Table 1. Grade of Chest X-ray in Swine Lung Graft

Grade	Findings
0	No infiltrates
1	Bronchovascular shadow
2	Bronchovascular shadow with partial lung area of aeration loss
3	A large part of grafted side demonstrates loss of aeration; only a part of it contains aeration
4	Total loss of aeration in grafted side

tional hematoxylin-and-eosin (H&E) stain. Acute rejection was scored on a grade of 0 to 4, based on Boston group modifications of the 1996 revision of the formulation promulgated by the Lung Rejection Study Group (Table 2).[29] After POD 14, chest radiography and open lung biopsy were performed weekly.

End-point

When chest radiography showed Grade 4, the thoracic cavity was entered to observe the lung. When gross inspection revealed a loss of aeration and compliance, autopsy was performed. As with open biopsy, histologic analyses were performed.

Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted from the freshly prepared PBMCs (4×10^6) or the lung tissues. The conventional rejection marker genes (*perforin, Fas-L, IP-10*) and *FOXP3* were quantified by real-time polymerase chain reaction (PCR) using gene-specific primer and the SYBR-Green PCR Master Mix (Applied Biosystems). The nucleotide sequences of each primer are indicated in Table 3.

Samples were run in triplicate using a real-time PCR thermocycler (Model 7500 Fast Real-Time PCR System; Applied Biosystems). The relative expression of each was determined by normalizing to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression.[32]

Table 2. Grade of Acute Rejection in Swine Lung Allograft

Grade	Histology
0	No interstitial filtrate, hemorrhage or necrosis
1	Minimal—scattered infrequent perivascular mononuclear infiltrates (not obvious at ×40)
2	Mild—frequent perivascular mononuclear infiltrates (easily seen at ×40)
3	Moderate—dense mononuclear cuffing with expansion into alveolar septa; endothelialitis
4	Severe—as above, but with necrosis and hemorrhage

Acute rejection was scored on a scale of of 0 to 4, based on Boston group modifications of the 1996 revision of the formulation promulgated by the Lung Rejection Study Group.[29]

Table 3. Sequences of Primers

Gene	Sequence
FOXP3	Sense: TTCCCAGACTTTCTTTCACAACAT
	Anti-sense: GCTGCTTCTCTG GAGCCTCCAG
Perforin	Sense: ACCGCTTCAGCCTGGACTCAG
	Anti-sense: GAAGTGGGTGCGT AGCTG
Fas-L	Sense: TGAACCATGAGATGAACGAG
	Anti-sense: TGCGGATCTCCTCCAAA CG
IP-10	Sense: TACTGATAAGGGATGGGCCG
	Anti-sense: GATGAACCATCTGCTG CCCTA
GAPDH	Sense: GTGGAGTCCACTGGTGTCTTCACG
	Anti-sense: AACTCCCTCTAA CAGTATGAAGAG

Immunosuppression

Methylprednisolone and tacrolimus were diluted in distilled water. In the induction immunosuppression group, tacrolimus was given continuously and intravenously, using an infusion pump (Baxter, Deerfield, IL) from POD 1 to 12. In the POD 4 immunosuppressive rescue group, 10 mg/kg/day methylprednisolone was given intravenously as a single injection on POD 4, 5 and 6. Tacrolimus was given in the same manner as in the induction immunosuppression group from POD 4 to 9. In the POD 6, immunosuppressive rescue group, similar immunosuppression regimens (steroid pulse + tacrolimus) were administered from POD 6 to 11. Tacrolimus was started at the dose of 0.15 mg/kg/day, and then adjusted to maintain trough levels (determined by microparticle enzyme immunoassay by a clinical laboratory testing service [SRL, Inc., Tokyo]) of 35 to 50 ng/ml.

Statistical Analysis

The Kolmogorov-Smirnov test was applied to test for a normal distribution. Data analyses were performed by the log-rank test, repeated-measures analysis of variance (ANOVA), Pearson's correlation coefficient and simple regression analysis. $p < 0.05$ was considered significant.

RESULTS

Chest Radiography and Graft Survival Rates

Chest radiography in the clamp reperfusion group exhibited grade 2.5 (mean) on POD 3, which declined to grade 1.7 by POD 6 (Figure 1a). The grade in this group remained low indefinitely (survival of the lung >140 days; Table 4). In contrast, the immunosuppression-free rejection group exhibited Grade 1.7 on POD 3, which increased gradually thereafter. From POD 3 to 5, the grade between the clamp reperfusion and immunosuppression-free rejection groups did not differ (not statistically significant [NS]). Finally, on POD 6, the grade in the immunosuppression-free rejection group became significantly

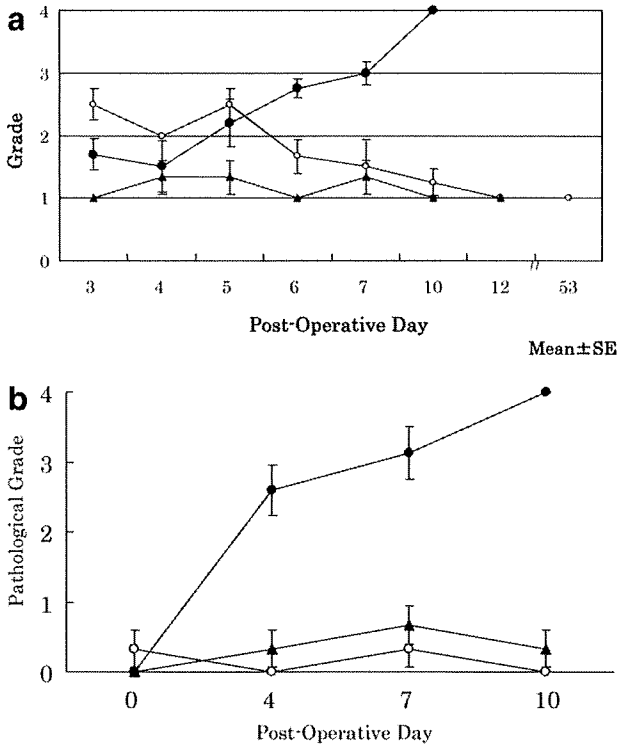

Figure 1. Radiologic and pathologic findings. Chest radiography was evaluated according to our grading system (see Table 1). Filled circle: immunosuppression-free rejection group (n = 10); open circle: the clamp reperfusion group (n = 4). From post-operative day (POD) 3 to POD 5, the grade between the clamp reperfusion and immunosuppression-free rejection groups did not differ (NS). On POD 6, the grade for the immunosuppression-free rejection group became significantly higher than that of the clamp reperfusion group (n = 4) filled triangle induction immunosuppression group (n = 3) (a) Radiological (clamp reperfusion and immunosuppression-free rejection groups: 1.7 ± 0.3 and 2.8 ± 0.2, respectively, $*p < 0.05$). The grade remained higher in the immunosuppression-free rejection group than in the clamp reperfusion group after POD 7 ($*p < 0.05$, $**p < 0.005$). The filled triangle indicates the induction immunosuppression group (n = 3). The grade of this group remained low throughout 12 days after transplantation. (b) Biopsy of the lung was performed on PODs 0, 4, 7 and 10. Following Allan's grading system (see Table 2), pathologic findings were evaluated. Filled circle: immunosuppression-free rejection group (n = 10); open circle: clamp reperfusion group (n = 4); filled triangle: induction immunosuppression group. On POD 0, the grade among the clamp reperfusion, immunosuppression-free rejection and induction immunosuppression groups did not differ (clamp reperfusion, immunosuppression-free rejection, and induction immunosuppression groups: Grade 0.3 ± 0.3, grade 0 ± 0 and grade 0 ± 0, respectively, p = NS). On POD 4, the immunosuppression-free rejection group was grade 2.4 ± 0.34, whereas the clamp reperfusion group was grade 0 ± 0 ($*p < 0.05$). On POD 7, the immunosuppression-free rejection group exhibited grade 3.3 ± 0.3, whereas the clamp -reperfusion group exhibited grade 0.3 ± 0.3 ($*p < 0.05$). On POD 10, all swine in the immunosuppression-free rejection group exhibited grade 4, whereas the clamp reperfusion group exhibited grade 0 ± 0 ($**p < 0.005$). Similar to the clamp reperfusion group, the induction immunosuppression group exhibited a low grade throughout 10 days.

higher than that of the clamp reperfusion group (the clamp reperfusion and immunosuppression-free rejection groups were 1.7 and 2.8, respectively, $p < 0.05$). This implies that rejection was suspected by chest radiography not before POD 5, but after POD 6. In 3 of 10 recipients in the immunosuppression-free rejection group, Grade 4 was reached on POD 7, and in 7 on POD 10. Autopsy was performed and rejection was suspected on gross inspection in all the cases. Thus, graft survival in the immunosuppression-free rejection group was 9.1 (mean) days ($p < 0.005$ versus clamp reperfusion group; Table 4). In the induction immunosuppression group, Grade 1 was detected on POD 3, which remained low throughout the first 12 days. The animals in this group succumbed to rejection on PODs 14, 20 and 24.

Pathologic Findings

The specimens of zero biopsy in the clamp reperfusion and immunosuppression-free rejection groups exhibited grade 0.3 and 0 (mean), respectively (p = NS) (Figure 1b). The specimens of open lung biopsies on POD 4 in the immunosuppression-free rejection group exhibited grade 2.4, whereas they POD 4 biopsies in the clamp reperfusion group exhibited grade 0 ($p < 0.05$). Thus, rejection was histologically proven on POD 4 in the immunosuppression-free rejection group. On POD 7, the specimens derived from 3 animals at autopsy uniformly exhibited grade 4 and biopsy specimens (n = 7) exhibited grades 1 to 3 in 4 animals and grade 4 in 3 animals whereas the grade of the clamp reperfusion group remained low (grade in immunosuppression-free rejection and clamp reperfusion groups: 3.3 and 0.3, respectively, $p < 0.05$). On POD 10, the specimens derived at autopsy (n = 7) uniformly exhibited Grade 4, whereas the clamp reperfusion group exhibited Grade 0 ($p < 0.005$). In the induction immunosuppression group, biopsy specimens exhibited Grade 0 at transplantation. The grade in this group remained low (0 or 1) throughout the first 10 days ($p < 0.05$ vs the immunosuppression-free rejection group, and p = NS versus clamp reperfusion group, on PODs 4, 7 and 10).

FOXP3 and Conventional Rejection Markers in Peripheral Blood

In the immunosuppression-free rejection group, *FOXP3* mRNA level reached its highest value as early as POD 4 ($p < 0.0001$) before showing a decline by POD 6. Figure 2). It remained low thereafter. In contrast, *FOXP3* mRNA remained low at all the time-points in the clamp reperfusion and induction immunosuppression groups. In the immunosuppression-free rejection group, the *perforin* mRNA level was slightly increased until POD 7

Table 4. Graft Survival

Group	*n*	Survival (POD)	POD (mean ± SD)	*p*-value
Clamp-reperfusion group	4	>140 × 4	>140	
Immunosuppression-free rejection group	10	7 × 3, 10 × 7	9.1 ± 0.5	$p < 0.005$ vs clamp-reperfusion group
POD 4 rescue immunosuppressive therapy group	6	7 × 1, 14 × 2, 24 × 1, 42 × 1, 88 × 1	27.2 ± 6.5	$p < 0.001$ vs immunosuppression-free rejection group
POD 6 rescue immunosuppressive therapy group	4	10 × 1, 11× 2, 14 × 1	11.5 ± 1.5	NS vs immunosuppression-free rejection group
Induction immunosuppression group	3	14, 20, 24		

and its peak was seen on POD 8 ($p = 0.0516$), whereas the perforin mRNA level in the clamp reperfusion and induction immunosuppression groups remained low at all time-points. The immunosuppression-free rejection group did not show any significant fluctuation in interferon-inducible protein-10 (IP-10) mRNA or Fas-ligand (Fas-L) mRNA at any time-point, similar to the clamp reperfusion and induction immunosuppression groups (p = NS).

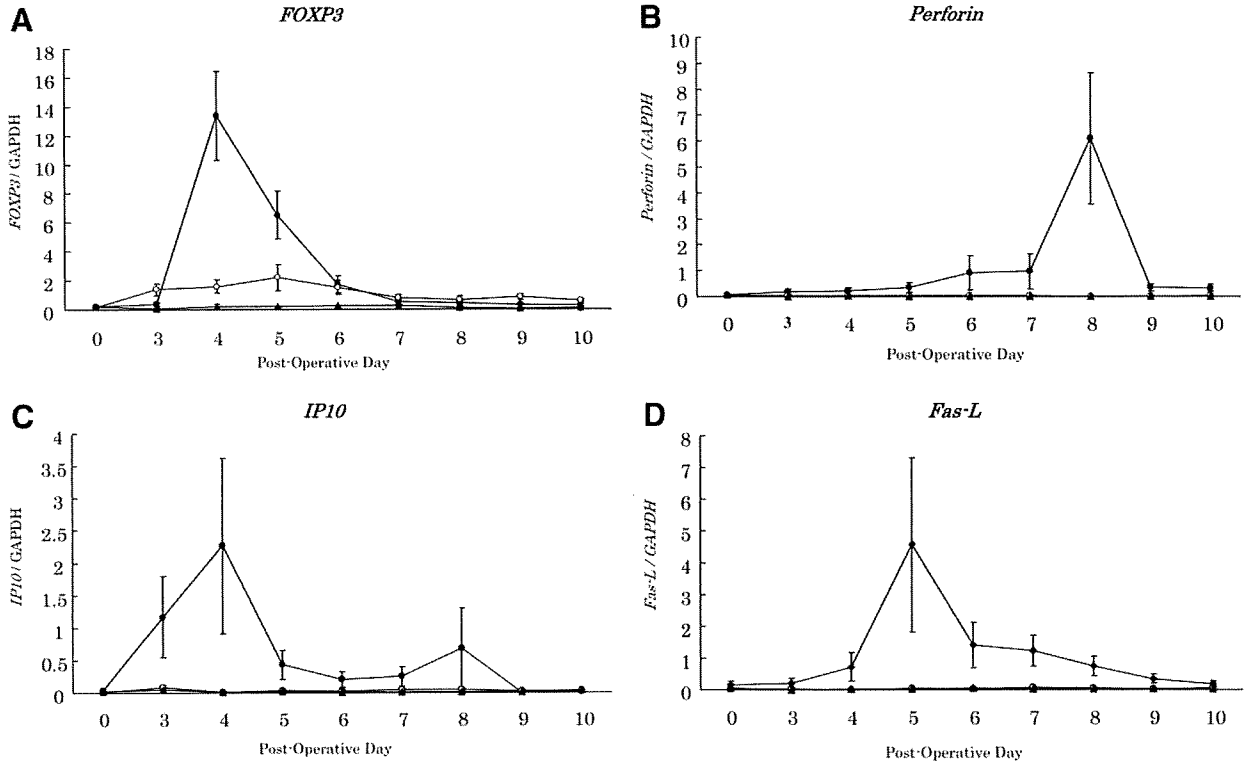

Figure 2. Expression of *FOXP3* mRNA *versus* conventional rejection markers in peripheral blood mononuclear cells (PBMCs). PBMCs were obtained on PODs 0 and 3 to 10. Total RNA was extracted from the freshly prepared PBMCs (4 × 106) using RNeasy Mini or Micro kits and reverse-transcribed into cDNA by QuantiTect Reverse Transcription kit (Qiagen). The conventional rejection marker genes (*perforin, Fas-L, IP-10*) and *FOXP3* were quantified by real-time PCR using the gene-specific primer and SYBR-Green PCR Master Mix (Applied Biosystems). The nucleotide sequences of each primer are indicated in Table 3. PCR amplification conditions were as follows: 95° C for 10 minutes, then 40 cycles of 95° C for 15 seconds and 60° C for 1 minute. Samples were run in triplicate using a real-time PCR thermocycler (Model 7500 Fast Real-Time PCR System, Applied Biosystems). The relative expressions of *FOXP3, perforin, Fas-L* and *IP-10* genes were determined by normalizing to *GAPDH* expression. Filled circle: the immunosuppression-free rejection group ($n = 5$); open circle: the clamp reperfusion group ($n = 3$); filled triangle: the induction immunosuppression group ($n = 3$). Data were normalized to *GAPDH.* In the immunosuppression-free rejection group, the *FOXP3* mRNA level reached its highest value as early as POD 4 (*$p = 0.0001$), before an abrupt decline. It remained low after POD 6. By contrast, *FOXP3* mRNA level remained low at all time-points in the clamp reperfusion group. In the induction immunosuppression group, elimination of rejection by immunosuppression resulted in the lack of an increase in *FOXP3* mRNA level at all time-points. In the same manner as *FOXP3*, the mRNA levels of the conventional rejection markers in the PBMCs were quantified. Filled circle: the immunosuppression-free rejection group ($n = 5$); open circle: the clamp reperfusion group ($n = 3$); filled triangle: the induction immunosuppression group ($n = 3$). In the immunosuppression-free rejection group, *perforin* level increased on POD 8 ($p = 0.0516$), but not in the clamp reperfusion group or the induction immunosuppression group. In the immunosupression-free rejection group, no significant fluctuation was observed in the level of *Fas-L* or *IP-10* at any time-point (p = NS). The levels of *perforin, Fas L* and *IP-10* remained low in the clamp reperfusion group and the induction immunosuppression group at all time-points.

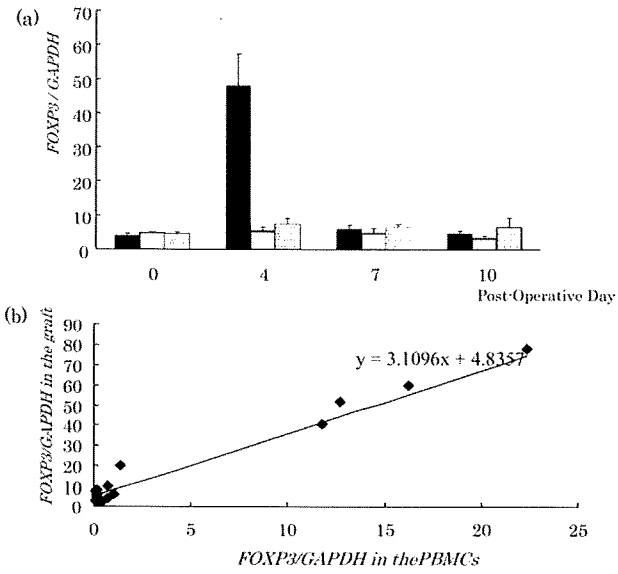

Figure 3. Intragraft *FOXP3* mRNA expression and its correlation with *FOXP3* mRNA expression in the peripheral blood mononuclear cells (PBMCs). (a) The tissues of grafts and lungs (2 mm × 2 mm × 2 mm) were obtained at transplant, at clamp reperfusion, at open lung biopsy, or at autopsy on PODs 0, 4, 7 and 10. The frozen lung (1 to 10 mg) tissue was put into 350 μl of RLT buffer (MM300; Qiagen, Tokyo). The mixture was crushed for 2 minutes at room temperature. Total RNA was extracted from lung tissues using in the same manner as the PBMCs. The expression of *FOXP3* was quantified by real-time PCR using the same primer as for the PBMCs (see Table 3). PCR amplifiction conditions were similar to those for the PBMCs. Samples were also run in triplicate using a real-time PCR thermocycler (Model 7500 Fast Real-Time PCR System; Applied Biosystems). The relative expression of *FOXP3* was determined by normalizing to *GAPDH* expression. Filled bar: immunosuppression-free rejection group ($n = 5$); open bar: the clamp reperfusion group ($n = 3$); shaded bar: the induction immunosuppression group ($n = 3$). Similar to the PBMCs, the immunosuppression-free rejection group exhibited its highest value on POD 4 (*$p = 0.0005$). The *FOXP3* mRNA level in the clamp reperfusion group and induction immunosuppression group remained low at all time-points (NS). (b) The dots indicate the data for PODs 4, 7 or 10. In the immunosuppression-free.

Intragraft FOXP3 mRNA Level

The immunosuppression-free rejection group displayed the highest value for intragraft *FOXP3* mRNA level on POD 4 ($p < 0.0005$), before a decline on POD 7. In contrast, its expression in lungs of the clamp reperfusion group and grafts of the induction immunosuppression group remained low at all time-points (Figure 3a).

Correlation Between Peripheral and Intragraft FOXP3 mRNA Levels

In the immunosuppression-free rejection group, there was a definite correlation between expression levels of *FOXP3* mRNA in the PBMCs and in grafts on PODs 4, 7 and 10 ($p < 0.0001$, $R = 0.961$) (Figure 3b).

Rescue Immunosuppressive Therapy

Rescue immunosuppressive therapy was started on POD 4, when the immunosuppression-free rejection group exhibited upregulation of *FOXP3* mRNA in the peripheral blood, or POD 6 when rejection was suspected by chest radiography. At POD 4, in the rescue immunosuppressive therapy group, graft survival was prolonged to POD 27.2 ± 6.5 ($p < 0.001$ vs the immunosuppression-free rejection group), but not at POD 6 in the rescue immunosuppressive therapy group (POD 11.5 ± 1.5, p = NS vs the immunosuppression-free rejection group) (Figure 4).

DISCUSSION

The paradoxical clinical evidence that, during rejection, the expression of *FOXP3* mRNA or protein is upregulated within cardiac grafts or grafts and urine of renal transplantation has been documented by several groups.[21–24] The Rotterdam group recently examined *FOXP3* mRNA level in the peripheral blood during rejection after human cardiac transplantation. However, they found no correlation between peripheral and intragraft *FOXP3* levels.[33] They concluded that *FOXP3*

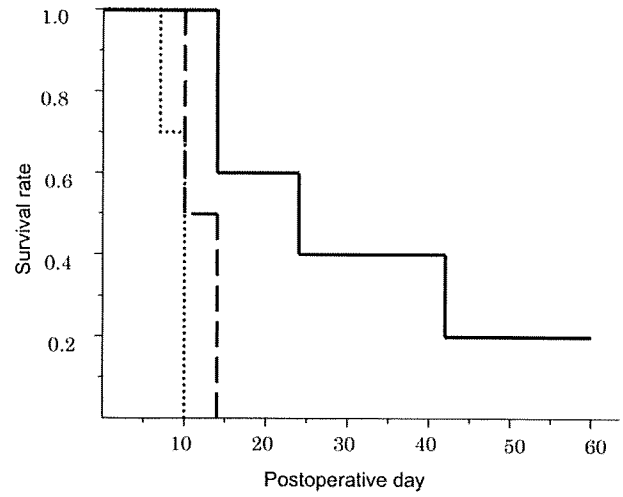

Figure 4. Rescue immunosuppressive therapies from POD 4 vs POD 6. Rescue immunosuppressive therapy (steroid + tacrolimus) was started on POD 4, when the immunosuppression-free rejection group exhibited the highest level of peripheral *FOXP3* mRNA level (the POD 4 rescue immunosuppressive therapy group) or POD 6, when rejection was suspected by chest radiography (the POD 6 rescue immunosuppressive therapy group). A rescue immunosuppressive therapy, which was started on POD 4, significantly prolonged graft survival to POD 27.2 ± 6.5 (solid line: the POD 4 rescue immunosuppressive therapy group, $n = 6$), comapred with that in the immunosuppression-free rejection group ($p = 0.001$ vs the immunosuppression-free rejection group, 9.1 ± 0.5 days [dotted line], $n = 10$). However, rescue immunosuppresive therapy, which was started on POD 6, did not prolong graft survival (11.5 ± 1.5 days, p = NS versus the immunosuppression-free rejection group; the POD 6 rescue immunosuppressive therapy group [dashed line], $n = 4$).

level in the peripheral blood does not provide the necessary evidence for non-invasive detection of rejection. In our study, the *FOXP3* mRNA levels were upregulated in the peripheral blood and within grafts at an early phase of rejection only in the immunosuppression-free rejection group and not in the clamp reperfusion control or induction immunosuppression groups. This implies that the upregulation of *FOXP3* is caused by rejection, excluding the effects of ischemia-reperfusion injury, surgery and rejection-free allo-lung transplantation on *FOXP3*. Thus, our study is the first demonstration of a definite correlation between peripheral and intragraft *FOXP3* mRNA levels during rejection after solid-organ transplantation.

The Stanford group reported that FOXP3 protein increased within the rejecting grafts after human liver transplant, but, in contrast to our data, it decreased in the peripheral blood during rejection.[34] They hypothesized that the decrease in peripheral FOXP3 level was a consequence of localization of the Tregs into grafts. They proposed that assessment of the peripheral Tregs is a useful tool for monitoring rejection. There is the possibility that the difference in peripheral FOXP3 levels between our study and the Rotterdam and Stanford groups could pertain to whether immunosuppression was used. The Rotterdam group demonstrated that calcineurin inhibitors inhibited in vitro-induced *FOXP3* mRNA expression.[21] In addition, in the clinical setting, it was reported that calcineurin inhibitors reduced the percentage of $FOXP3^+$ cells in the peripheral blood after renal transplantation.[35] Of note, in our study, lung transplantation was performed in an immunosuppression-free environment, whereas clinical transplantations were performed in the presence of immunosuppression by the Rotterdam and Stanford groups. Therefore, in our laboratory, FOXP3 expression is currently being measured in the peripheral blood of clinical lung transplantation patients to determine whether measurement of FOXP3 level is useful for monitoring rejection even in the presence of immunosuppression.

The presence of interleukin-2 (IL-2) in allografts, which is produced by the effector cells (i.e., the non-Tregs), has been associated with acute rejection.[36] Recent studies, however, have shown that IL-2 is also critically required for activation and maintenance of the Tregs.[37,38] Thus, the Rotterdam group speculated that the non-Tregs and the Tregs, both of which were IL-2 dependent, were recruited within rejecting grafts simultaneously in the presence of allo-antigens, and that the upregulation of the intragraft *FOXP3* mRNA level was caused by the latter.[22] Unlike Foxp3 in mouse expressed exclusively by the Tregs, recent in vitro studies have reported that human $CD4^+CD25^-$ and $CD8^+CD25^-$ non-Tregs, when activated, "transiently" (48 to 72 hours) express *FOXP3* mRNA and FOXP3 protein at a high level, followed by gradual downregulation.[39,40] Of note, this is in accordance with our findings that *FOXP3* mRNA levels in the peripheral blood and grafts were upregulated "transiently" only at an early stage of rejection. Therefore, there is the possibility that the source of FOXP3 during rejection may be the activated non-Tregs, which were responsible for rejection rather than the Tregs. Of note, upregulation of FOXP3 within rejecting grafts has been seen in humans, whereas such paradoxical evidence has never been documented in rodents. If an increase in FOXP3 level during rejection is caused by the non-Tregs, this may be human-specific. In this context, the immune responses of miniature swine would be similar to those in humans and data obtained from that model may be clinically relevant.

In the clinical setting, rejection after lung transplantation is currently monitored by chest radiography rather than transbronchial lung biopsy, because biopsy can cause several complications, such as bleeding and pneumonia, with a high incidence rate.[41-43] However, as shown in our study, an early phase of rejection could be misdiagnosed by chest radiography. Therefore, it should be noted that, in our study, the *FOXP3* level in the peripheral blood increased dramatically before rejection was suspected by chest radiography. In addition, our study suggested that *FOXP3* may be superior to conventional rejection markers, such as *perforin*, *Fas-L* and *IP-10*, in terms of detecting early rejection.[44-47] The possibility of *FOXP3* being a marker for an early phase of rejection was supported by our finding that a rescue immunosuppressive therapy, which was started when peak *FOXP3* level was noted, significantly prolonged graft survival, but not in a therapy started when rejection was suspected by chest radiography. However, because the expression of *FOXP3* was upregulated only early post-transplantation, a combination of *FOXP3* and chest radiography may detect rejection more efficiently than *FOXP3* alone or chest radiography alone. Finally, before the value of *FOXP3* is validated, it must be known whether the measurement of *FOXP3* excludes infection in our clinical lung transplantation setting, because differentiation between infection and rejection is especially crucial after lung transplantation.

In our miniature pig lung transplantation model, the *FOXP3* mRNA level in the peripheral blood was upregulated at an early stage of rejection. It must be elucidated whether measurement of FOXP3 is useful as a minimally invasive method for detection of early rejection in clinical lung transplantation.

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The Presence of Foxp3 Expressing T Cells Within Grafts of Tolerant Human Liver Transplant Recipients

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Background. Some experimental transplant-tolerance models have shown that the presence of regulatory T cells within grafts is important for the development of tolerance (Tol).
Methods. To determine if the presence of regulatory T cells correlates with graft acceptance in living-donor liver-transplantation tolerance, the expression of *Foxp3* mRNA and the presence of $CD4^+$, $CD8^+$, and $Foxp3^+$ cells were quantified in biopsies from tolerant recipients by real-time polymerase chain reaction and by immunohistochemistry and immunofluorescent staining (Gr-Tol). The results were compared with biopsies from the recipients on maintenance immunosuppression (Gr-IS), grafts removed because of chronic rejection (Gr-CR), or normal liver (Gr-NL).
Results. The expression of *Foxp3* mRNA in Gr-Tol was higher than that in Gr-IS ($P=0.07$) and Gr-NL ($P<0.0001$), but equivalent to that in Gr-CR. In Gr-Tol, $Foxp3^+$ cells were detectable within the clustered $CD4^+$ and $CD8^+$ cells in the portal areas. Ninety-two percent of those $Foxp3^+$ cells were $CD4^+$, whereas 8% were $CD8^+$. The number of $Foxp3^+$ cells was significantly increased in Gr-Tol, compared with that in Gr-IS ($P<0.05$), although the number of $CD4^+$ or $CD8^+$ cells did not differ between the two. $Foxp3^+$ cells were hardly detectable in Gr-CR or -NL.
Conclusions. This is the first report showing that $CD4^+Foxp3^+$ cells are present within grafts in a subset of tolerant patients after human liver transplantation. A prospective study is needed to elucidate whether the assessment of intragraft expression of Foxp3 protein, but not *Foxp3* mRNA, can aid the identification of living-donor liver-transplantation recipients who can successfully withdraw IS.

Keywords: Tolerance, Foxp3, Liver transplantation, Human, Regulatory T cells.

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The $CD4^+CD25^{high+}$ regulatory T cells (Tregs) have been shown to play a pivotal role in transplant (Tx) tolerance (*1*). In mice, Foxp3 has been shown to be a transcription factor exclusively expressed by Tregs (i.e., not expressed by non-Tregs) (*2*). Additionally, recent studies have suggested that Foxp3 (Foxp3 in humans) may not only be a molecular marker of Tregs but also a necessary molecule for the development and the maintenance of regulatory function (*3*). Consistent with this, some rodent Tx models have demonstrated that high levels of *Foxp3* mRNA and a substantial number of Foxp3 protein expressing cells are found within tolerant, but not rejecting grafts (*4*). These data have suggested that the presence of Tregs within grafts may be crucial for the establishment and the maintenance of tolerance. However, to date this has not been studied in detail in humans.

In our previous study, analyses of the peripheral blood of immunosuppression (IS) free living-donor liver-transplantation (LDLT) recipients demonstrated that T cells potentially reactive to donor antigens remained physically in the immune repertoire, but that such T cells were suppressed by Tregs in an antigen-specific manner in a subset of our tolerant patients (*5*, *6*).

Part of this work was presented at the World Transplant Congress (WTC) 2006, July 22–27, 2006, Boston, Massachusetts, USA, and at the American Transplant Congress (ATC) 2007, May 5–9, 2007, San Francisco, California, USA, and selected as one of the top abstracts for presentation at the XXII International Congress of The Transplantation Society, August 10–14, 2008, Sydney, Australia; the invited manuscript was peer reviewed and accepted for publication in the special issue dedicated to the XXII Congress.

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TABLE 1. Characteristics of study groups

Gender	Original diseases	ABO matching	Age at Tx (yr)	Years post-Tx	Age at biopsy (yr)	Reasons for weaning IS	Duration after cessation of IS	Donor	Donor age (yr)
Tolerance group (n=28) (Gr-Tol)									
F (n=21)	BA (n=24)	Identical (n=19)	1±2	10±4	12±4	Prot (n=23)	47±29 mo	F (n=18)	33±6
M (n=7)	FHF (n=1)	Compatible (n=5)				EBV (n=3)		M (n=10)	
	Alagille (n=1)	Incompatible (n=4)				Side effect (n=1)			
	Budd-chiari (n=1)					Cholangitis (n=1)			
	Metabolic disease (n=1)								
Maintenance immunosuppression group (n=29) (Gr-IS)									
F (n=17)	BA (n=21)	Identical (n=15)	4±4	4±2	9±5			F (n=9)	34±6
M (n=12)	FHF (n=3)	Compatible (n=11)						M (n=20)	
	PSC (n=1)	Incompatible (n=3)							
	Wilson's disease (n=3)								
	Veno-occlusive disease (n=1)								
Chronic rejection group (Gr-CR)									
n=7 F (n=4)	BA (n=3)	Identical (n=5)	15±17	1±2	17±17			F (n=4)	44±13
M (n=3)	FHF (n=2)	Compatible (n=1)						M (n=3)	
	PSC (n=1)	Incompatible (n=1)							
	PBC (n=1)								
P value Gr-Tol vs. Gr-IS, -CR, NS			Gr-Tol vs. Gr-IS, $P=0.06$, Gr-CR vs. Gr-Tol, -IS, $P<0.0001$	Gr-Tol vs. Gr-IS, -CR, $P<0.0001$, Gr-IS vs. Gr-CR, $P=0.02$	Gr-IS vs. Gr-CR, $P<0.01$ Gr-Tol vs. Gr-IS, -CR, NS			Gr-Tol vs. Gr-IS, -CR, NS	Gr-CR vs. Gr-Tol, -IS, $P<0.005$ Gr-Tol vs. Gr -IS, NS
Normal liver group (n=12) (Gr-NL)									
F (n=7)									33±7
M (n=5)									

Recipients' age at Tx and at sampling, gender, original disease, years post-Tx, and ABO matching are indicated.
Donors' age and gender are also indicated. Whether IS was electively weaned by protocol (prot) or because of other reasons is indicated.
IS, immunosuppression; BA, Biliary atresia; FHF, fulminant hepatic failure; PSC, primary sclerosing cholangitis; PBC, primary biliary cholangitis; EBV, Epstein-Barr virus.

In the next phase of this study, we have investigated the hypothesis that $Foxp3^+$ cells would exist in grafts in operationally tolerant LDLT recipients and explored whether this parameter might be useful as a relevant indicator of operational tolerance. We compared *Foxp3* mRNA and protein expressions within biopsies from liver-Tx recipients who were IS-free (Gr-Tol), receiving IS (Gr-IS), exhibiting chronic rejection (Gr-CR), and normal liver (Gr-NL). This is the first report providing detailed evidence that $Foxp3^+$ cells are present within grafts of operational tolerance after LDLT.

MATERIALS AND METHODS

Experimental Design and Samples Collection

All the patients underwent LDLT. Operational tolerance was defined as long-term stable normal graft function in the total absence of a requirement for maintenance IS (*7, 8*). Experimental groups consisted of IS-free recipients exhibiting operational tolerance (Gr-Tol) (n=28), recipients receiving maintenance IS (Gr-IS) (n=29), recipients with clinical evidence of chronic rejection (Gr-CR) (n=7), and samples of the normal liver (Gr-NL) (n=12). In Gr-IS, the patients were taking tacrolimus or cyclosporine A two times per day and fulfilled the criteria to start IS weaning protocol (*9*). For sampling, percutaneous liver biopsy was performed using an automated biopsy gun (Monopty, Bard) under the guidance of ultrasonography. The tolerant recipients exhibited no evidence of acute or chronic rejection. In Gr-CR, the liver tissues were obtained from seven grafts that had been removed after the diagnosis of chronic rejection. The normal liver tissues were obtained from 12 donors during LDLT by zero biopsy. This study was approved by Ethical Committee of Graduate School of Medicine, Kyoto University, and informed consent was obtained after the Declaration of Helsinki (*10*).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from liver tissue samples using RNeasy Mini kit or Micro kit (Qiagen) and synthesized for cDNA using QuantiTect Reverse Transcription kit (Qiagen). The level of *Foxp3* was quantified by quantitative real-time polymerase chain reaction (PCR) by standard curve methods and normalized to *HPRT*, multiplied by 10^6, and log transformed. The primers and probes for human *Foxp3* (Hs00203958_m1) and human *HPRT* (4326321E) were purchased from Applied Biosystem (Foster City, CA). Reactions were conducted in triplicate after the predeveloped TaqMan assay reagents protocol using Applied Biosystems 7500 real-time PCR system.

Immunohistochemistry and Immunofluorescent Staining

Four micrometer paraffin-embedded sections were deparaffinized and rehydrated, and antigen was retrieved using retrieval solution (Dakocytomation, Glostrup, Denmark). For immunohistochemistry, a 1:200 diluted anti-Foxp3 monoclonal antibody (mAb) (Abcam ab22510), a 1:200 diluted anti-CD4 mAb (clone 4B12, MBL, Nagoya, Japan), and an anti-CD8 mAb (clone C8/144B, Dakocytomation) were used. The slides were incubated with the primary antibody overnight at 4°C and incubated with HRP-conjugated goat anti-mouse-Ig, followed by DAB chromogen as a substrate. The sections were counterstained with Mayer's hematoxylin (Wako, Richmond, VA) and mounted. $CD4^+$, $CD8^+$, and $Foxp3^+$ cells were counted in all the portal areas. The size of the portal area was measured by computerized calculation using ImageJ software (Image processing and analysis in Java [http://rsb.info.nih.gov/ij/]). The number of $CD4^+$, $CD8^+$, and $Foxp3^+$ cells/mm^2 was assessed, dividing the number of $CD4^+$, $CD8^+$, and $Foxp3^+$ cells, respectively, by the size of each portal area.

For immunofluorescent staining of CD4 versus Foxp3, the sections were incubated overnight with a 1:20 diluted anti-CD4 mAb (K0003–1B, MBL, Nagoya, Japan) and stained using PE-conjugated streptavidin (Vector Laboratories, Burlingame, CA). The sections were further incubated with a 1:150 diluted fluorescein isothiocyanate-conjugated anti-Foxp3 mAb (Bioscience, San Diego, CA) at room temperature for 1 hr. For immunohistochemistry and immunofluorescent staining of CD8 versus Foxp3, the sections stained with the anti-Foxp3 mAb were further incubated with a 1:50 diluted anti-CD8 mAb (M7103, Carpinteria, CA) for 16 hr. The signal was visualized with DAB. The immunofluorescent staining or immunohistochemistry and immunofluorescent staining for CD4 or CD8, versus Foxp3 were overlaid as described previously (*11*).

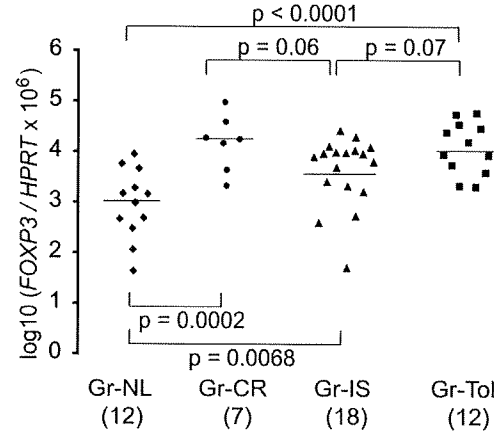

FIGURE 1. The expression of *Foxp3* mRNA in tolerance group (Gr-Tol), maintenance IS group (Gr-IS), chronic rejection group (Gr-CR), and normal liver group (Gr-NL).The level of *Foxp3* mRNA was quantified by RT-PCR in 12, 18, and 7 frozen biopsy samples from the following groups, operational tolerance (Gr-Tol), maintenance immunosuppression (Gr-IS), chronic rejection (Gr-CR) respectively, together with 12 samples of the normal liver (Gr-NL). The value was normalized to *HPRT*, multiplied by 10^6, and log transformed. The expression of *Foxp3* mRNA tended to be higher in tolerance group, compared with those in maintenance immunosuppression and normal liver groups. The level of *Foxp3* mRNA in chronic rejection group was as high as that in tolerance group (log10 [*Foxp3/HPRT*$\times 10^6$]) Gr-Tol, Gr-IS, Gr-CR and Gr-NL: 4.1 ± 0.5, 3.6 ± 0.7, 4.2 ± 0.6, and 3.0 ± 0.7; Gr-Tol vs. Gr-IS $P=0.07$, Gr-CR vs. Gr-IS $P=0.06$, Gr-Tol, Gr-IS, Gr-CR vs. Gr-NL $P<0.005$, Gr-Tol vs. Gr-CR NS). The number of examined samples is indicated in parenthesis.

Statistical Analysis

Results were presented as mean±SD for variables. Data analysis was performed by one-way analysis of variance followed by Bonferroni's post-hoc comparison. Statistical significance was set at P less than 0.05. All data were analyzed using Statview version 5.0 for Windows software.

RESULTS

Patient Characteristics

In tolerance group (Gr-Tol), all the patients discontinued IS by Kyoto University weaning protocol (*9*) or as a result of infection or side effects (Table 1). The graft survival after cessation of IS was 47±29 months. The age of the donor at Tx in Gr-Tol was comparable with that in maintenance immunosuppression group (Gr-IS), but younger than that in chronic rejection group (Gr-CR) (Gr-Tol, -IS, and -CR: 33±6, 34±6, and 44±13 yr; Gr-Tol vs. Gr-IS, NS). The recipient age at Tx in Gr-Tol was younger than those in Gr-IS and -CR (Gr-Tol, -IS, and -CR; 1±2, 4±4, and 15±17 yr; Gr-Tol vs. -IS P=0.06, Gr-Tol vs. Gr-CR P<0.0001). The recipient age at biopsy in Gr-Tol was comparable with those in Gr-IS and Gr-CR (Gr-Tol, -IS, and -CR; 12±4, 9±5, and 17±17 yr; Gr-Tol vs. Gr-IS, -CR NS). The interval between Tx and biopsy was 10±4, 4±2, and 1±2 yr in Gr-Tol, Gr-IS, and Gr-CR, respectively (Gr-Tol vs. Gr-IS, -CR P<0.0001, Gr-IS vs. Gr-CR P=0.02). In the normal liver group (Gr-NL), the donor age at zero biopsy was 33±7 yr.

Up-regulation of *Foxp3* mRNA in Rejecting Grafts and Tolerant Grafts

A trend toward increased expression of *Foxp3* mRNA was observed in the biopsy samples from Gr-Tol, compared with that in Gr-IS (Fig. 1). Of note, the expression of *Foxp3* mRNA in Gr-CR tended to be more increased, compared with that in Gr-IS and it did not differ between Gr-Tol and Gr-CR. The expression of *Foxp3* mRNA was significantly increased in all of the biopsy samples taken after Tx, compared with that in the normal liver biopsies (log10 [*Foxp3*/*HPRT*×10^6]) Gr-Tol, -IS, -CR, and -NL: 4.1±0.5, 3.6±0.7, 4.2±0.6, and 3.0±0.7; Gr-Tol vs. Gr-IS P=0.07, Gr-CR vs. Gr-IS P=0.06, Gr-Tol, -IS, -CR vs. Gr-NL P<0.01, Gr-Tol vs. Gr-CR NS) (Fig. 1).

In Tolerant Grafts, Foxp3 Protein Expressing Cells (Foxp3$^+$ Cells) are Present Within the Clusters of CD4- and CD8-Positive Cells and most Foxp3$^+$ Cells are CD4 Positive

In tolerant grafts, CD4-positive (CD4$^+$) cells and CD8-positive (CD8$^+$) cells were focally present in the portal areas, but were hardly detectable within the parenchyma or the perivenular space of the grafts (Fig. 2A,B). Cells expressing Foxp3 protein (Foxp3$^+$ cells) were present within these clusters (Fig. 2A–C).

Nine biopsy samples from tolerance group were used for immunofluorescent staining. A total of 5.1 Foxp3$^+$ cells/sample (between 1 and 12 cells) were detected in each slide of the nine samples. In total, 46 Foxp3$^+$ cells were observed in all of the nine samples analyzed. The overlay of immunofluorescent staining for CD4 versus Foxp3 and immunohistochemistry and immunofluorescent staining for CD8 versus Foxp3 revealed that 92% (42 of 46) of Foxp3$^+$ cells were CD4$^+$, whereas 8% (4 of 46) were CD8$^+$ (Fig. 2D–I).

The Number of Foxp3$^+$ Cells was Significantly Increased Compared With Grafts of LDLT Recipients Receiving Maintenance IS or Those Removed Because of Chronic Rejection

Like tolerant graft, Foxp3$^+$ cells were present within the clusters of CD4$^+$ and CD8$^+$ cells in the portal areas of grafts in recipients who were receiving maintenance IS (data not shown). The number of Foxp3$^+$ cells detected in Gr-Tol was significantly increased, compared with that in Gr-IS (Fig. 3C), although CD4$^+$ and CD8$^+$ cells were present within the portal areas to the same degree between the two groups (Fig. 3A,B). By contrast, in grafts removed as a consequence of the diagnosis of chronic rejection, no Foxp3$^+$ cell was detected in the portal areas (Fig. 3C), although CD8$^+$ cells were observed to have massively infiltrated in the portal areas and CD4$^+$ cells were present to the same degree as was observed in the normal liver (Fig. 3A,B). (Foxp3 cells in Gr-Tol, -IS, -CR, and -NL: 51.5±70.0, 23.3±31.1, 0±0, and 6.8±7.9 cells/mm^2, Gr-Tol vs. Gr-IS P=0.0292, Gr-Tol vs. Gr-CR P=0.0128, Gr-Tol vs. Gr-NL P=0.0131, Gr-CR,-IS vs. Gr-NL NS.)

DISCUSSION

Our study is the first demonstration of the presence of Foxp3$^+$ cells within grafts of LDLT recipients exhibiting operational tolerance. Similar to the evidence from rodent Tx tolerance models, some Foxp3$^+$ cells were present within grafts at least in a subset of the tolerant LDLT recipients, whereas a lesser number of Foxp3$^+$ cells were found in the functioning grafts of recipients still receiving maintenance IS or liver allografts that had been removed after a diagnosis of chronic rejection. Immunohistochemistry and immunofluorescent staining demonstrated that Foxp3$^+$ cells were present within the clusters of CD4$^+$ and CD8$^+$ cells in the portal areas of grafts and most of them were CD4$^+$. This is in accordance with the existing hypothesis that the Foxp3$^+$CD4$^+$ Tregs exert their immune suppressive property in a cytokines and cell–cell contact-dependent manner (*12*, *13*).

We and others have shown previously that up-regulation in the frequency of CD4$^+$CD25^{high+} T cells persists in the peripheral blood of tolerant liver-Tx recipients (*6*, *14*). Additionally, our previous in vitro functional study demonstrated that CD4$^+$CD25^{high+} T cells in the peripheral blood of tolerant LDLT recipients exerted their suppressive property against other T cells responding to donor alloantigens (*5*). Collectively, these data support a hypothesis that the Foxp3$^+$CD4$^+$ cells identified within the allografts of IS-free LDLT recipients may facilitate the development of operational tolerance by protecting the graft against rejection in situ. As the age at Tx was significantly younger and the interval between Tx and biopsy was significantly longer in tolerant recipients compared with immunosuppressed or rejecting recipients in the current study, further work using biopsies from LDLT patients who were matched in time after Tx is required to validate this hypothesis. We cannot entirely exclude a possibility that the highest expression of Foxp3 among the groups is merely a time-dependent consequence of inflammation and thereby, not specific to tolerance.

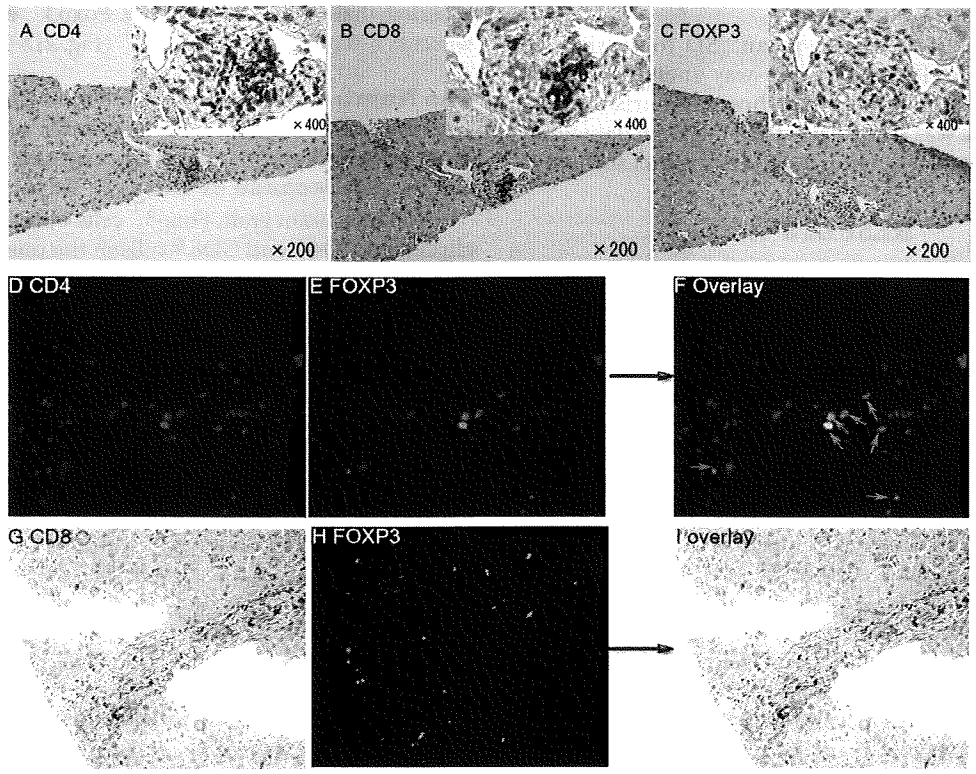

FIGURE 2. Immunohistochemistry and Immunofluorescent staining for CD4, CD8, and Foxp3 in tolerant grafts. (A) A cluster of $CD4^+$ cells was present in the portal area in a tolerant graft (brown). (B) A cluster of $CD8^+$ cells was present in the same portal area as $CD4^+$ cells in a tolerant graft (brown). (C) $Foxp3^+$ cells were present within the clusters of $CD4^+$ cells and $CD8^+$ cells in the tolerant graft (brown). Twenty-eight tolerant grafts were examined and similar results were obtained. (D and E, indicated by arrows) Immunofluorescent stainings for CD4 (red D) and Foxp3 (green E). (F) Double-positive cells are seen as greenish-yellow. (G and H) Immunohistochemistry for CD8 (brown G) and immunofluorescent staining Foxp3 (green H). (I) Double-positive cells are indicated by an arrow. Ninety-two percent (42 of 46) of $Foxp3^+$ cells were $CD4^+$, whereas 8% (4 of 46) were $CD8^+$. Original magnification ×400. Nine individuals were examined (mean; 5.1 cells, ranging 1–12 cells/sample) and totally, 46 $Foxp3^+$ cells were observed.

One discrepancy between rodents and humans was that the expression of *Foxp3* mRNA in grafts removed because of chronic rejection was as high as that in tolerant functioning liver grafts (Fig. 1). In the previous rodents study, the expression of *Foxp3* mRNA was exclusively up-regulated in tolerant grafts, that is, not in rejecting grafts (*4*). Consistent with our data, it has been reported that the expression of *Foxp3* mRNA is up-regulated within human rejecting cardiac grafts (*15–17*). However, this study did not look at the expression of Foxp3 protein by immunohistochemistry. Therefore, it was impossible to determine whether a discrepancy existed between the levels of *Foxp3* mRNA and protein. A question arises as to why the expression of *Foxp3* mRNA in human rejecting liver grafts analyzed here was up-regulated, although no $Foxp3^+$ cells were detectable in the rejecting grafts.

One explanation of the lack of correlation between the levels of *Foxp3* mRNA and Foxp3 protein is simply that it is due to the lower sensitivity of immunohistochemistry. Unlike Foxp3 in mouse, expressed exclusively by Tregs, the recent in vitro studies have reported that virtually all the human $CD4^+CD25^-$ and $CD8^+CD25^-$ non-Tregs, transiently (48–72 hr) express *Foxp3* mRNA and Foxp3 protein at a high level when they are activated, and that this is followed by gradual decrease and low plateauing with time after activation (*18, 19*). By contrast, $CD4^+CD25^+$ Tregs stably express *Foxp3* mRNA and protein at a high level (*18, 19*). We found that the number of $CD8^+$ cells was significantly increased in samples from the chronic rejection group (Fig. 3B) and that the level of *CD25* mRNA was significantly up-regulated in these samples (data not shown). These data suggest that a plenty of activated effector T cells were present within rejecting grafts. In theory, such activated effector T cells could express *Foxp3* mRNA and protein. Even if the expression of *Foxp3* mRNA by effector T cells was low at the single-cell level, a low Foxp3 expression in a large population of effector T cells could result in an increase in intragraft *Foxp3* mRNA, as was detected in the PCR analysis performed for this study (Fig. 1). However, immunohistochemistry can only detect relatively high levels of expression of Foxp3 protein because of the lower sensitivity of the technique. More recently, Baron et al. (*20, 21*) reported that the expression of Foxp3 in Tregs was stabilized by the DNA demethylation in the Foxp3 locus and that this epigenetic modification discriminated Tregs from activated conventional T cells. Therefore, in the future, investigation of DNA demethylation in the Foxp3 locus may also facilitate the discrimination of tolerance from rejection.

In reviewing the literature, we found that after the completion of transcription, posttranscriptional processes, in-

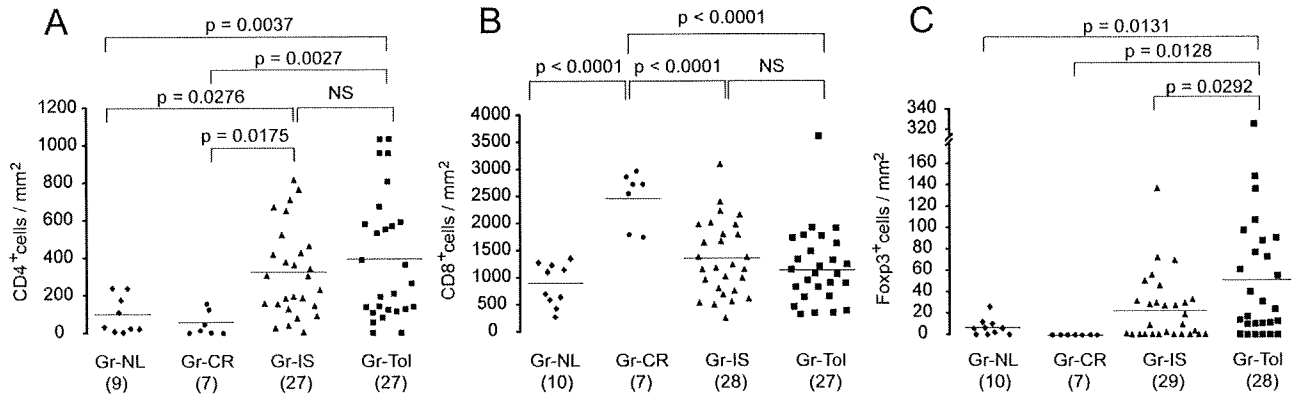

FIGURE 3. The number of CD4 positive ($CD4^+$) cells, CD8 positive ($CD8^+$) cells, Foxp3 expressing ($Foxp3^+$) cells in tolerance group (Gr-Tol), maintenance IS group (Gr-IS), chronic rejection group (Gr-CR), and normal liver group (Gr-NL). Immunohistochemistry was performed for CD4, CD8, and Foxp3. The numbers of $CD4^+$, $CD8^+$, and $Foxp3^+$ cells in all the portal areas were counted. The size of the portal area was measured by computerized calculation. Each number was divided by the size of the portal area to assess the number/mm^2. The number of examined samples is indicated in parenthesis. (A) The number of $CD4^+$ cells. The numbers of $CD4^+$ cells which infiltrated into the portal areas in tolerance and maintenance IS groups were significantly increased, compared with those in chronic rejection and normal liver groups. However, the number of $CD4^+$ cells did not differ between tolerance and maintenance IS groups, or between chronic rejection and normal liver groups (Gr-Tol, Gr-IS, Gr-CR and Gr-NL: 396.9±337.8, 320.8±238.1, 49.5±65.5 and 93.3±98.8 cells/mm^2, Gr-Tol, Gr-IS vs. Gr-CR and Gr-NL $P<0.05$, Gr-Tol vs. Gr-IS NS, Gr-CR vs. Gr-NL NS). (B) The number of $CD8^+$ cells. The number of $CD8^+$ cells which infiltrated into the portal areas in chronic rejection group was significantly increased, compared with those in tolerance, maintenance IS and normal liver groups. However, it did not differ between tolerance and maintenance IS groups, or between tolerance and normal liver groups (Gr-Tol, Gr-IS, Gr-CR and Gr-NL: 1179.5±699.5, 1346.2±688.1, 2487.5±501.7 and 871.0±389.5 cells/mm^2, Gr-CR vs. Gr-Tol, Gr-IS,and Gr-NL $P<0.0001$, Gr-Tol vs. Gr-IS, Gr-NL NS, Gr-IS vs. Gr-NL NS). (C) The number of $Foxp3^+$ cells. The number of $Foxp3^+$ cells in the portal areas was significantly increased in tolerance group, compared with those in maintenance immunosuppression, chronic rejection and normal liver groups (Gr-Tol, Gr-IS, Gr-CR and Gr-NL: 51.5±70.0, 23.3±31.1, 0±0 and 6.8±7.9 cells/mm^2, Gr-Tol vs. Gr-IS $P=0.0292$, Gr-Tol vs. Gr-CR $P=0.0128$, Gr-Tol vs. Gr-NL $P=0.0131$, Gr-CR vs. Gr-NL NS).

cluding splicing, export, RNA stability, and translation, can determine the expression profiles of proteins in eukaryotic cells, such as yeast and mammalian cells (*22–25*). The other explanation of the lack of correlation between levels of *Foxp3* mRNA and protein is that production of Foxp3 protein may be inhibited at the level of posttranscription exclusively in rejecting grafts.

This is the first report providing detailed evidence that $Foxp3^+$ cells were present in human liver allografts in operationally tolerant recipients. Our findings raised a hypothesis that the analysis of intragraft expression of Foxp3 protein detectable by immunohistochemistry may aid identifying LDLT recipients who can be successfully withdrawn from IS. However, because $Foxp3^+$ cells were absent within grafts in a subset of tolerant patients, a prospective study is mandatory to elucidate whether this hypothesis is valid. Moreover, further studies are needed to elucidate whether those $Foxp3^+$ cells function as suppressors of rejection within grafts in situ.

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綜　説

制御性T細胞による拒絶反応抑制
—免疫抑制剤からの解放—

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Regulatory T cell based transplant tolerance — freedom from immunosuppression —

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Abstract

Tolerance after clinical transplantation (Tx) is still extremely rare. However, Kyoto elective protocol enabled a substantial number of patients to weaned off immunosuppression after liver Tx. This is referred to as an immunoprivilege. Nevertheless, the operating mechanisms for liver Tx tolerance remain elusive. The authors demonstrated that regulatory T cells (Tregs) are likely to play an important role in liver Tx tolerance. In addition, we found that precursor like Tregs exist in the human peripheral blood. This can propagate upon stimulation with allo-antigen, in contrast to anergic property of Tregs. Thus, the exploitation of precursor like Tregs as a cellular source of *ex vivo* and *in vivo* expansion may lead to the widespread clinical use of Tregs for Tx.

Key words: tolerance, regulatory T cells

1. 臓器移植における免疫寛容の必要性

各種臓器移植において拒絶反応を防ぐために免疫抑制剤が投与される．しかし，免疫抑制剤による非特異的な免疫の抑制下に，患者は感染症に罹患する危険にさらされるだけではなく，免疫抑制剤そのものの副作用も大きな弊害である．したがって，免疫抑制剤を投与されなくても免疫が制御され拒絶が起こらない状態，すなわち免疫寛容が成立することは臓器を問わず患者にとって大変に好ましい[1]．

とりわけ小腸移植と肺移植の移植後の5年生存率は50％前後と他の臓器の移植より低く，**図1**に示すように肺癌を除く主要な悪性腫瘍の予後よりも悪い．したがって，これらの臓器の移植の予後を改善することは，緊急の課題である．小腸移植と肺移植では拒絶反応が強く起こるため，これまで免疫抑制剤が強力に使用されてきた．しかし，その結果，免疫抑制剤の弊害が強く現れ，患者が死亡することが多い．したがって，今後，特にこのような臓器で免疫寛容が誘導されれば患者の生存率は著しく向上する

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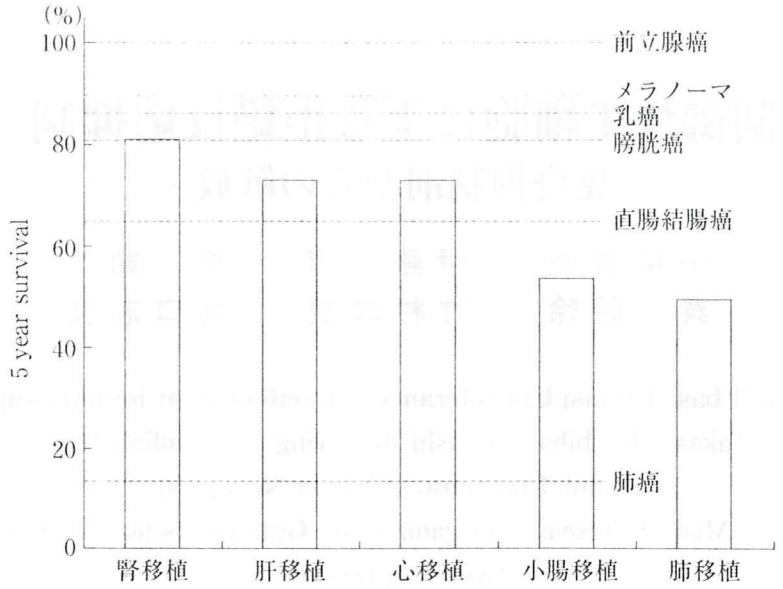

図1　5年生存率—臓器移植 対 悪性腫瘍—

小腸移植と肺移植の5年生存率は50%前後と腎臓・肝臓・心臓の移植の5年生存率と比較して悪い．また，小腸移植と肺移植の5年生存率を悪性腫瘍と比較した場合，肺癌を除く主要な悪性腫瘍(前立腺癌・メラノーマ・乳癌・膀胱癌・直腸結腸癌)の5年生存率を下回る．

2005 OPTN/SRTR Annual Report(Organ Procurement and Transplant Network) Cancer statistics 2006(American Cancer Society)

と期待される．

また，**図1**が示すように肝移植の場合，全体的な予後は良好であるが，原疾患により予後が異なる．欧米，日本どちらにおいても現在，成人の肝移植の適応の一番はC型肝硬変である．ガンマグロブリンなど特効薬のないC型肝炎ウイルスは移植を行っても，血液中に残存したウイルスが移植肝に再感染を起こすことは必発である．更に移植後の免疫抑制剤の使用により，ウイルスの増殖は促進され，移植肝のウイルスによる変化が急速に進行すると考えられている[2]．事実，移植後，短期間で移植肝が肝硬変となることはまれではない．したがって，C型肝硬変で移植を受けた患者で仮に免疫寛容が成立すれば移植肝のウイルスによる変化を遅らせることができるのではないかと予想される．

2. 肝移植における免疫寛容

マウス，ラット，ブタを用いた動物実験では，ドナーとレシピエントの主要組織適合性抗原の違いを超えて移植肝が免疫抑制剤を使用しなくても拒絶されない場合がある[3-5]．ところが，同じ系のドナーとレシピエントの組み合わせで腎臓や心臓の移植をすれば拒絶が起こる．この現象は肝臓のimmuno-privilegeと呼ばれる．同様に，ヒトの腎臓移植や心臓移植で免疫寛容が成立することは例外的である．しかし，京都大学で生体肝移植を受けた小児の患者においては，免疫抑制剤によるEBウイルスの再燃や，免疫抑制剤の副作用が原因で，免疫抑制剤を中止したところ拒絶が起きず免疫寛容の成立した患者がみられた．これらの経験から，京都大学では小児の患者に限って肝機能の安定している場合には，計画的に免疫抑制剤を中止してきた[6]．これまで80例以上の患者に免疫寛容が成立した(小児の肝移植患者は総計およそ600例)．しかし，このimmuno-privilegeという現象を十分に説明する機序は明らかにされていない．

3. 肝移植後の免疫寛容における制御性T細胞の役割

CD4陽性CD25強陽性のフェノタイプを有す

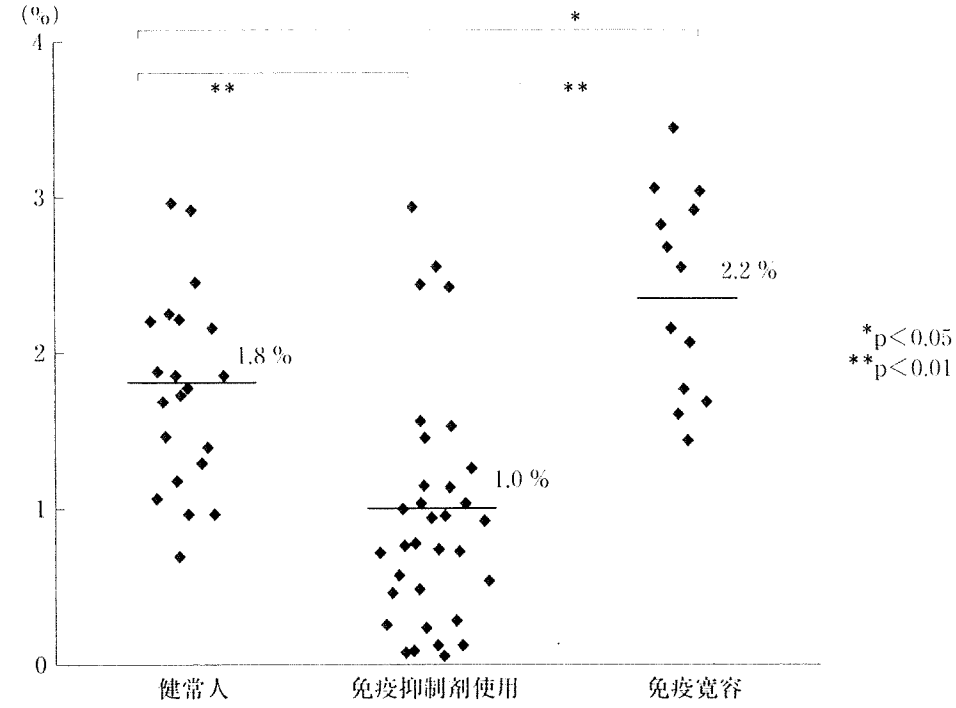

図2　免疫寛容患者におけるCD4陽性CD25強陽性細胞cellsの増加

フローサイトメータにて京都大学で生体肝移植を受けた後に免疫寛容となった患者，免疫抑制剤が必要な患者，同年代の健常人の3群で，制御性T細胞の分画とされるCD4陽性CD25強陽性細胞の割合が検討された．免疫寛容となった患者のCD4陽性CD25強陽性細胞の割合はほかの2群に比較して有意に増加していた．

る制御性T細胞は，生理的には自己免疫疾患の発症を抑制しているが，臓器移植後の免疫寛容にも重要な役割を果たしているとの知見が動物実験のレベルで集積されつつある[7]．京都大学で生体肝移植後に免疫寛容の成立した小児患者の末梢血から単核球を分離し，フローサイトメータにてリンパ球の中のCD4陽性CD25強陽性細胞の割合を検討した．**図2**に示すように，現在免疫抑制剤を使用中の患者，同年代の健常人と比較して，免疫寛容の患者のCD4陽性CD25強陽性細胞の割合は有意に増加していた[8]．また，免疫寛容の患者のCD4陽性CD25強陽性細胞とCD4陽性CD25陰性細胞を細胞分離装置にて分離して，CD4陽性CD25陰性細胞の数を一定にして，そこに混在させるCD4陽性CD25強陽性細胞の量を変えて，ドナー，3rdパーティの単核球に対するリンパ球混合試験を施行した．**図3**に示すようにCD4陽性CD25陰性細胞とCD4陽性CD25強陽性細胞を1：1に混合するとドナー，3rdパーティに対するCD4陽性CD25陰性細胞の増殖はCD4陽性CD25強陽性細胞に抑制されている．CD4陽性CD25陰性細胞に対してCD4陽性CD25強陽性細胞の割合を減らしていくと，CD4陽性CD25強陽性細胞の割合が1/9となった段階で3rdパーティに対する反応の抑制は解除される．一方，ドナーに対してはCD4陽性CD25強陽性細胞の割合が1/9の段階では抑制が効いており，1/27となったときに初めて抑制が解除される．これらの所見は，CD4陽性CD25強陽性細胞の抑制効果が，3rdパーティの抗原よりドナーの抗原に対して強いことを意味しており，制御性T細胞のドナー抗原特異的免疫抑制効果を示すものである．制御性T細胞のドナー抗原特異的免疫抑制効果は調べられた5人の免疫寛容患者のうち4人で認められた[9,10]．以上の結果から，生体肝移植後の免疫寛容のメカニズムはドナー抗原反応性のエフェクター細胞が除去されるdeletional toleranceではなく，患者のリンパ球のレパートリーにドナー抗原反応性のエフェク

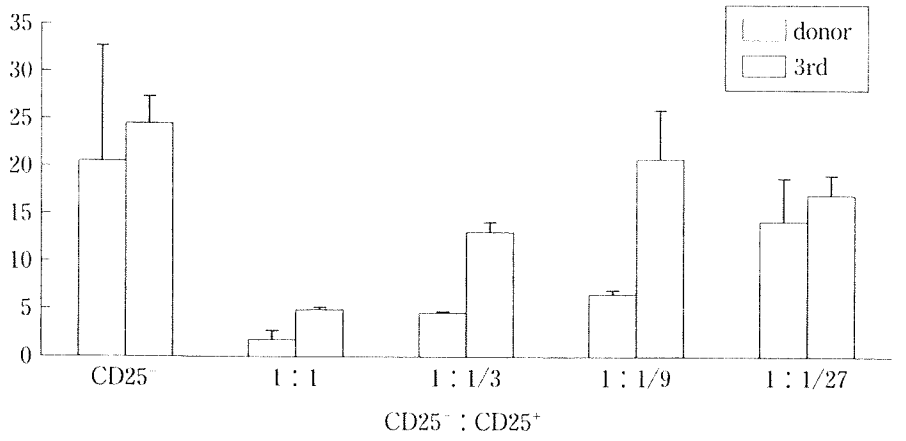

図3 免疫寛容患者における$CD4^+CD25^{hi}$ cellsによるドナー抗原に特異的な免疫制御

免疫寛容の患者の末梢血から，CD4陽性CD25陰性(CD25⁻)細胞とCD4陽性CD25陽性($CD25^+$)細胞を分離して，以下の割合でCD25⁻細胞と$CD25^+$細胞を混合した．

CD25⁻：CD25⁻：$CD25^+$=1：0

1：1; CD25⁻：$CD25^+$=1：1

1：1/3; CD25⁻：$CD25^+$=1：1/3

1：1/9; CD25⁻：$CD25^+$=1：1/9

1：1/27; CD25⁻：$CD25^+$=1：1/27

おのおのに，ドナーの単核球(□)，3rdパーティの単核球(□)を添加して，リンパ球混合試験を行った．1：1，1：1/3の段階ではドナー，3rdパーティに対する反応はともに抑制されているが，1：1/9の段階では3rdパーティに対する反応性の抑制が解除される．一方，ドナーに対しては，1：1/9の段階では反応が抑制されており，1：1/27の段階で初めて解除される．これらより，CD25⁻細胞の抑制は3rdパーティに対して比較して，ドナーに対してより強いことが示された．5人の免疫寛容の患者が調べられ，うち4人が同様の結果であった．

ター細胞が存在しており，このエフェクター細胞が末梢で抑制されるnon-deletional toleranceに制御性T細胞が重要な働きをしている場合が多いと考えられる．それでは，制御性T細胞によるドナー抗原反応性のエフェクター細胞の抑制は末梢のどこで起こるのであろうか？ 著者らはプロトコールバイオプシーにより免疫寛容の患者から得られた移植肝組織内における*FOXP3*(制御性T細胞特異的遺伝子)，T細胞関連の遺伝子(CD4，CD8，CD25)の発現をリアルタイムPCRの手法を用いて調べた．各種遺伝子の発現量は，慢性拒絶で喪失した移植肝，正常肝を代表する移植時にドナーから取られたゼロバイオプシーの組織の遺伝子発現と比較された．**図4**に示すように*FOXP3*の発現は免疫寛容，慢性拒絶のいずれにおいても正常肝に比較して増加していた．ただし，慢性拒絶の移植肝では*FOXP3*の発現の増加に伴い，CD4，CD8，CD25の発現の増加が認められたが，一方，免疫寛容の移植肝におけるこれらの遺伝子の発現は正常肝と差がなかった．これらの結果から，免疫寛容の移植肝内には制御性T細胞が存在し$CD4^+$および$CD8^+$エフェクター細胞の増殖・浸潤と活性化を移植肝の局所で抑制している可能性が強く示唆される．ところが，慢性拒絶の移植肝内では，制御性T細胞が存在するにもかかわらず，$CD4^+$および$CD8^+$エフェクター細胞の増殖・浸潤と活性化が抑制されていないため拒絶を起こしたと考えられる．すなわち，慢性拒絶では，制御性T細胞の制御能を上回るエフェクター細胞の攻撃が移植肝局所で起こっていると考えられる[11]．このように，著者らは，生体肝移植の免疫寛容はnon-deletional toleranceの範疇に属し，その成立に制御性T細胞が重要な役割を果たしているらしいことを突き止めたが，制御性T細胞のメカニズムが肝臓のimmuno-privilegeを説明するのに有力な因子であるのかどうか，今後の検討を要する．

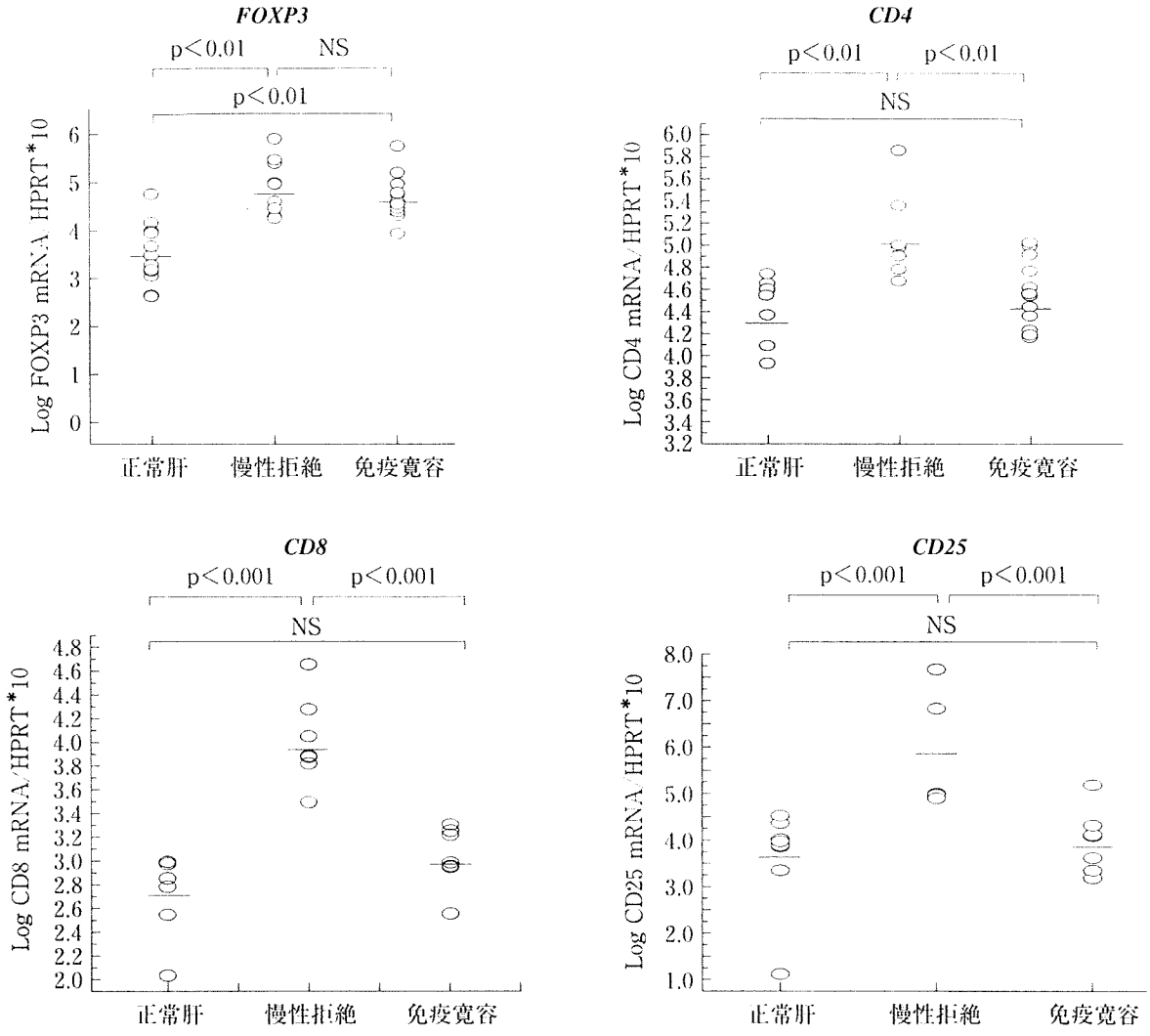

図4　免疫寛容移植肝における*FOXP3*(T細胞関連遺伝子)の発現

免疫寛容の移植肝，慢性拒絶の移植肝における*FOXP3*の発現は，ともに正常肝に比べて上昇していた．慢性拒絶では，*FOXP3*の発現の上昇に伴い*CD4*，*CD8*，*CD25*の発現がすべて正常肝に比べて増加していた．一方，免疫寛容では，*FOXP3*の発現のみが増加しており，*CD4*，*CD8*，*CD25*いずれの発現も増加していなかった．

FOXP3：制御性T細胞のマスター・コントロール遺伝子．

4. 制御性T細胞による免疫寛容の誘導を目指して

冒頭で触れたように，臓器のいかんを問わず，積極的な免疫寛容の誘導は重要な課題である．しかし，どのような手段を用いればそのことが可能かという質問に対する回答は得られていない．著者らは，既に自然に免疫寛容の成立した京都大学の患者の免疫学的な特徴を明らかにすることが，上記の質問に対する回答を得るうえで重要であると考えている．先に示したデータによれば，ヒトの臓器移植後の免疫寛容の成立に制御性T細胞が重要な働きをしている可能性が高い．そうであれば，制御性T細胞をうまく利用して免疫寛容を誘導することができるのではないか？　具体的な方法としては著者らは少なくとも次の2つのストラテジーがあると考えている．①患者の末梢血から分離した細胞からドナー抗原に対して抑制効果をもつ制御性T細胞を培養・増殖させ，再度患者の体内に戻す(細胞養子免疫)．②制御性T細胞が体内で増殖するために最適化された免疫抑制療法を考案す

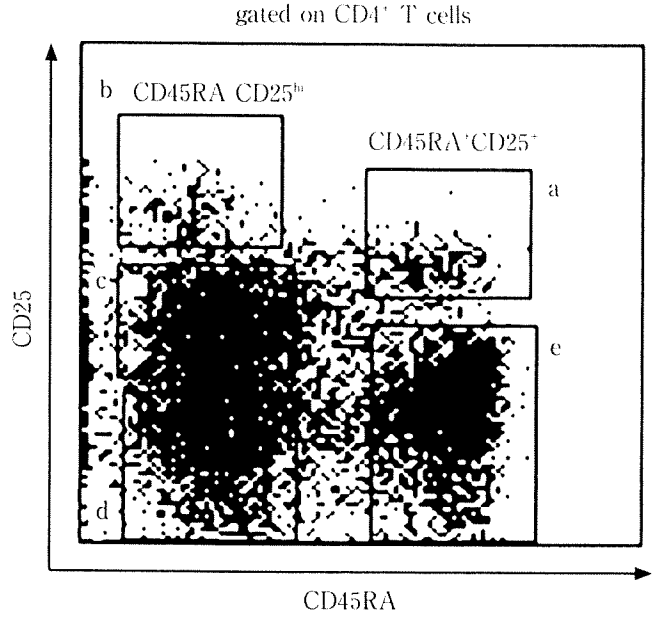

図5 制御性T細胞(Tregs)はメモリー表現系を有する

CD4でゲートした細胞を，CD25とCD45RAで展開したフローサイトメータの図である．bに示される，CD45RA陰性CD25強陽性の細胞(CD45RA$^-$ CD25^{hi})が制御性T細胞とされている．aに示されるCD45RA陽性CD25陽性細胞(CD45RA$^+$CD25$^+$)とは明確に区別される．

る．あるいは，制御性T細胞の体内での増殖を促す新規薬剤を開発する[12]．以上のことを実現するため，著者らの研究室ではヒト制御性T細胞の試験管内での培養系を確立するための研究を進めてきた．

ヒト制御性T細胞は胸腺で作られ末梢へ分布してその機能を果たしていると考えられているが，その分化様式は不明である．ヒト制御性T細胞はそれ自体不応答性であるにもかかわらずどのようにして体内で増加するのであろうか？

ヒト制御性T細胞は**図5-b**に示すように，CD4陽性CD25陽性細胞の中でもメモリー表現系を有する分画であり，CD45RO陽性またはCD45RA(ナイーヴ表現系)陰性でCD25を強く発現する(CD45RA$^-$CD25^{hi})．一方，**図5-a**が示す細胞は，CD4陽性CD25陽性の中でもCD45RA(ナイーヴ表現系)が陽性(CD45RA$^+$CD25$^+$)で制御性T細胞(CD45RA$^-$CD25^{hi})とは異なる[13]．これまで，CD45RA$^+$CD25$^+$細胞とCD45RA$^-$CD25^{hi}制御性T細胞との関係は不明であった．ところが，著者らは，**図6**に示すようにCD45RA$^+$CD25$^+$細胞が制御性T細胞に特異的に発現する*FOXP3*を強発現している事実を突き止め，この細胞の性質を詳細に調べた．**図7**に示すようにCD45RA$^-$CD25^{hi}制御性T細胞とは異なり，CD45RA$^+$CD25$^+$細胞はアロ抗原の刺激により弱いながらも増殖活性を示した．また，全CD4$^+$細胞の増殖に弱い抑制効果を示した．CD45RA$^+$CD25$^+$細胞はアロ抗原の存在下にIL-2を添加すると強い増殖を示し，40日の間に100倍以上に増加した．CD45RA$^-$CD25^{hi}制御性T細胞とCD45RA$^+$CD25$^+$細胞をそれぞれHLAA2$^-$/A24$^+$および，HLAA2$^+$/A24$^-$の異なる2人の健常人から分離して1：1に混合してCD3・CD28抗体の刺激下にIL-2を添加して14日間培養したところ**図8**に示すようにCD45RA$^+$CD25$^+$細胞のみ増殖し，CD45RA$^-$CD25^{hi}制御性T細胞は増殖しなかった．**図9**に示すようにCD45RA$^+$CD25$^+$細胞は増殖の後では，不応答性と増殖に際して刺激に用いたアロ抗原に，特異的な強い免疫抑制効果を獲得した．また，増殖した細胞株のフェノタイプはCD45RO陽性であった．**図10**に示すように小児の健常人においてCD4$^+$細胞の中のCD45RA$^+$