Value of FOXP3 Expression in Peripheral Blood as Rejection Marker After Miniature Swine Lung Transplantation

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Background: Outcome for highly immunogenic lung transplantation remains unsatisfactory despite the development of potent immunosuppressants. The poor outcome may be the result of a lack of minimally invasive methods to detect early rejection. There is emerging clinical evidence that, paradoxically, expression of forkhead box P3 (FOXP3, a specific marker for the regulatory T cells) is upregulated within rejecting grafts.

Methods: Orthotopic lung transplantation was performed using miniature swine without immunosuppression. Rejection was monitored by chest radiography and open lung biopsy. Expressions levels of *FOXP3, perforin, Fas-L* and *IP-10* mRNA were quantified in the peripheral blood. In addition, rescue immunosuppressive therapy (steroid plus tacrolimus) was administered on post-operative day (POD) 4 or 6.

Results: Early rejection was detected by open lung biopsy, but misdiagnosed by chest radiography on POD 4. Expression of *FOXP3* in the peripheral blood reached its highest value as early as POD 4, followed by a decline. Such an increase of *FOXP3* was not observed in recipients given high-dose tacrolimus. Neither *perforin, Fas-L* or *IP-10* in the peripheral blood exhibited significant fluctuations in the early phase of rejection. Rescue immunosuppressive therapy from POD 4, when peak *FOXP3* was seen, prolonged graft survival (27.2 days, versus 9.1 days without immunosuppression, $p < 0.001$), in contrast to POD 6, when rejection was suspected by chest radiography (11.5 days, p = not statistically significant [NS]).

Conclusions: In a miniature swine lung transplantation model, the *FOXP3* mRNA level in the peripheral blood was upregulated at an early phase of rejection. The clinical implication of this finding remains to be elucidated. J Heart Lung Transplant 2008;27:1293–301. Copyright © 2008 by the International Society for Heart and Lung Transplantation.

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In the era of potent immunosuppressants, the incidence of acute rejection has been dramatically reduced after clinical organ transplants.[1] The 5-year survival rate has reached 70% to 80% for most solid organ transplants.[2–4] Unfortunately, improvement in survival rate for lung transplantation has not been as dramatic as compared with other organs, with a 5-year survival rate of <50%.[5] This is due to the fact that the lung, which is the most immunogenic organ to transplant, can generate extremely vigorous rejection.[6,7] Nevertheless, a reliable, non-invasive method to detect early rejection has not been established,[8–12] although some approaches have been proposed.[13,14] In clinical lung transplantation, profound immunosuppression is uniformly administered, taking precautions against rejection with attending complications.[5] Infections represent the leading cause of death in the early post–lung transplantation period.[5] If a reliable, non-invasive method becomes available, then clinicians could decrease the baseline immunosuppression and better tailor the immunosuppression for the individual patient, which may decrease the rate of death from infections.

Studies in mice demonstrated that forkhead box P3 (Foxp3) was a master control gene, exclusively ex-

pressed by regulatory T cells (Tregs) (i.e., not expressed by non-Tregs).[15-17] In some rodent transplant models, high levels of *Foxp3* mRNA and a substantial number of Foxp3 protein–expressing cells were found in tolerant grafts, but not in rejecting grafts.[18-20] Thus, in rodents, *Foxp3* mRNA and Foxp3 protein may be reliable biomarkers of tolerance. However, it has been reported that *FOXP3* mRNA and protein levels are paradoxically increased within rejecting cardiac grafts in humans or grafts and urine specimens in renal allograft recipients.[21-24] Therefore, the question arises as to whether FOXP3 is a biomarker of tolerance, or whether FOXP3 can also be useful to detect rejection.

We quantified the *FOXP3* mRNA level in the peripheral blood during acute rejection using a miniature pig lung transplantation model. The *FOXP3* mRNA level in the peripheral blood was upregulated at an early phase of rejection. It must be determined whether measurement of peripheral blood FOXP3 level would be useful as a minimally invasive method to detect the early phase of rejection in clinical lung transplantation.

METHODS
Animals

Male or female swine leukocyte antigen (SLA)-unknown Clawn miniature swine (22 to 30 kg, 6 to 10 months old), were purchased from the Japan Farm CLAWN Institute (Kagoshima, Japan). The pigs were raised in conventional housing facilities and free of foot-and-mouth disease, hog cholera, vesicular disease, African swine fever and hepatitis E. All animal care and procedures were in compliance with the "Principles of Animal Care," formulated by the animal facility of Kyoto University.

Experimental Groups

Five groups were studied that were differentiated according to the type of operation and whether and when immunosuppressants were administered:

Immunosuppression-free rejection group (*n* = 10): Lung transplantation, no immunosuppression.

Clamp reperfusion group (*n* = 4): Clamp reperfusion, no transplantation, no immunosuppression.

Induction immunosuppression group (*n* = 3): Lung transplantation, high-dose tacrolimus from post-operative day (POD) 1 to 12.

POD 4 rescue immunosuppressive therapy group (*n* = 6): Lung transplantation, rescue immunosuppressive therapy from PODs 4 to 9.

POD 6 rescue immunosuppressive therapy group (*n* = 4): Lung transplantation, rescue immunosuppressive therapy from PODs 6 to 11.

Isolation of PBMCs

Blood samples were collected in heparinized test tubes. The peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation.[25]

Mixed Lymphocyte Culture

For responders, $CD4^+$ cells were positively selected on a LS separation column by the MACS system (magnetic cell sorting; Miltenyi Biotech).[26] The medium used for culture was RPMI with 10% fetal calf serum and 2-mercaptoethanol. Cells were seeded in 96-well microtiter plates in replicates of four at a concentration of 5×10^4 cells/well, 1×10^5 cells/well-irradiated (25-Gy) stimulator PBMCs were added to the culture for 96 hours, and ^{3}H-thymidine uptake was determined using standard techniques.[27] For lung transplantation, reactive donor–recipient pairs were used. Reactivity was defined as a tritiated thymidine incorporation value (cpm) of >5,000.

Lung Transplantation

Orthotopic left lung transplantation was performed as previously described.[28,29] ET-Kyoto solution (Otsuka Pharmaceutical, Tokushima, Japan [not for sale]) was used to flush and preserve the graft.[30] During the operation, two catheters were placed into the bilateral external jugular veins. One catheter was used to administer fluid, antibiotics and immunosuppressants, and the other was to obtain blood.

Clamp Reperfusion Model

After heparinization (300 U/kg), the vessels of the left lung were clamped. After the pulmonary artery was catheterized and the pulmonary vein was incised for fluid drainage, the lung was topically cooled with iced saline and flushed in situ with 4 liters of cold ET-Kyoto solution. The lung was left in situ and cooled for 1.5 hours (comparable to the cold ischemic time of lung transplantation) with iced saline. The iced saline was then removed and the lung was kept at room temperature for 1.5 hours (comparable to the warm ischemic time of lung transplantation). During this time, the catheter for the artery was removed and the incisions in the artery and the vein were closed by sutures. Finally, the vessels were declamped and the lung was reperfused.[31]

Monitoring Rejection

Chest radiography was performed on POD 3 to 7, and 10. The findings were scored on a grade of 0 to 4, based on our grading system (Table 1). Under general anesthesia, open lung biopsy was performed on PODs 4, 7 and 10. The specimens were evaluated using conven-

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

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

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]

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
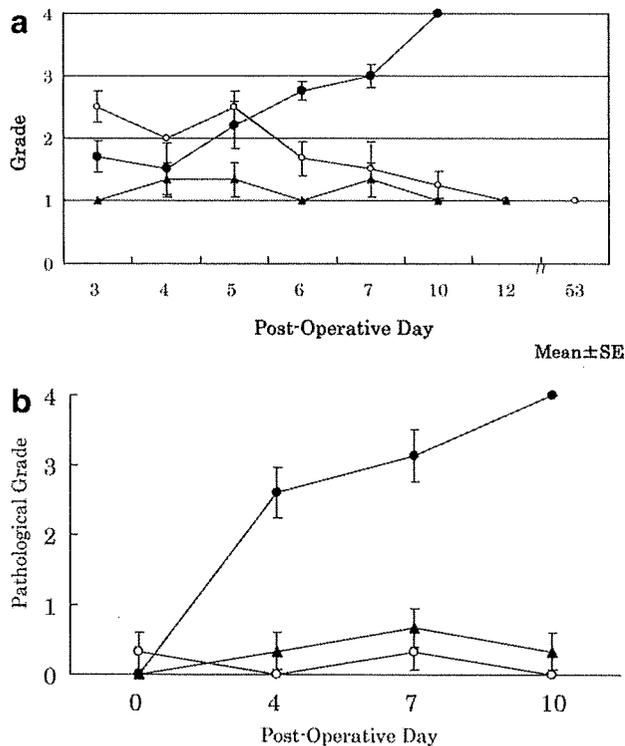
Figure 1. Radiologic and pathologic findings. Chest radiography was evaluated according to our grading system (see Table 1). Filled circle: immunosuppression-free rejection group ($n = 10$); open circle: the clamp reperfusion group ($n = 4$). From post-operative day (POD) 3 to POD 5, the grade between 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).

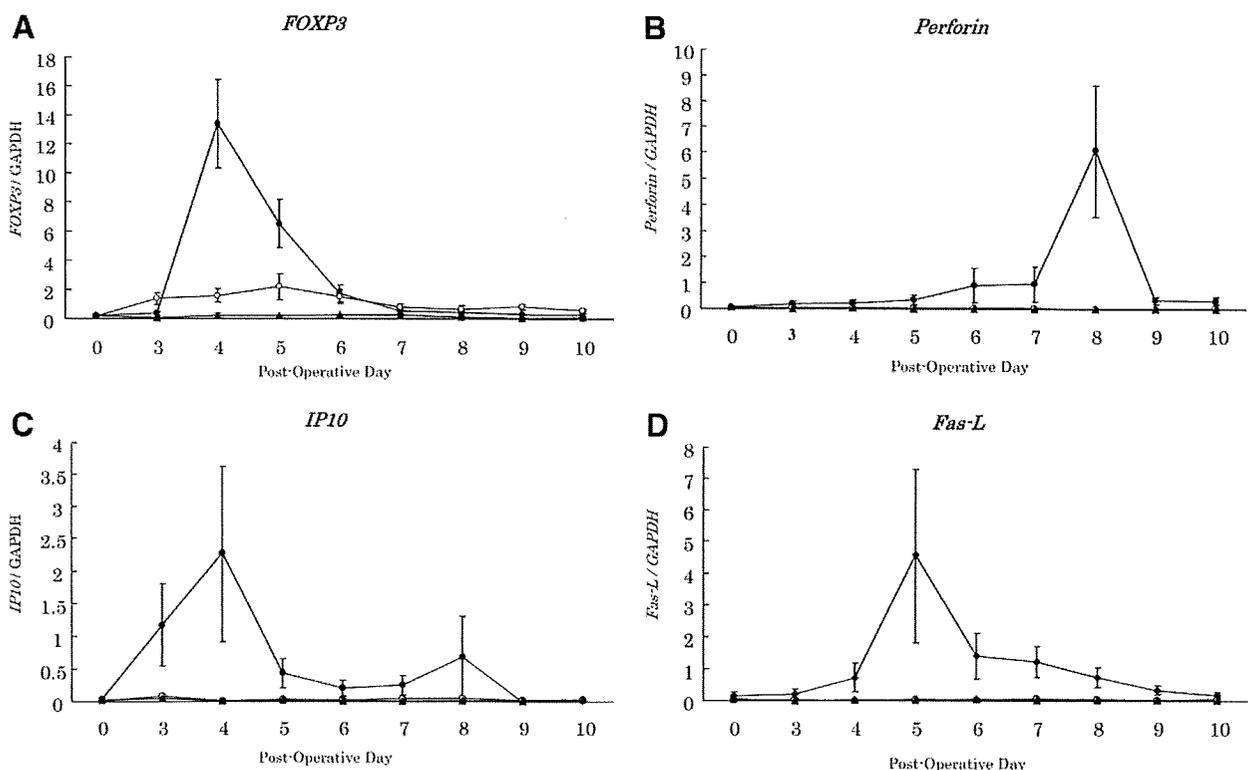## *FOXP3* and Conventional Rejection Markers in Peripheral Blood

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 $\pm$ 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.
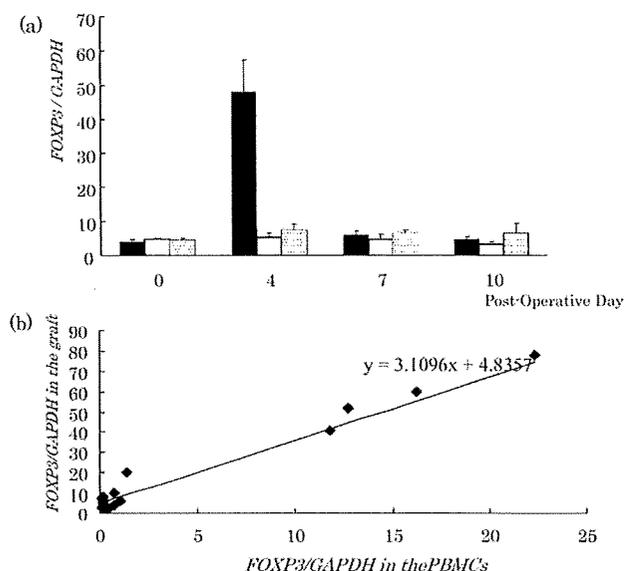
Figure 3. Intragraft *FOXP3* mRNA expression and its correlation with *FOXP3* mRNA expression in the peripheral blood mononuclear cells (PBMCs). (a) The tissues of grafts and lungs (2 mm × 2 mm × 2 mm) were obtained at transplant, at clamp reperfusion, at open lung biopsy, or at autopsy on PODs 0, 4, 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*
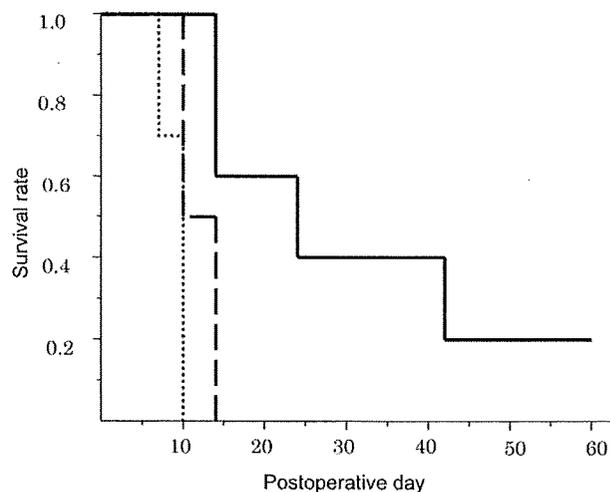
Figure 4. Rescue immunosuppressive therapies from POD 4 vs POD 6. Rescue immunosuppressive therapy (steroid + tacrolimus) was started on POD 4, when the immunosuppression-free rejection group exhibited the highest level of peripheral *FOXP3* mRNA level (the POD 4 rescue immunosuppressive therapy group) 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.

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*).

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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, Gr-Tol vs. -CR, 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, Dakocytoma-

tion) 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).
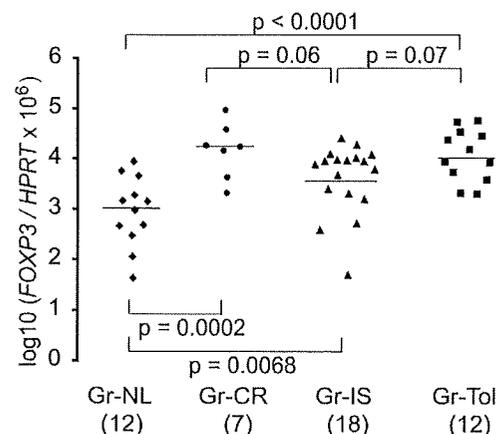
FIGURE 1. The expression of *Foxp3* mRNA in tolerance group (Gr-Tol), maintenance IS group (Gr-IS), chronic rejection group (Gr-CR), and normal liver group (Gr-NL). The level of *Foxp3* mRNA was quantified by RT-PCR in 12, 18, and 7 frozen biopsy samples from the following groups, operational tolerance (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*×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×10⁶]) 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/mm², 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⁺CD25ʰⁱᵍʰ⁺ T cells persists in the peripheral blood of tolerant liver-Tx recipients (*6, 14*). Additionally, our previous in vitro functional study demonstrated that CD4⁺CD25ʰⁱᵍʰ⁺ 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.
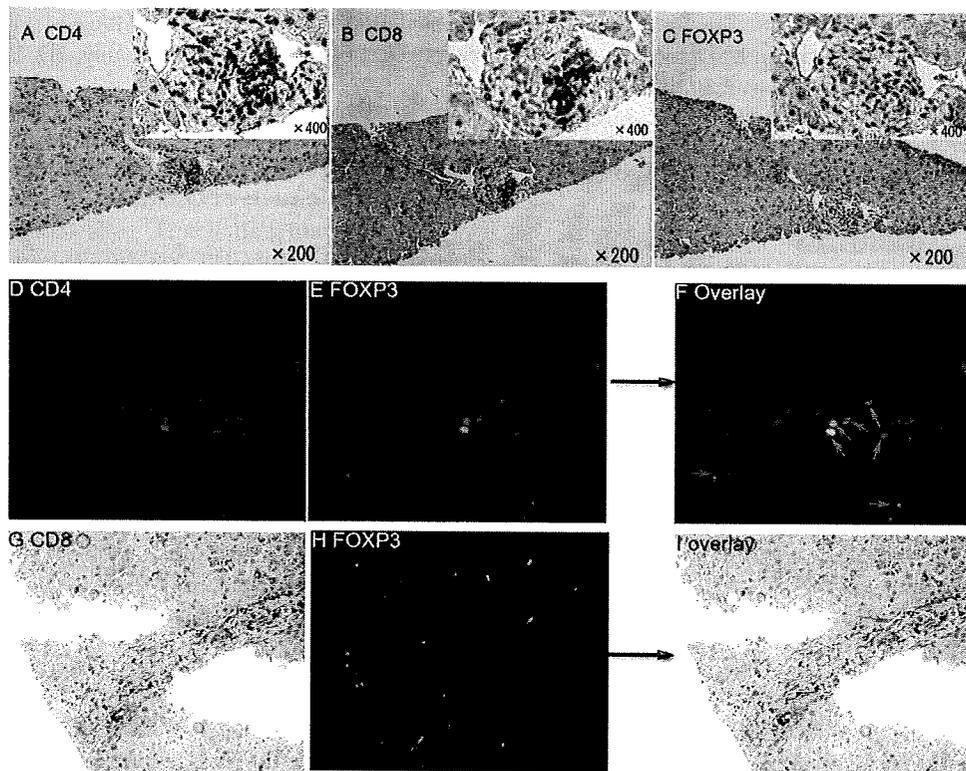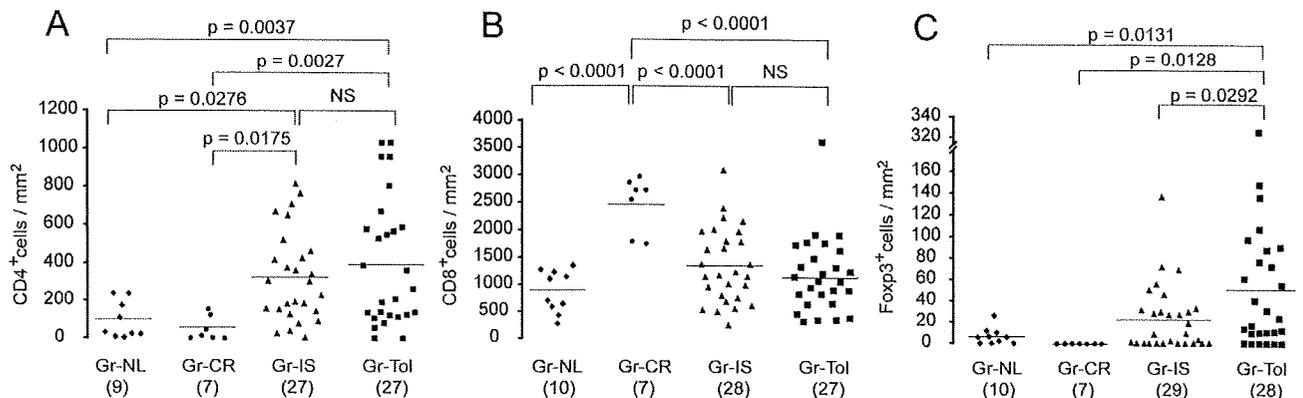
FIGURE 2. Immunohistochemistry and Immunofluorescent staining for CD4, CD8, and Foxp3 in tolerant grafts. (A) A cluster of CD4^{+} cells was present in the portal area in a tolerant graft (brown). (B) A cluster of CD8^{+} cells was present in the same portal area as CD4^{+} cells in a tolerant graft (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 activa-

tion (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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