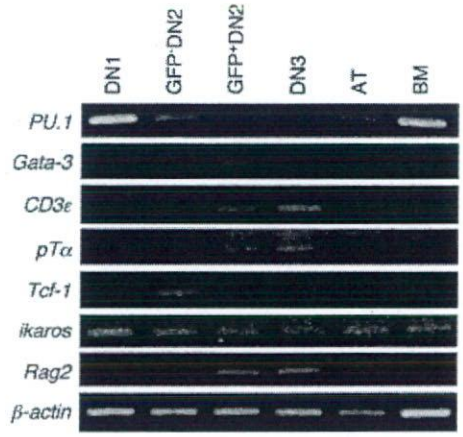

FIGURE 3. Expression profiles of developmentally regulated genes in FT subpopulations. mRNA was prepared from the indicated subpopulations of cells of 15 dpc FT. mRNA from AT and bone marrow (BM) cells were used as controls. Samples equivalent to 300 cells were analyzed by RT-PCR.

myeloid transcription factor PU.1 is expressed in cells at the GFP$^-$DN2 stage, although it is significantly down-regulated compared with the DN1 stage. The expression of PU.1 becomes undetectable at the GFP$^+$DN2 stage, consistent with our finding that DC potential was completely lost at this stage. Reciprocally, expression of GATA3, a zinc finger transcription factor crucial for T cell development, is up-regulated at the GFP$^+$DN2 stage. Expression of Rag2 at the GFP$^+$DN2 stage was found to be as high as at the DN3 stage. Of note, molecules that are known to be required at the DN3 stage to proceed further to the DN4 stage, e.g., CD3ε and pTα, have already begun to be highly expressed at the GFP$^+$DN2 stage. Therefore, the shutoff of DC potential in progenitors seems to take place concurrently with up-regulation of T cell lineage-specific genes.

Several cell divisions take place between the DC shutoff point and TCRβ *gene rearrangement*

The above findings indicated that the DC potential of thymocytes is shutoff within DN2 cells, a stage during which there is extensive proliferation. However, it seemed unclear whether the shutoff of DC potential takes place during or after the proliferative phase. Thus, we investigated whether GFP$^+$DN2 cells undergo cell division before the TCRβ rearrangement.

We first analyzed the configuration of D-J loci of the *TCRβ* gene, which is the first gene where rearrangement of the TCR gene occurs, by genomic PCR in cells in the FT subpopulations. Previous reports suggested that D-J rearrangement of the TCRβ chain gene is initiated at the DN1 or DN2 stage in a small proportion of cells (19–21). Indeed, Dβ1-Jβ1 (D1-J1) rearrangements were detected in both GFP$^-$ and GFP$^+$DN2 cells, but the *D1-J1* locus remained mostly unrearranged at this stage (Fig. 4*A*). Rearrangements of the Dβ2-Jβ2 (*D2-J2*) locus were already detected in DN1 cells, but again the frequency of rearranged loci remained low up to the GFP$^+$DN2 stage (Fig. 4*B*). In both D1-J1 and D2-J2 loci, rearranged constructs dominate the unrearranged germline configuration at the DN3 stage. Thus, GFP$^+$DN2 cells are closer to GFP$^-$DN2 cells than to DN3 cells in terms of their *TCRβ* gene rearrangement status.

Because some D-J rearrangements were detected in DN1 cells and GFP$^-$DN2 cells, we examined whether the DC generated from these cells harbor rearranged D-J constructs. DC generation was induced from DN1 and GFP$^-$DN2 cells and from Lin$^-$c-*kit*$^+$Sca-1$^+$ 15 dpc fetal liver cells by culturing these cells in the same manner as in Fig. 1*C*, and it was found that the DC derived from these cells carry the unrearranged germline configuration in both *D1-J1* and *D2-J2* loci (Fig. 4, *C* and *D*).

We then investigated whether GFP$^+$DN2 cells undergo proliferation before they become DN3 cells. Analysis of the forward and side scatter characteristics of cells in these FT subpopulations indicated that GFP$^+$DN2 cells are somehow smaller than GFP$^-$DN2 cells, but still much larger than DN3 cells (Fig. 4*E*). Cell cycle analysis demonstrated that the GFP$^+$DN2 fraction contains even more cycling cells than DN1 or GFP$^-$DN2 fractions ($p < 0.05$) (Fig. 4, *F* and *G*). These findings suggested that the GFP$^+$DN2 cells undergo proliferation to some extent before entering the DN3 stage.

We then cultured GFP$^+$DN2 cells on TSt-4/DLL1. Virtually all cells differentiated into CD44$^-$CD25$^+$ cells within 1 day, and a 5-fold expansion in cell number was observed during 3 days of culture (Fig. 4, *H* and *I*). These data strongly suggested that GFP$^+$DN2 cells undergo cell divisions before the TCRβ rearrangement.

To obtain more direct evidence that a proliferative phase exists between the DC shutoff and TCRβ rearrangement-initiation points, we cultured single progenitors with dGuo lobes and investigated whether diverse D-J rearrangements of the *TCRβ* locus were formed in the progeny T cells. The formation of Dβ-Jβ rearrangement diversity in this clonal culture indicates that the seeded cell has proliferated before the initiation of *TCRβ* gene rearrangement (5). Representative flow cytometric profiles of T cells generated from single progenitors in various FT subpopulations are shown in Fig. 5*A*. Virtually all clones contained CD4$^+$CD8$^+$ (double-positive (DP)) cells, CD4$^+$CD8$^-$ (CD4 single-positive (SP)) cells, and

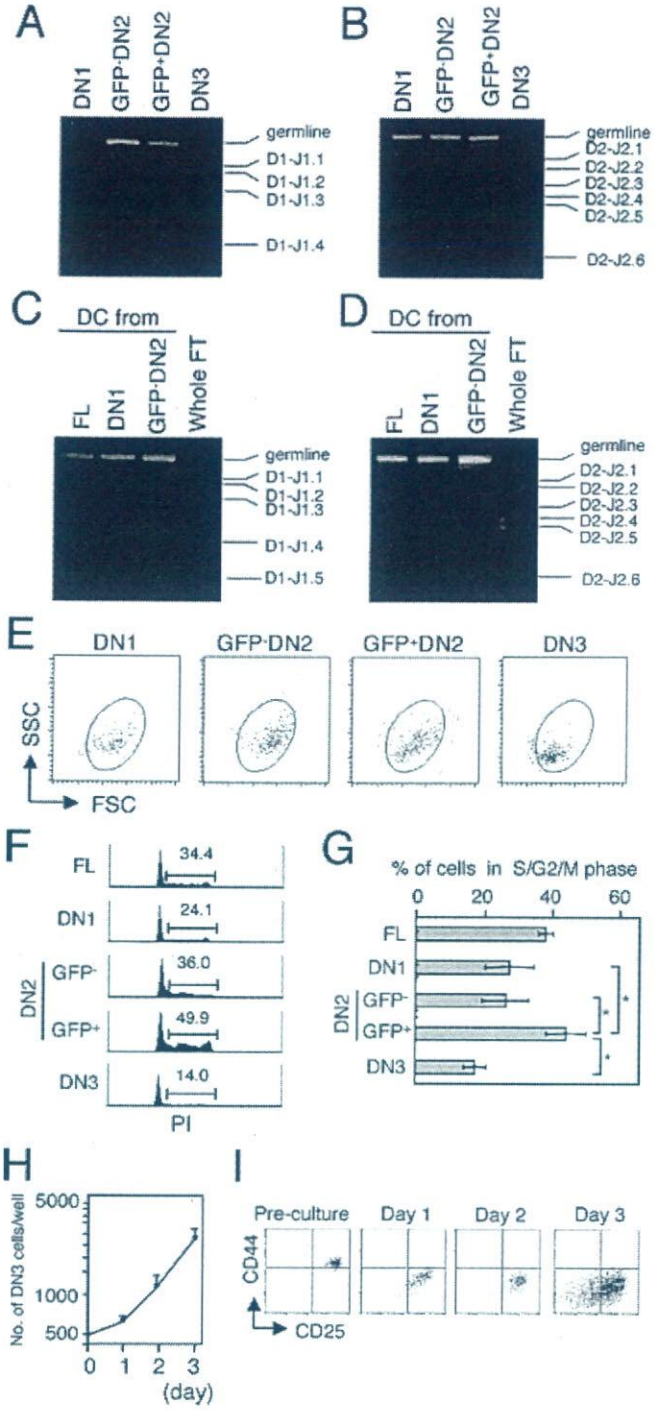

FIGURE 4. Thymic progenitors proliferate after losing DC potential. *A* and *B*, Rearrangement status of *Dβ-Jβ* loci in FT subpopulations. Genomic DNA was prepared from the cells of the indicated subpopulations of 15 dpc FT. Each sample (equivalent to 3000 cells) was PCR amplified using primers for Dβ1-Jβ1.5 (*A*) or Dβ2-Jβ2.6 (*B*). *C* and *D*, Rearrangement status of *Dβ-Jβ* loci in DC generated in vitro from FT subpopulations. DC were identically induced as in Fig. 1*C* from DN1 cells, GFP$^-$DN2 cells, or Lin$^-$c-*kit*$^+$Sca-1$^+$ cells of 15 dpc fetal liver (FL) as a control. Genomic DNA was prepared from the recovered cells and from whole 17 dpc FT cells as a control, and each sample (equivalent to 3000 cells) was PCR amplified using primers for Dβ1-Jβ1.5 (*C*) or Dβ2-Jβ2.6 (*D*). *E*, Scatter characteristics of cells in the indicated subpopulations from 15 dpc FT. *F* and *G*, Cell cycle analysis of subpopulations of 15 dpc FT cells. Cells collected from FT subpopulations and Lin$^-$c-*kit*$^+$ cells from 15 dpc FL were fixed and stained by PI. Representative histograms for each subpopulation are shown in *D*. Proportions of cells in S/G_2/M phase (mean ± SD of percentages from five independent experiments including those shown in *D*) are shown in (*E*). *, $p < 0.05$. *H* and *I*, A total of 500 cells of GFP$^+$DN2 population were cultured on a monolayer of stromal cells TSt-4/DLL1. Cells were harvested at the indicated day of cultivation, counted, and analyzed for the expression of CD44 vs CD25.

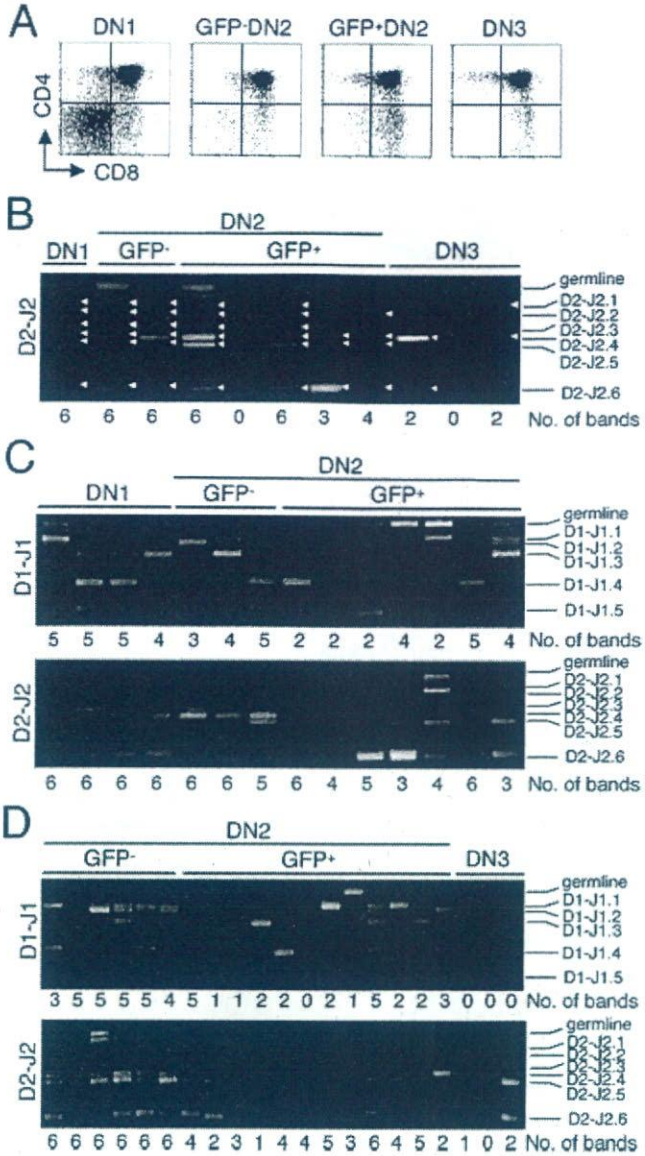

FIGURE 5. Proliferation of thymic progenitors before the onset of TCRβ chain gene rearrangement as evidenced by formation of diversified D-Jβ rearrangement in clonal cultures. *A*, Representative flow cytometric profiles of cells generated from a single progenitor of the indicated FT subpopulations. *B*, Diversified Dβ2-Jβ2 rearrangements formed in T cells generated from single GFP$^+$DN2 cells. Cells of various subpopulations from 15 dpc FT were individually cultured under high oxygen submersion conditions for 12 days. Genomic DNA was prepared from the cells generated in each well. Each sample (equivalent to 1000 cells) was PCR amplified using primers for Dβ2-Jβ2.6. Representative results are shown. *C* and *D*, Diversified Dβ1-Jβ1 and Dβ2-Jβ2 rearrangements formed in T cells generated from single GFP$^+$DN2 cells. Experiments were performed in the same manner as in *B*. Genomic DNA prepared from the cells generated in each well (equivalent to 1000 cells) was PCR amplified using primers for Dβ1-Jβ1.5 or Dβ2-Jβ2.6. Samples were pooled from several similarly designed experiments. Plating efficiency in DN1 and DN2 subpopulations was 30–50% and that of DN3 was 10–20%.

CD4$^-$CD8$^+$ (CD8SP) cells. As expected, clones generated from more differentiated progenitors tended to contain more SP cells and fewer DN cells (DN3>DN2>DN1), although no significant

Table I. *Pre-β proliferation of T cell progenitors in subpopulations of FT cells*

Progenitor Source	No. of Clones	Analyzed Average No. of D2-J2 Bands Per Clone	Predicted Expansion (-Fold)[a] (Range)
DN1	10	6.0	>63.8 (>46.7)
GFP$^-$DN2	10	5.6	42.2 (33.9–88.9)
GFP$^+$DN2	16	3.75	15.3 (14.1–16.7)
DN3	10	1.1	3.2 (3.0–3.3)

[a] The expansion of progenitors prior to the initiation of TCRβ rearrangement was estimated from the average number of D2-J2 bands (5). Samples were pooled from several similarly designed experiments. Plating efficiency in DN1 and DN2 subpopulations was 30–50%, and that of DN3 was 10–20%. See *Materials and Methods* for the estimation of the range of expansion.

difference was seen between clones originating from GFP$^-$DN2 and GFP$^+$DN2 progenitors.

PCR analysis of Dβ2-Jβ2 rearrangements in cells of each clone detected the rearranged D2-J2 constructs (maximum six) formed in each clonal culture. It was seen that most of the clones from the GFP$^+$DN2 population exhibited multiple bands (Fig. 5*B*). The number of bands differs among GFP$^+$DN2 clones, reflecting the heterogeneity in proliferation potential of these progenitors. The average number of bands in 16 GFP$^+$DN2 clones was 3.75 (Table I). According to a mathematical prediction based on simulation of TCRβ chain gene rearrangement (5), it is estimated that these clones have produced an average of 15.3 cells before the *TCRβ* gene rearrangement. This result indicates that about four cell divisions on average have occurred between the shutoff of DC potential and the initiation of TCRβ rearrangement.

Previous studies have suggested that the D1-J1 rearrangement precedes the D2-J2 rearrangement (14, 21). This leaves the possibility remaining that D1-J1 rearrangement occurs before the time point to shutoff the DC potential. We therefore decided to analyze the rearrangement status of the *D1-J1* locus together with the *D2-J2* locus in cells generated in clonal cultures. A total of 9 GFP$^-$DN2 clones and 19 GFP$^+$DN2 clones were analyzed (Fig. 5, *C* and *D*). Multiple D1-J1 bands were seen in most of the GFP$^+$DN2 clones. The average number of D1-J1 bands among the five visible rearrangements in 19 GFP$^+$DN2 clones was 2.47, while that of D2-J2 bands in six constructs was 3.89 (Table II). According to the mathematical prediction (as described in the *Materials and Methods*), seeded GFP$^+$DN2 cells were estimated to have produced 17.6 cells on average before the initiation of D1-J1 rearrangement. Using the same sample, it was also predicted that 16.3 cells were formed before D2-J2 rearrangement. This value (16.3), which was quite similar to the value (15.3) obtained from D2-J2 PCR data in the previous experiment (Table I), was very close to the value (17.6) obtained from D1-J1 data. These results thus clearly indicated that these progenitors shutoff their DC potentials just before the initiation of rearrangement in either the *D1-J1* or *D2-J2* locus. The results also indicated that the timing to start the rearrangement of *D1-J1* and *D2-J2* loci is almost the same between the two.

The presence of a proliferation phase between lineage restriction and TCRβ rearrangement suggests that the shutoff of DC potential does not directly interact with the mechanisms that drive the *TCRβ* gene rearrangement and vice versa.

Discussion

It has been unclear at which stage the intrathymic progenitors become completely determined to the T cell lineage. By using plck-GFP mice as the progenitor source, the present study clarified that the T cell lineage determination occurs at late DN2 stage, which is before the initiation of *TCRβ* gene rearrangement, and that a proliferation phase exists between the stage of T cell lineage determination and that of *TCRβ* gene rearrangement.

Fig. 6 schematically illustrates the timetable of early intrathymic T cell development before the TCRβ rearrangement. During DN1 and DN2 stages in FT, T cell progenitors undergo extensive (>10) cell divisions (4). Such an extensive proliferation before TCRβ rearrangement may be important in generating a diversity of TCRβ chains. This pre-β proliferation phase can be divided into two phases, i.e., the phase of T cell lineage specified progenitors and that of T cell lineage determined progenitors that have lost DC potential. In this context, it can be said that the ~16 fold-expansion (i.e., four round cell divisions) revealed in the present study is the first task of the T cell lineage-determined progenitors for producing an extensively diversified T cell repertoire. Although this timetable of T cell lineage determination and proliferation is based on the findings on FT progenitors, our experimental results indicated that the timetable was almost the same in AT progenitors (data not shown), except that the earliest AT progenitors may undergo at least three times more cell divisions than the FT progenitors at DN1 stage (22).

We have previously reported that the DN1 population of the AT contains a small fraction of GFP$^+$ cells, and these cells were considered to belong to a more advanced stage toward T cells, because they have lost the potential to generate B cells, NK cells, and DC (17). Such GFP$^+$ DN1 cells were rarely seen in FT (Fig. 1, *A* and *B*). We speculate that GFP expression at the DN1 stage represents a noncanonical pathway occurring only in AT, and that in both the fetus and adult, the GFP expression at the DN2 stage represents the major developmental pathway toward αβ T cells.

Previous studies showed that rearranged D-J constructs of the *TCRβ* gene are detectable in DN1 and/or DN2 cells (19–21). Hence, it has been proposed that D-J rearrangement of the *TCRβ* gene proceeds gradually, starting at the DN1 or DN2 stage. We also detected rearranged Dβ-Jβ constructs in DN1 or DN2 cells (Fig. 4*A*). However, detection of Dβ-Jβ constructs in cells at these

Table II. *GFP$^+$DN2 cells exhibit similar pre-β proliferation rates prior to D1-J1 and D2-J2 rearrangement*

Progenitor Source	No. of Clones Analyzed	Data from D1-J1 PCR		Data from D2-J2 PCR	
		No. of bands	Predicted expansion (range)[a]	No. of bands	Predicted expansion (range)
DN1	10	4.8	>82.6 (>62.7)	6.0	>63.8 (>46.7)
GFP$^-$DN2	9	4.3	52.6 (43.6–60.2)	5.89	62.2 (>42.1)
GFP$^+$DN2	19	2.47	17.6 (16.3–18.9)	3.89	16.3 (14.9–17.8)

[a] The expansion of progenitors prior to the initiation of TCRβ rearrangement was estimated from the average number of D1-J1 bands or D2-J2 bands. Data for GFP$^-$DN2 and GFP$^+$DN2 populations are from Fig. 5, *C* and *D*. Samples were pooled from several similarly designed experiments. Plating efficiency in DN1 and DN2 subpopulations was 30–50%, and that of DN3 was 10–20%. See *Materials and Methods* for the estimation of the range of expansion.

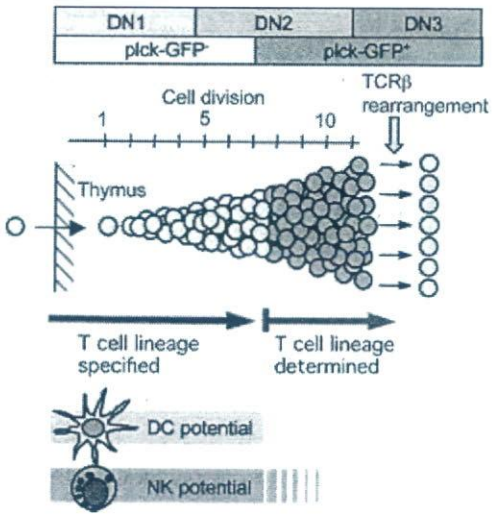

FIGURE 6. Schematic illustration of the early differentiation/proliferation of thymic T lineage cells. A single early thymic progenitor undergoes >10 cell divisions during the DN1 and DN2 stages to generate >1000 DN3 cells (5). The shutoff of DC potential occurs during the transition from the GFP$^-$DN2 to GFP$^+$DN2 stages and subsequently the T cell lineage determined progenitors undergo several cell divisions before they enter the DN3 stage and initiate *TCRβ* gene rearrangement.

early stages does not necessarily prove that some of the progenitors initiated their rearrangements of Dβ-Jβ at the DN1 or DN2 stages. As shown in the present study, even in GFP$^+$DN2 clones, at least 3 bands among 11 possible bands (by D1-J1.5 and D2-J2.6 PCR) were detected in all 19 clones analyzed (Fig. 5, *C* and *D*). The failure to detect clones that contain less D-J constructs than three is not because we picked up only the cultures derived from very proliferative progenitors, because the plating efficiency of clonal culture is always >30% for GFP$^+$DN2 cells, and all clones in which DP cell generation was observed were served for PCR. It might be still argued that D-J rearrangement at the DN1 or DN2 stages takes place solely on either the *D1-J1* locus or the *D2-J2* locus, thus allowing the possibility of generating diversified D-J rearrangements in progeny cells. However, our data do not support this possibility, because most GFP$^+$DN2 clones already contain at least two bands for both loci (Fig. 5, *C* and *D*). Thus, it is quite unlikely that these T cell clones were derived from cells that had already initiated partial D-J rearrangement. Therefore, the initiation of Dβ-Jβ rearrangement at the DN1 or DN2 stage, even if it occurs, represents a very rare event.

It has been shown that in some experimental settings, B cells or macrophages carrying rearranged Dβ-Jβ constructs are generated from thymic progenitors (14, 23). These results were interpreted to indicate that T cell progenitors that have undergone Dβ-Jβ rearrangement still retain the potential to give rise to other lineage cells than the T cell lineage. However, in the case of B cells, it could alternatively be argued that the Dβ-Jβ rearrangements occurred after progenitors were segregated from the main pathway for T cells, because Rag and various genes involved in gene rearrangement are also expressed during B cell development. In the case of macrophages, Balciunaite et al. (14) showed that the frequency of macrophage colonies generated from DN2 cells that harbor rearranged Dβ-Jβ construct is only 3%. Their data therefore are consistent with our data in which rearranged Dβ-Jβ constructs were not detected in DC generated from GFP$^-$DN2 cells (Fig. 4, *C* and *D*). Another interpretation, based on the finding that the Dβ-Jβ constructs in B cells or myeloid cells derived from thymic progenitors are always that of the *D1-J1* locus (14, 21, 23), proposed that the rearrangement of the *D1-J1* locus precedes that of the *D2-J2* locus. However, our present results did not support such a scenario, because GFP$^+$DN2 cells were estimated to proliferate at a similar rate before the rearrangement of the *D1-J1* and *D2-J2* loci (Table II). The selective rearrangement of the *D1-J1* locus in certain conditions may indicate that the *D1-J1* and *D2-J2* loci are differently regulated. Therefore, the possible explanation for selective D1-J1 rearrangement in thymus-derived nonthymic lineage cells is that the *D2-J2* locus is more strictly regulated than the *D1-J1* locus to prevent rearrangement in non-T lineage cells.

Another important characteristic of thymic T cell progenitors is whether the DC potential is retained in all T cell progenitors until they become GFP$^+$ or whether some T cell progenitors shutoff the DC potential as early as the DN1 stage. Our previous data indicated that the frequency of progenitors exhibiting DC potential among progenitors that showed T cell potential in the DN1 and DN2 populations was ~50–60% in the DN1 population and 10–20% in the DN2 population (9, 11, 22). However, considering that any clonal analysis has some limitation in sensitivity, the finding that over 50% of the T cell progenitors in the DN1 population have DC potential may imply that most DN1 T cell progenitors could have retained DC potential. Although our clonal analysis using a fetal thymic organ culture system showed that the DC-generating progenitors within the DN2 population are lower than that in the DN1 population, a more sensitive analysis by culturing cells in the presence of a cytokine mixture in Terasaki plates revealed that DN2 cells contain DC-generating precursors at a comparable frequency as DN1 cells (Fig. 1*D*). Therefore, it is conceivable that the potential of thymic progenitors to generate DC might be retained until they enter the GFP$^+$DN2 stage.

Collectively, we propose that the time point of T cell lineage determination precedes that of the initiation of TCRβ gene rearrangement by several cell divisions and the timings of these events are strictly regulated. In contrast, initiation of D-J rearrangement in the *IgH* gene in B lineage cells may not be so strictly regulated. Although it has been shown that the early B cell progenitors bear an unrearranged *D-J* locus in their *IgH* gene (3) as mentioned in the *Introduction*, some recent studies demonstrated that the early lymphoid progenitors in bone marrow express *Rag* genes and harbor the rearranged D-J construct in the *IgH* gene (24, 25). It was recently shown that non-B lineage cells, i.e., a subset of plasmacytoid DCs, bear a rearranged D-J *IgH* gene (26, 27). It might be interesting to study these differences in initiating the gene rearrangement between T and B cell lineages.

The DC potential retained in T cell progenitors may provide a clue as to the relationship among various hemopoietic cell lineages. We have previously shown that even after multipotent progenitors are segregated into branches for erythroid, T and B cell lineages, progenitors of each branch still retain myeloid potential (4, 28–30). This may indicate that the developmental program for erythroid, B and T cell lineages proceeds on a basis of an underlying prototypical myeloid program. From this perspective, the DC potential in T cell progenitors could represent a modified form of myeloid potential in a T cell branch. Thus, the shutoff of DC potential can be regarded as the final event for the progenitor to determine its identity as a T cell progenitor. Down-regulation of PU.1, a key transcription factor for the myeloid lineage (31, 32), was observed coincident with the shutoff of DC potential (Fig. 3). Therefore, it seems that the down-regulation of PU.1 at this transition point may account for the shutoff of myeloid potential in T cell progenitors. This transition step is also accompanied by the up-regulation of T cell lineage-specific molecules, e.g., pTα, CD3ε, which are essential to pass the DN3 stage checkpoint and to enter DN4 and DP stages.

In general, differentiation of cells occurs during their nonproliferating phase. In the case of T cell development, TCRβ and TCRα chain genes are rearranged at the DN3 and DP stages, respectively, when cells are in a quiescent status. Positive selection also proceeds at the DP stage. The shutoff of DC potential is also one of the dramatic events in thymic T cell development, but this event appears to occur in a midproliferation phase. Whether molecular mechanisms that actually shutoff DC potential take place in the G_1 phase of cycling cells or at some resting G_0 period within the DN2 stage remains to be clarified. In this context, the fact that GFP$^+$DN2 cells contain more cycling cells than GFP$^-$DN2 cells (Fig. 4, *D* and *E*) suggests that the GFP$^-$DN2 stage contains some resting cells in which the determination process may take place, whereas GFP$^+$DN2 cells contain only cells that have passed this critical step and are proliferating before they enter DN3 stage.

Although DC potential was found to be completely shutoff at the transition between the GFP$^-$DN2 and GFP$^+$DN2 stages, NK potential does not seem to be so completely lost at this step. It is probable that the NK program has to be more or less maintained beyond this step or even beyond the DN3 stage, because thymic precursors have to produce NKT cells, which share various characteristics with NK cells, after *TCR* gene rearrangements (33). Further studies are needed to clarify the molecular mechanisms by which NK-generating potential is shutoff in relation to the shutoff of DC potential during T cell development.

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Disclosures

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Operational Tolerance to Class I Disparate Lungs Can Be Induced Despite Pretransplant Immunization With Class I Allopeptides

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Background. Using a class I-disparate swine lung transplant model, we examined whether an intensive course of tacrolimus could induce operational tolerance and whether preoperative allopeptide immunization would prevent the development of tolerance.

Methods. Left lung grafts were performed using class I-disparate (class II-matched) donors. Recipients were treated with 12 days of postoperative tacrolimus. Three recipients were immunized prior to transplantation with class I allopeptides. Three other recipients were not immunized.

Results. The nonimmunized recipients maintained their grafts long term (>497, >451, and >432 days), without developing chronic rejection. The immunized swine also maintained their grafts long term (>417, >402, >401 days), despite developing a variety of in vitro and in vivo responses to the immunizing peptides, as well as having strong mixed lymphocyte reactions to donor cells prior to transplantation.

Conclusions. Using only a brief course of tacrolimus, we have been able to induce a state of operational tolerance in a class I-disparate preclinical lung transplant model. Moreover, preoperative alloimmunization did not block tolerance induction or induce chronic rejection. These data show that it is possible to create a state of operational tolerance to lung allografts even in the presence of donor-sensitized cells.

Keywords: Lung, Transplantation, Swine, Tolerance.

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As a result of advances in surgical techniques, immunosuppression, and postoperative management, lung transplantation has become an established therapeutic option for individuals with end-stage lung disease. The current 1-year actuarial survival rate after lung transplantation is approximately 75%. However, chronic lung allograft rejection, manifested principally by the histopathologic changes of obliterative bronchiolitis (OB), has limited the long-term success of lung transplantation. Almost 50% of lung transplant recipients develop OB within 5 years of transplantation, and OB is currently the leading cause of graft loss and mortality after the first posttransplant year (*1*). However, the mechanism and treatment of chronic rejection in pulmonary allotransplantation remain elusive, and overcoming graft loss due to chronic rejection may require the induction of a tolerant state in the recipient.

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Our previous study in a cyclosporine (CSA)-based major histocompatibility (MHC) class I-disparate swine lung transplantation model demonstrated that indirect recognition of class I allopeptides accelerates graft rejection (*2*). In this rejection model, preoperative immunization to class I allopeptides induced a proliferative response to donor peptides both in vitro and in vivo, and accelerated pulmonary allograft rejection, as compared to a non-immunized control group. The dominant histologic finding in the immunized group was that of a mononuclear infiltrate. The rapidity of this accelerated acute rejection (AR) precluded us from reaching conclusions as to whether immunization to MHC allopeptides had any effect on the development of chronic rejection.

In an attempt to isolate the effect of augmented indirect allorecognition on chronic rejection, we elected to employ a tacrolimus-based protocol developed in our center that is known to induce tolerance across strong allogeneic barriers in a renal allograft model (*3*). At issue was whether the induction of donor-specific hyporesponsiveness could prevent chronic allograft rejection and whether preoperative immunization with class I allopeptides could prevent the induction of this tolerant state, leading to accelerated chronic rejection.

MATERIALS AND METHODS

Animals

Transplant donors and recipients were selected from our herd of partially inbred miniature swine at 4–7 months of age. These swine have been inbred to homozygosity at the class I and class II MHC (termed swine leukocyte antigen [SLA] in pigs) loci, as described previously (*4*). At present, three homozygous haplotypes are available for study. In ad-

dition, a number of intra-MHC recombinant haplotypes (derived from spontaneous recombination events) are also available (5). In this study, the recipient haplotype was SLA^{dd} (homozygous class I^d and homozygous class II^d), and the donor haplotype was SLA^{gg} (homozygous class I^c and homozygous class II^d). Genotyping has been controlled by strict pedigree breeding and confirmed by microcytotoxicity testing using allospecific antisera. All transplants in this study were performed on swine that were MHC class II matched (i.e., MHC class I and minor antigen–disparate). All donor-recipient pairs were confirmed to be mutually reactive on a preintervention assay of cell-mediated lympholysis (CML). All animal care and procedures were in compliance with the "Principles of Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (revised 1996).

Experimental Design

Twelve swine were used in present experiments to conduct six class I-disparate orthotopic left lung allografts. Three recipient swine were immunized 3 weeks prior to transplantation with a mixture of three allogeneic MHC class I^c allopeptides (see Allopeptide Immunization below), and three recipients served as non-immunized controls. All recipients were treated with a 12-day course of postoperative tacrolimus (0.15 mg/kg/day, as a continuous intravenous infusion; target level: 35–50 ng/ml), as their only immunosuppression. Blood tacrolimus levels were monitored daily by a microparticle enzyme immunoassay (Tacrolimus II, IMX System; Abbott Laboratories, Abbot Park, IL) and the results were expressed in nanograms per milliliter. Tacrolimus was generously provided by Fujisawa Healthcare, Inc (Deerfield, IL).

In all experiments, lung allografts were monitored for rejection by a combination of chest radiography and serial open lung biopsies. Immune responses were monitored with a variety of in vitro and in vivo assays, as described below. The presence of high-grade histologic rejection and/or the loss of graft aeration, compliance, or perfusion were the principal study endpoints.

Allopeptide Immunization

Most of the polymorphic sites of the two known class I MHC loci in the pig (designated *P1* and *P14*) are contained within the hypervariable regions of the α_1 and α_2 domains, as determined by comparison of the MHC class I^c and MHC class I^d genetic sequences (6). Three MHC class I^c peptides spanning the full length of the hypervariable regions of the P14 α1 helix were synthesized and labeled as PC14-1 (amino acids [AA] 3–27), PC14-2 (AA 45–59), and PC14-3 (AA 60–85). Peptide purity was >90%, as verified by high-performance liquid chromatography and mass spectrometry. The length of the peptides was chosen to optimize binding to class II molecules. Previous studies have shown that the recipients rejecting lung grafts (either acutely or chronically) spontaneously develop T cell reactivity to these same peptides, and that preoperative immunization with these synthetic donor-derived peptides causes accelerated rejection in comparison to nonimmunized controls (2).

In this study, 500 μg of each peptide in 750 μL of complete Freund's adjuvant were injected subcutaneously 21 days prior to transplantation. One week prior to transplantation, peripheral blood mononuclear cells (PBMCs) from the prospective recipients were tested for in vitro proliferative responses against each individual allogeneic peptide (see below). At the same time, the immunized pigs were rechallenged with individual peptides to evaluate in vivo delayed-type hypersensitivity (DTH) responses (see below).

Orthotopic Lung Transplantation

Orthotopic left lung transplantation was performed as previously described (7). Briefly, under general anesthesia, two indwelling silastic central venous catheters were placed into external jugular veins. One catheter was used solely for the administration of tacrolimus and other medications. The other catheter was used to obtain blood for clinical monitoring and for use in various in vitro assays. The recipient's chest was entered through a left thoracotomy, and the hilar structures of the native lung were isolated. After heparinization (300 U/kg), the native lung was removed. The donor lung was approached through a median sternotomy. After heparinization (300 U/kg), the heart-lung block was topically cooled with iced saline and flushed in situ with 4 L of cold Euro-Collins solution (Fresenius Medical Care AG, Bad Homburg, Germany) containing prostaglandin E_1 (500 μg/L). Immediately after harvesting, the left lung was surgically prepared and transplanted. Bronchial anastomoses were performed using interrupted 4-0 polyglactin sutures (Vicryl; Ethicon, Inc., Somerville, NJ), and vascular anastomoses were performed using running 6-0 polypropylene (Prolene; Ethicon, Inc.) sutures. The pleural space was evacuated with a thoracostomy tube, which was removed after recovery from anesthesia. Total ischemic time averaged approximately 2 hours.

Delayed-Type Hypersensitivity (DTH) Responses

In the immunized group, DTH responses were evaluated 2 weeks after allopeptide immunization by injecting 100 μg of each individual PC14 peptide in 0.1 mL phosphate-buffered saline (PBS) intradermally into separate sites on the neck of the pig. PBS (0.1 mL) was used as a negative control, and 100 μg of *Mycobacterium tuberculosis* H37 RA (MTB) was used as a positive control. Induration was measured 48 hr after injection by blinded observers using calipers. Positive responses were defined as having a diameter of induration greater than 10 mm. Induration between 5 and 10 mm was considered to be an intermediate response, and negative responses had less than 5 mm of induration.

Isolation of Peripheral Blood Mononuclear Cells

PBMCs were prepared from freshly collected, heparinized whole blood diluted approximately 1:2 with Hanks' balanced salt solution (HBSS; GIBCO Invitrogen, Carlsbad, CA). Mononuclear cells were obtained by gradient centrifugation using Lymphocyte Separation Medium (Organon, Teknika, Durham, NC), washed once with HBSS, and contaminating red cells were lysed with ACK Buffer (B&B Research Laboratory, Fiskeville, RI). Cells were then washed with HBSS and resuspended in tissue culture medium. All cell suspensions were stored at 4°C until utilized in cellular assays. Antigen presenting cell (APC) and T-cell preparations were isolated from PBMCs by nylon wool passage.

Primary Cell-Mediated Lympholysis Assay

Primary CML assays were performed as previously described (*8*). In brief, tissue culture media used for CML assays consisted of Roswell Park Memorial Institute (RPMI) 1640 (GIBCO Invitrogen) supplemented with 6% fetal calf serum (Sigma, St. Louis, MO), 100 U/mL penicillin (GIBCO Invitrogen), 135 μg/mL streptomycin (GIBCO Invitrogen), 50 μg/mL gentamicin (GIBCO Invitrogen), 10 mM HEPES (Fisher Scientific, Pittsburgh, PA), 2 mM L-glutamine (GIBCO Invitrogen), 1 mM sodium pyruvate (BioWhittaker, Walkersville, MD), nonessential amino acids (BioWhittaker), and 5×10-5M β-2 mercaptoethanol (Sigma). The effector phase of the CML assay was performed using Basal Medium Eagle (GIBCO Invitrogen) supplemented with 6% CPSR-3 (Sigma) and 10 mM HEPES (Fisher Scientific). Lymphocyte cultures containing 4×10^6 responder and 4×10^6 stimulator PBMC (irradiated with 2500 cGy) were incubated for 6 days at 37°C in 7.5% CO_2 and 100% humidity. Bulk cultures were harvested, and effectors were tested for cytotoxic activity on ^{51}chromium (Amersham, Arlington Heights, IL)-labeled lymphoblast targets. Effector cells were incubated for 5.5 hr with target cells at E/T ratios of 100:1, 50:1, 25:1, and 12.5:1. Three target cells were tested in each assay; MHC-matched PBMC to the effectors, donor-matched PBMC, and third-party PBMC. Supernatants were then harvested using the Skatron collection system (Skatron, Sterling, VA) and ^{51}chromium release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as percent specific lysis (PSL), calculated as: PSL=([experimental release (cpm)–spontaneous release (cpm)]/[maximum release (cpm)–spontaneous release (cpm)])×100%.

Secondary Coculture Cell-Mediated Lympholysis Assay

As previously described (*8*), lymphocyte cultures from experimental animal containing 4×10^6 were primed with donor-type 4×10^6 PBMC (irradiated with 2500 cGy) and incubated as a regulator for 6 days at 37°C in 7.5% CO_2 and 100% humidity. Then regulator cultures were harvested, and 2×10^6 regulator cells and naïve matched type 2×10^6 PBMC (responder) and 4×10^6 donor-matched stimulator PBMC (irradiated with 2500 cGy) were incubated for 6 days at 37°C in 7.5% CO_2 and 100% humidity. Bulk cultures were incubated with and effectors were tested for cytotoxic activity on ^{51}chromium (Amersham, Arlington Heights, IL)-labeled lymphoblast targets. Effector cells were incubated for 5.5 hr with target cells at E/T ratios of 100:1, 50:1, 25:1, and 12.5:1. Three target cells were tested in each assay; SLA-matched PBMC to the effectors, donor-matched PBMC, and third-party PBMC. Supernatants were then harvested using the Skatron collection system (Skatron, Sterling, VA) and ^{51}chromium release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as PSL, calculated as above.

Mixed Lymphocyte Reaction Assay

Mixed lymphocyte reaction (MLR) assays were performed by plating 4×10 (5) responder PBMC in triplicate in 96-well flat-bottom plates (Costar, Cambridge, MA). Cells were stimulated with 4×10^5 stimulator PBMC irradiated with 2500 cGy. Medium consisted of RPMI 1640 supplemented with 6% fetal pig serum,10 mM HEPES, 1 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/mL penicillin, 135 μg/mL streptomycin, 50 μg/mL gentamicin, and 2×10^{-5} M β-2mercaptoethanol. Cultures were incubated for 5 days at 37°C in 6% CO_2 and 100% humidity. [^{3}H] thymidine was added for an additional 6 hr of culture. Wells were then harvested onto Mash II glass fibers and counted for beta emission. A stimulation index (SI) for each reaction was expressed as experimental counts per minute divided by media control counts per minute.

Peptide Proliferation Assay (PPA)

To evaluate the ability of a recipient to mount a proliferative T-cell response to an indirectly presented peptide antigen, a peptide proliferation assay (PPA) using thymidine incorporation was performed as previously described (*7*). In brief, 4×10^5 recipient-matched PBMCs were cultured with 50 μg/mL of individual allopeptides for 5 days in triplicate plates. The culture was then pulsed with [^{3}H]thymidine (1 μCi/well) for 5 hr, and [^{3}H]thymidine incorporation was measured by β-scintillation counting. An SI for each peptide was expressed as experimental counts per minute divided by media control counts per minute. Based on historical data from 30 naïve pigs tested against each of three allogeneic class I PC14 peptides (*9*), the average SI of all 30 naïve responses was 1.2. Adding three standard errors resulted in an SI of 2.2. Therefore, SIs greater than 2.3 are deemed to be significant.

Flow Cytometry

Sera from animals were tested for the presence of anti-donor IgM and IgG antibodies by indirect flow cytometric assay, as previously described (*7*).

ELISPOT Assay

To evaluate the ability of production of cytokine in immunized recipients, an ELISPOT assay was performed. ELISPOT plates (Multiscreen, Millipore Corporation, Bedford, MA) were coated with antiswine interferon (IFN)-γ capture antibodies (Biosource, Cammarillo, CA). After blocking and washing, responder cells (3×10^5) were added to triplicate wells with or without stimulators or antigens for 48 hr. After washing, the anti-swine IFN-γ -biotinylated mAb detection antibody (Biosource, Cammarillo, CA) was added, followed by streptavidin-horseradish peroxidase. The plates were developed by using AEC Substrate Set (BD Biosciences Pharmingen, San Diego, CA), and the resulting spots were counted on a computer-assisted ELISPOT image analyzer (Cellular Technologies).

Skin Grafts

Split-thickness skin grafts (4×4 cm) were harvested from donors with Zimmer dermatome and placed on a graft beds on the dorsum of recipients. Donor and donor-matched skin were SLA^{gg}. Third-party class I-mismatched skin was SLA^{hh} (homozygous class I^a and homozygous class II^d), and third-party fully mismatched skin was SLA^{cc} (homozygous class I^c and homozygous class II^c). Skin grafts were assessed for viability by color, warmth, and softness to touch. They were assessed daily and were regarded as rejected when less than 10% of the graft appeared to be viable.

TABLE 1. Graft survival and histology of class I-disparate lung allografts

Animal	Postoperative day								Following skin grafting	Survival (days)
	20	50	100	150	200	250	300	350		
Nonimmunized										
15622										
AR	0	0	0	0		0		0	0	>497
OB	0	0	0	0		0		0	0	
15669										
AR	2	0	0	0		0		0	0	>432
OB	0	0	0	0		0		0	0	
15792										
AR	0	0	2	0		0		0	0	>451
OB	0	0	0	0		0		0	0	
Immunized										
15678										
AR	3	0	2	0	2	2	0	0	0	>417
OB	0	0	0	0	0	0	0	0	0	
15839										
AR	1	2	0	0	0	0	0	2	1	>402
OB	0	0	0	0	0	0	0	0	0	
15894										
AR	2	2	0	0	0	0	0	0	0	>401
OB	0	0	0	0	0	0	0	0	0	

All animals were sacrificed with a viable graft.

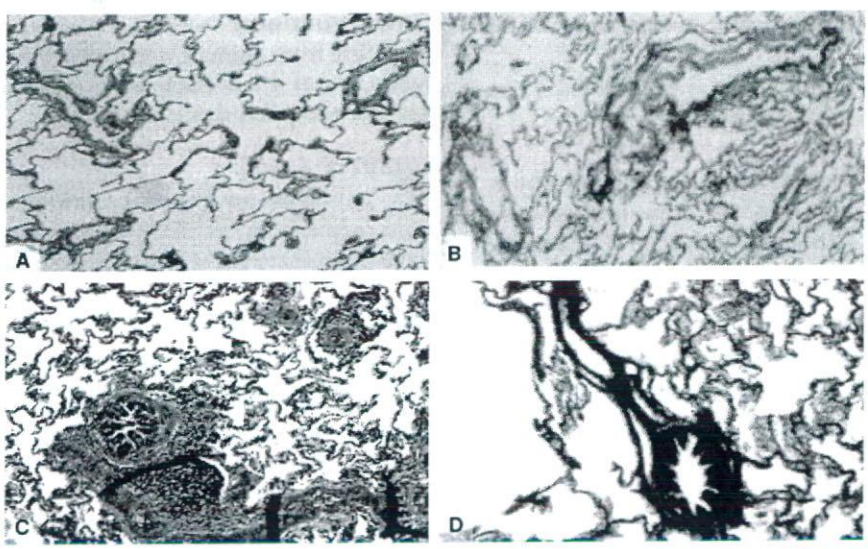

FIGURE 1. Histology of control lung allograft from a non-immunized recipient (A and B) and from a recipient immunized with donor-derived MHC-peptides (C and D). (A) Graft from nonimmunized recipient #15669 on POD 389 (H&E, ×100) shows no mononuclear cell infiltrate. (B) Lung from nonimmunized recipient #15669 on POD 389 (trichrome, ×100) shows no evidence of OB. (C) Graft from immunized recipient #15678 on POD 104 (H&E, ×100) shows a perivascular mononuclear cell infiltrate, but no alveolar infiltration, edema, or necrosis. These findings are consistent with moderate (ISHLT grade 2/4) AR. (D) Lung allograft from immunized recipient #15678 on POD 361 (trichrome, ×100) shows no evidence of ACR or OB.

Histology

Open lung biopsies were performed under general anesthesia at intervals as frequent as three weeks. To minimize sampling error, relatively large specimens (i.e., 30–50 g) were obtained. At necropsy, histologic analysis was performed on the entire explanted lung. Tissues were fixed in 10% formalin and evaluated with hematoxylin and eosin (H&E) stain, Masson's trichrome stain, and Verhoeff's elastic stain. Each biopsy was evaluated for AR, OB, airway inflammation, interstitial fibrosis, and vascular sclerosis based on criteria established in

TABLE 2. DTH responses (mm of induration) in pigs immunized with donor-derived MHC class I allopeptides

Animal	PC14-1	PC14-2	PC14-3	Saline	MTB
15678					
Preoperative	9	0	21	0	23
POD 354	0	0	19	0	17
15839					
Preoperative	15	0	13	0	18
POD 305	0	0	15	0	14
15894					
Preoperative	0	0	22	0	12
POD 193	0	0	19	0	14

MTB, *Mycobacterium tuberculosis* H37 RA; POD, postoperative day.

the 1996 revision of the formulation promulgated by the Lung Rejection Study Group of the International Society for Heart and Lung Transplantation (ISHLT) (*10*).

RESULTS

Tacrolimus-Induced Operational Tolerance of Lung Allograft in Both Nonimmunized Controls and Immunized Recipients

Both nonimmunized controls and immunized recipients maintained their lung grafts long-term, without any requirement for immunosuppression after the initial 12-day course of high-dose tacrolimus (Table 1).

After transplantation, lung grafts in nonimmunized recipients exhibited little, if any, mononuclear cellular infiltrate during entire postoperative period (Fig. 1A and B). Two of the animals in nonimmunized group showed a mild mononuclear infiltrate at only one time point during entire postoperative period (postoperative days [POD] 20 and 102, respectively). The other animal (#15622) never exhibited any histologic evidence of AR (Table 1).

In contrast, all lung grafts in immunized recipients exhibited a mild to moderate mononuclear cellular infiltrate during the early postoperative period (Fig. 1C) at multiple time points. However, this infiltrate subsequently subsided (Fig. 1D), and all recipients maintained their lung grafts in the long term (Table 1).

None of the animals (immunized or nonimmunized) ever showed any histologic evidence for OB on multiple open lung biopsies and upon postmortem evaluation (Table 1).

In Vivo DTH Responses to Class I Allopeptides

DTH responses to each class I^c allopeptide were analyzed 14 days after immunization (prior to transplantation). In the three pigs that were immunized, two showed positive or intermediate responses to PC14-1; none responded to PC14-2; and all developed responses to PC14-3. These results confirm the immunogenicity of our synthetic MHC class I allopeptides. After transplantation, immunized recipients maintained their DTH responses to the immunizing peptides, despite graft acceptance (Table 2).

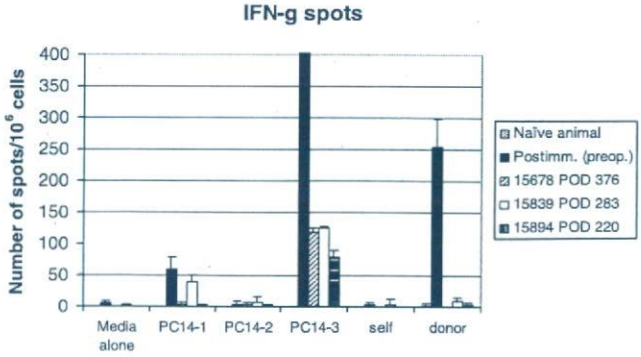

FIGURE 2. IFN-γ production in immunized recipients. The production of IFN-γ by peptide stimulation and donor cell stimulation after immunization was augmented after peptide immunization on ELISPOT assay. The production of IFN-γ by peptide stimulation was maintained after graft acceptance, while IFN-γ production from donor-cell stimulation subsided.

In Vitro Responses to Class I Allopeptides

The ability of recipient T cells to proliferate in vitro to individual class I allopeptides was analyzed by PPA 14 days after immunization (prior to transplantation). Prior to immunization, there was no T cell proliferative response to any of the donor-derived class I MHC allopeptides. After immunization, T cell responses to immunized peptides were consistently present and correlated with positive DTH reactions.

ELISPOT assays were performed with PBMCs from the peptide-immunized recipients to assess for Th1 cytokine production from recipient T cells. As shown in Figure 2, IFN-γ production was upregulated in response to peptide stimulation developed after immunization.

In Vitro Assays of Cellular Immune Responses

To assess in vitro reactivity to donor cells, MLR assays were performed with PBMCs from the peptide-immunized recipients bearing class I disparate lungs. Prior to immunization, there was no MLR response to any of the donor cells. After immunization, high MLR responses to donor cells developed. After transplantation, immunized recipients lost their MLR responses to donor cells.

On primary CML assays, all recipients developed donor-specific hyporesponsiveness after transplantation. (Fig. 3) This hyporesponsiveness persisted until the animals rejected skin grafts bearing donor-class I and third-party class II antigens (Fig. 3).

On secondary coculture CML assays after transplantation, recipient PBLs were able to suppress the normally robust response of naïve recipient-matched cells to donor targets (Fig. 4).

Skin Grafting Induced CML Responses, But Had No Effect on the Lung Allograft

After exhibiting long-term lung graft acceptance (>350 days), all recipients in both groups were challenged with grafts of 1) donor skin, 2) donor-matched skin, and 3) skin bearing donor class I and third-party class II antigens. Donor and donor matched skin typically showed prolonged survival (mean survival 41.2 days), whereas the skin bearing donor

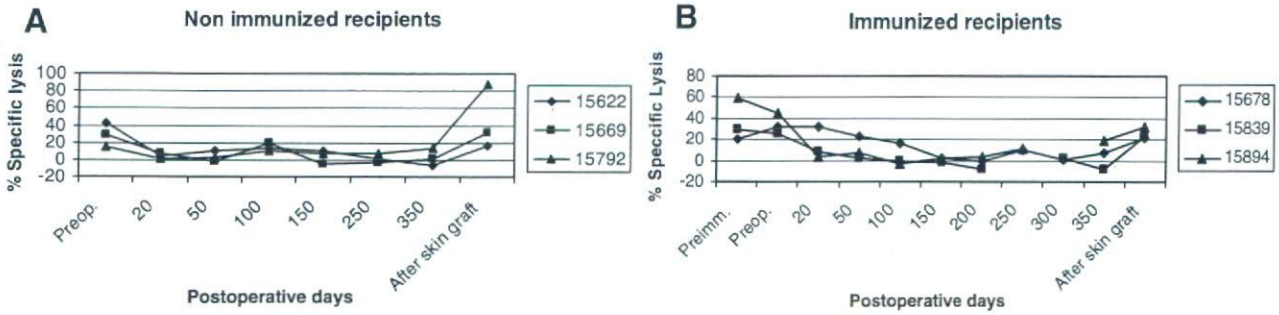

FIGURE 3. Comparison of CML antidonor reactivity in lung transplant recipients. (A) Nonimmunized recipients. (B) Immunized recipients. PSL (effector/target ratio 100:1) decreased after transplantation in all recipients, with return of CML response after skin grafting.

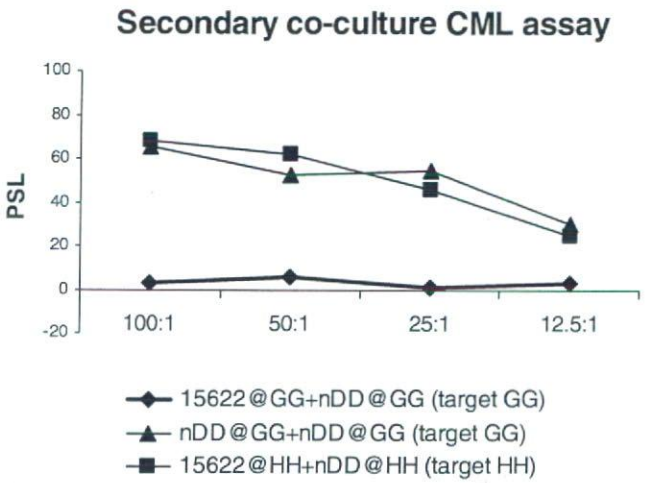

FIGURE 4. Secondary coculture CML demonstrates the ability of PBLs from tolerant recipients to suppress the normally robust response of naïve recipient matched cells to donor-type cells. PSL at various effector/target ratios in tolerant nonimmunized recipient #15622 on POD 103.

class I and third-party class II antigens rejected more promptly (mean survival 13.5 days). As mentioned above, rejection of skin bearing donor class I and third-party class II antigens, induced antidonor CML responses. However, none of the animals rejected its lung allograft, despite ultimate skin graft rejection.

Production of Alloantibody Was Not Observed

Sera collected from nonimmunized and peptide-immunized animals were analyzed for the presence of donor-specific IgM and IgG by flow cytometry. Pretransplant immunization did not induce the formation of antidonor antibody, as measured on the day of transplantation. Also, there was no evidence of antidonor antibody during the postoperative course, even after donor and/or donor-matched skin graft rejection was observed.

DISCUSSION

In the present study, the recipients of MHC class I-disparate lung allografts demonstrated indefinite graft survival after receiving only a brief postoperative course of high-dose tacrolimus. Moreover, none of these animals developed chronic allograft rejection on multiple open lung biopsies and upon postmortem evaluation. Finally, all animals studied developed and maintained donor-specific hyporesponsiveness in CML and MLR assays after transplantation. While CML responses were ultimately restored by the grafting of skin bearing donor class I plus third-party class II antigens, tolerance to the original lung allograft was not broken. These observations apply to both the nonimmunized control swine and the peptide-immunized swine in this study. A similar pattern has been observed in an analogous model using class I-disparate renal allografts in miniature swine (*3*). To our knowledge, this is the first report of long-term engraftment of MHC-disparate pulmonary allografts using only a brief course of a conventional immunosuppressive drug.

Since the porcine MHC has been fully characterized for a variety of haplotypes, we were able to synthesize peptides derived from the genetic sequence of the donor haplotype (MHC class I^c) to use as an experimental tool, as described above. We know from previous studies in heart and lung allograft models that reactivity to these peptides naturally develops when swine reject class I-mismatched allografts (*2, 9*). Furthermore, we know that pretransplant immunization with these MHC-derived allopeptides will accelerate rejection in CsA-based class I-mismatched transplant models (*2, 9*). In this study, we chose to use a 12-day postoperative course of tacrolimus on the belief that this regimen might induce tolerance to class I-disparate lung allografts, as had been seen previously in fully mismatched renal allografts (*3*). However, at the outset of this experiment, the effect of pretransplant alloimmunization was less certain.

Several experimental studies suggest that memory T cells both foster acute and chronic rejection, and impede the induction of tolerance. In a murine model, Valusjkikh and colleagues found that the adoptive transfer of primed donor-reactive T cells interferes with the prolongation of cardiac allograft survival induced by treatment with an anti-CD40L monoclonal antibody and donor spleen cell transfusion (*11*). In a similar rodent model based on anti-CD40L therapy, Zhai and colleagues showed that presensitization with skin grafts from the donor can block tolerance induction (*12*). Finally, another study suggested that virally induced alloreactive memory T cells can interfere with the robust tolerance generally seen after the generation of a mixed chimeric state (*13*).

Based on this body of evidence, it was surprising to see that pretransplant allopeptide immunization did not pre-

clude the induction of tolerance in our tacrolimus-based model. Also of note is the fact that there was no evidence of chronic rejection (OB) despite our priming of the indirect pathway by pretransplant immunization with biologically relevant allopeptides. These findings suggest our short course of tacrolimus was not only sufficient to suppress deleterious lymphocyte responses to the graft (both in vivo and in vitro), but also to protect the graft from rejection due to the presence of a T-cell population that was persistently sensitized to donor MHC antigen. Likewise, posttransplant skin grafting did not break tolerance to the graft or induce alloantibody, despite the return of an in vitro CML response to donor cells.

We anticipate that the induction of transplantation tolerance will be an important therapeutic strategy in the elimination of chronic allograft rejection and in the long-term maintenance of grafts without the requirement for generalized immunosuppression.

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