Diverse Effects of FK506 on the apoptosis of hepatocytes and infiltrating lymphocytes in an allografted rat liver.

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ABSTRACT

Background. The current study investigated whether FK506 (FK) regulates the apoptotic systems in allografted rat liver and the contribution of Fas/Fas-ligand system and Bcl-2 family during acute rejection.

Materials and Methods. The recipients were divided into three groups, the allo, the allo-FK and the syn group. Each rats were euthanized 1, 3, 5, and 7 days after OLT. The apoptotic activity was explored using TUNEL assay. The expression of Fas/Fas-ligand and Bcl-2/Bax in the grafted livers was investigated by Western blotting and immunohistochemistry.

Results. The apoptotic index (AI) of hepatocytes in the allo-FK group was less than that in the allo group. Fas in the allo group was more intense than that in the allo-FK group in the periportal areas on days 1 and 3, while Bcl-2 in the allo group was less intense than that in the allo-FK group in the pericaval areas at all time-points after OLT.

Conclusion. FK provides beneficial anti-apoptotic effects on hepatocytes in the grafted rat livers through both the down-regulation of Fas expression in the periportal areas and the up-regulation of Bcl-2 expression in the pericaval areas.

Key words: FK506; apoptosis; liver transplantation; Fas; Fas ligand; Bax; Bcl-2.

INTRODUCTION

Apoptosis occurs in various organs to maintain normal development and homeostasis. Moreover, apoptosis is involved in several human diseases, including autoimmune diseases, viral hepatitis, hepatocellular carcinoma, ischemia-reperfusion injury (I/R injury), and gastro-intestinal malignancies (1-5). In the case of liver transplantation, apoptosis in liver allografts was first reported in a porcine model in 1974 (6). Recent studies suggest that apoptosis of infiltrating lymphocytes plays an important role in acquiring spontaneous tolerance to liver allografts (7, 8).

FK506 (FK), like cyclosporine A, is a widely accepted immunosuppressant. The use of FK is well standardized in liver transplantation. It belongs to the family of calcineurin inhibitors, which reduces IL-2 production through NF-AT in T lymphocytes (9, 10). In addition, FK enhances apoptosis of activated T cells *in vivo* (11).

Apoptosis is regulated by several factors including the Fas/Fas ligand (FasL) system, and Bcl-2 related proteins. However, in liver transplantation, the relationship between FK and apoptosis associated factors remains unclear, especially in hepatocytes.

The present study investigated the effects of FK on the induction of apoptosis in hepatocytes and infiltrating lymphocytes, and their correlation with the expression of those apoptosis-related molecules using rat models of allogeneic OLT.

MATERIALS AND METHODS

Animals

Male Dark Agouti (RTI^a), and Lewis (RTI^l) rats were purchased from SLC (Shizuoka, Japan) and Charles River Japan (Atsugi, Japan), respectively. All animals were housed in a climate controlled room with a 12 hr light, 12 hr dark schedule, and maintained in the pathogen-free facility at the Nagasaki University School of Medicine. They were provided rat chow (Oriental Koubo Kougyo, Tokyo, Japan) and tap water ad libitum, and were used as donors or recipients for OLT at a weight 250-300 g All experiments were performed in accordance with the University of Nagasaki Research Animal Resources guidelines.

Orthotopic liver transplantation

Donor livers were harvested with flushing by 5 ml of lactated Ringer's solution and preserved in a bath of lactated Ringer's solution at 0-4℃ for 30 min. The liver was transplanted orthotopically, using the cuff technique for the anastomosis of the portal vein, infrahepatic vena cava and bile duct. The hepatic artery was not reconstructed, as previously described (12). The transplant procedures were finished within 15 min in all instances.

Experimental design

The recipients were divided into three groups, 1) allo group; allogeneic combination without any immunosuppressant (DA to LEW, n=20), 2) the allo-FK group; allogeneic combination with FK (0.2 mg/kg per day) in a subcutaneous injection (s.c.; DA to LEW, n=20), 3) syn group; syngeneic combination (LEW to LEW, n=12). In the allo-FK group, FK was dissolved in normal saline at a concentration of 0.1 mg/ml and kept at 4℃. Other groups received normal saline only at the same amount used for the allo-FK group. Five animals from each group were euthanized and the grafted liver was harvested 1, 3, 5, and 7 days after liver transplantation, respectively. Harvested livers were frozen in liquid nitrogen for protein extraction, or fixed in 10% formalin. The tissues were embedded with paraffin, sectioned at 4 μ m and placed on silane-coated glass slides for hematoxylin and eosin (H.E.) staining, immunohistochemistry, and the DNA double-strand break assay. Peripheral blood was obtained for measurements of the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T.Bil) levels.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL)

TUNEL was performed to detect DNA fragmentation according to the method of Hakuno N. et al. (13), using proteinase K (Wako Pure Chemicals, Osaka, Japan),

terminal deoxynucleotidyl transferase (TdT) buffer (Roche Diagnostics, Mannheim, Germany), and biotin-16-dUTP (Roche Diagnostics). Lastly, the sites of horseradish peroxidase (HRP) were visualized by 3, 3‘-diaminobenzidine tetrachloride (DAB; Wako Pure Chemicals, Osaka, Japan) and H_2O_2 with nickel and cobalt enhancement (14) for 5 min. Thymidine triphosphate (TTP) was used instead of biotin-16-dUTP as a negative control.

Apoptotic index

The apoptotic index (AI) was calculated in each grafted liver. More than 5,000 cells were counted in one section of the grafted liver and the number of TUNEL positive hepatocytes or lymphocytes per 1,000 each cells was expressed as an AI.

Western blot analysis

Total proteins were extracted from the frozen livers in liquid nitrogen according to the previously described method (13, 15). Briefly, the tissue specimens were homogenized in a lysis buffer (5 mM phosphate buffer with 0.1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 1 μ g/ml leupeptin, 1 μ g/ml chymostatin pH 7.2) at 0-4℃ using a Potter type homogenizer. The concentration of protein samples was measured by the Bradford method (16), using bovine serum albumin as a standard. Protein extracts were mixed with a loading buffer (0.25 M Tris-HCl, 2% SDS, 30%

glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue pH 6.8) at the rate of 1:1 and then heated for 5 min at 95℃. The sample proteins (10 μ g) per lane were separated in 10-20% gradient gels by SDS-PAGE, as described by Laemmli (17). Proteins were electrophoretically transferred onto polyvinylidine difluoride membranes (Immobilon P, Millipore Corp, Bedford, MA) by the semi-dry method using a blotting apparatus. Then, the membranes were soaked in 5% nonfat dry milk in PBS overnight at 4℃. The washed membranes were incubated for 2 hr with a 1:800 dilution of rabbit anti-Fas (P4; 13) or anti-FasL (P5; 13), or anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA) antibody at room temperature. After washing in T-PBS three times (10 mM phosphate-buffered saline with 0.1% Tween 20 pH 7.4), the membranes were incubated with a 1:1000 dilution of HRP conjugated goat anti-rabbit IgG F(ab')2 (MBL, Nagoya, Japan) as a second antibody for 1 hr. The membranes were washed three times with T-PBS and then were visualized by DAB and H_2O_2 with enhancement of nickel and cobalt for 5 min (14).

Immunohistochemical staining

Immunohistochemical staining of Fas, FasL, Bcl-2, and Bax were performed, according to the previous paper (18). The primary antibodies were as follows; the polyclonal antibodies for anti-mouse Fas (diluted 1:200, P4), anti-rat FasL (diluted

1:100, P5), anti-Bcl-2 (diluted 1:200), and anti-Bax (diluted 1:200) derived from rabbits described above (13). In the case of Bcl-2 staining, the sections were immersed in 0.01 M citrate buffer (pH 6.0), and autoclaved at 121℃ for 10 min (19). The sections were reacted with 500 μg/ml normal goat IgG in 1% BSA/PBS at room temperature for 1 hr, and were incubated with the primary antibody solution overnight for anti-Fas and anti-Bcl-2 antibodies, or for 2 hr for anti-FasL and anti-Bax antibodies. All slides were washed with 0.075% Brij 35 in PBS three times, and were incubated with HRP-goat anti-rabbit IgG (1:200) diluted in 1% BSA/PBS at room temperature for 1 hr. HRP was added for visualization with DAB and H_2O_2 for Fas and FasL staining. On the other hand, for Bcl-2 and Bax staining, nickel and cobalt enhancement (14) with H_2O_2 and DAB were performed. Normal rabbit serum was used in place of the specific primary antibody as a negative control. For Fas and FasL staining, the sections were counterstained with methyl green. In addition, immunohistochemical staining of T cell receptor was performed to identify the infiltrating cells in the periportal areas, using monoclonal antibody R73 (αβ T cell receptors; Serotec, Oxford, UK) as the primary antibody, according to the previous paper (20).

Statistical analysis

The statistical analysis between the two groups was carried out using Mann-Whitney *U* test. *P*-values less than 0.05 were considered to indicated a significant difference. All data are presented as the means ± SD.

RESULTS

Survival time. The survival time of the allo-FK group rats were significantly prolonged, in comparison to the allo group rats (10.4±1.52 vs. 42.8±11.8 days, $P<0.01$).

Alteration of the serum levels of ALT, AST, and T.Bil. The levels of AST in the allo-FK group tended to be lower than that of the allo group after liver transplantation, but not significantly. On the other hand, there was a significant difference on day 7 between the allo group and the allo-FK group in levels of ALT (**Fig. 1**). The elevation in the T.Bil level was suppressed in the allo-FK group after liver transplantation. In the allo group, the T.Bil level significantly increased on days 5 and 7 (**Fig. 1**).

Histological assessment of the grafted liver. The clearly defined diagnosis criteria according to the Banff schema for grading liver allograft rejection was used to evaluate acute rejection in grafted livers (21), which suggests a global assessment of the

grafted liver. The grading of acute rejection into the Indeterminate, Mild, Moderate, and Severe category, was based on the overall appearance of the specimens according to the criteria. Portal inflammation, perivenular inflammation, and bile duct damage were main features of that's criteria. Acute rejection was observed to be more severely advanced in the allo group in comparison to the other groups (**Fig. 2**). In the allo-FK group, the grade of acute rejection was lower than that of the allo group on days 3, 5, and 7. Meanwhile, there were no cases with acute rejection in the syn group (**Fig. 2**). In addition, most of the infiltrating cells in the periportal areas (zone 1) were T lymphocytes, as assessed by T cell receptor staining (data not shown).

Apoptosis in the grafted liver. TUNEL positive cells, which included hepatocytes, infiltrating cells, and sinusoidal endothelial cells, were observed in all posttransplant groups. There was no significant change in the number of TUNEL positive infiltrating T lymphocytes during the posttransplant period in the syn group. TUNEL positive T lymphocytes were observed to have markedly increased in the other groups. The AI of infiltrating T lymphocytes in the periportal areas (zone 1) reached a maximum on days 3-5 in the allo-FK group, while there was no significant change in the allo group (**Fig. 3, 4a**). On the other hand, hepatocyte apoptosis increased and maximized on day 7 in the allo group, while the number of hepatocyte apoptosis was

less in the allo-FK group than that in the allo group (**Fig. 4b**). TUNEL positive hepatocytes were mainly located in the periportal areas (zone 1) in both groups (**Fig. 3**). In the syn group, the number of TUNEL positive hepatocytes was smaller than in the other groups after OLT, while it was not significantly changed over time (**Fig. 3**).

Western blot analysis for Fas, FasL, Bcl-2, and Bax in the grafted liver. Bands of the appropriate size were respectively visualized at 45 kD (Fas; 13), 31 kD (FasL; 13), 25 kD (Bcl-2; 22, 23), and 21 kD (Bax; 22, 23). The expression of Fas in the allo group was more intense than that in the allo-FK group on days 1 and 3 after liver transplantation. Bcl-2 was strongly expressed in the allo-FK group during the posttransplant days, but the expression was slightly decreased on day 7. The expression of Bcl-2 in the allo group was less intense than that in the allo-FK group at any time-point after liver transplantation. However, the expression level of Bcl-2 in the allo group was temporarily increased just on day 3. On the other hand, the expression of FasL and Bax were almost constant and there was essentially no significant difference in the intensity between the allo group and the allo-FK (**Fig. 5**).

Immunohistochemical staining of Fas and FasL. Fas positive hepatocytes as well as Fas positive infiltrating lymphocytes were detected mainly in the periportal areas (zone 1) in the allo and the allo-FK group posttransplantation. The

immunohistochemical reaction for Fas positive hepatocytes was stronger in the allo group than that in the allo-FK group on days 3, 5, and 7, while there was little difference between the allo group and the allo-FK group on day 1. In the syn group, Fas positive hepatocytes were also observed in all samples, while the expression levels of Fas remained the same throughout the course (data not shown).

Immunohistochemical staining of Bcl-2 and Bax. **Figure 6** shows that most of the immunohistochemical results were consistent with that of the Western blot analysis. Bcl-2 positive hepatocytes were not found in the pericaval area (zone 3) in the normal LEW rats (**Fig. 6**). Bcl-2 positive hepatocytes in the syn group were mainly detected in pericaval area (zone 3). The expression level of Bcl-2 in the allo group was lower than that in the allo-FK group on days 1, 3, and 5 after OLT. However, there was little difference between the groups only on day 3 in the immunoreactivity of Bcl-2. Interestingly, the expression level of Bcl-2 in the syn group was lower than that in the allo-FK group on day 1 after OLT (**Fig. 6**). On the other hand, Bcl-2 positive hepatocytes were detected more frequently in the pericaval area (zone 3) in the allo-FK group in comparison to the allo group (**Fig. 6**). Bax positive hepatocytes were distributed evenly in grafted livers in the syn group. The expression of Bax was

always detected in the hepatocytes of both the allo and the allo-FK groups, and there was no difference in the expression among the three groups (data not shown).

DISCUSSION

The present study investigated the effects of FK on the grafted liver in the acute rejection phase after allogeneic liver transplantation in rats, and successfully demonstrated a significant decrease in the number of infiltrating lymphocytes and damaged hepatocytes in the FK treated grafts. Moreover, the results showed that FK acts on infiltrating T lymphocytes and rescues hepatocytes from apoptosis. The present results reinforce the efficacy of FK as an immunosuppressant in liver transplantation.

It was suggested that FK could thus have dual actions on both infiltrating T lymphocytes and hapatocytes in our model by mediating apoptosis. In solid organ transplantation, activated T lymphocytes decrease after suppression of IL-2 production (11, 24), but the relationship between FK and T lymphocyte apoptosis has not been sufficiently clarified *in vivo*. The present study clearly demonstrated that apoptotic T lymphocytes were abundant in the periportal area (zone 1) in the allo-FK group in accordance with the inhibitory effect of FK on IL-2 expression. On the other hand, FK

reduces the neutrophil accumulation, including the serum cytokine levels, while attenuating the I/R injury of the liver (25-27). In addition, since FK in itself can protect hepatocytes from apoptosis induced by ischemia-reperfusion (28, 29), FK may rescue hepatocytes from apoptosis through the induction of infiltrating T lymphocyte apoptosis, as well as directly making hepatocytes resistant to apoptosis.

The current study showed that FK reduced the expression of Fas in the grafted liver, where the expression in hepatocytes around the periportal area seemed to be closely associated with apoptosis. Apoptosis is regulated by several molecules including Fas and FasL (30, 31). The expression of Fas is directly suppressed by FK in cultured hepatocytes and the suppression of Fas expression leads to the inhibition of apoptosis (32). However, considering that FK suppresses hepatocyte apoptosis in either the grafted liver or the injured liver through the reduced expression of several cytokines, such as IL-4, IFN γ , and TNF α (33-35), as well as the inhibition of the caspase cascade reaction (36), FK apparently plays multifunctional roles in the blockade of Fas-dependent hepatocyte apoptosis. In contrast, FasL expression was not affected by FK, indicating that FK treatment affected the expression of Fas in hepatocytes but not the expression of FasL in rat OLT.

Moreover, the Bcl-2 family such as Bcl-2 and Bax is another major group of regulators of apoptosis (37-39). In this study, there was a significant difference in the expression of Bcl-2 between the FK treated group and the untreated group, while Bax expression showed no difference in the allografted groups. The most striking finding was the zonal expression of Bcl-2 in hepatic lobules. The immune reaction following liver transplantation is observed mostly in the periportal area (zone 1). Bcl-2 was expressed largely in the pericaval area (zone 3). In the syn group, the Bcl-2 expression level in the pericaval area was less than that of the allografts with FK. In addition, FK allowed the allografts to maintain the same level of Bcl-2 expression, even though its expression in the pericaval area was markedly reduced in the allografts. Pericaval hepatocytes (zone 3) are responsible for liver specific functions and unfortunately they are less tolerant of cellular stress, such as I/R injuries (40-42). Therefore, if zone 3 is severely injured, then the liver loses most of its specific functions (40). In addition, the apoptosis of the hepatocytes was frequently detected in the centrilobular zones after ischemia-reperfusion in human liver allografts (41). Although the transplantation was completed immediately after graft harvesting in our study, the liver graft was damaged by I/R injury on day 1 after OLT (43, 44). Accordingly, the allo-FK group showed a greater Bcl-2 expression in the pericaval hepatocytes than in the syn group on day 1

after OLT. As a result, FK was thus suggested to ameliorate the I/R injury after OLT via mediating the Bcl-2 expression on hapatocytes in zone 3. The observation that the liver function was better preserved in the FK group and that the area (pericaval area) of Bcl-2 expression was different from the area with T lymphocyte accumulation, FK seemed to maintain the cellular viability of zone 3 hepatocytes via enhanced Bcl-2 expression (37-41, 45), independently of T lymphocyte action. This is the first report regarding to FK and Bcl-2 expression in liver transplantation.

Several approaches to avoid of hepatocyte apoptosis have been explored recently, by either the administration of FK or the genetic modification of apoptosis regulating factors (28, 29, 36, 46-50). Moreover, several attempts have successfully achieved better protection of transplanted liver, including: 1) The use of antibodies to neutralize Fas action (51, 52). 2) Overexpression of Bcl-2 by gene transfer (47). As summarized in **Figure 7**, however, FK plays multifunctional roles in the protection of hepatocytes at different points and also is widely accepted for clinical use. Therefore, FK could be a better candidate as a therapeutic strategy against hepatocyte apoptosis.

In conclusion, the current study suggested that FK has beneficial anti-apoptotic effects on hepatocytes in the allografted rat livers through both the down-regulation of Fas expression in the periportal areas and the up-regulation of Bcl-2 expression in the

pericaval areas. Although further studies are required, particularly to elucidate the molecular mechanism underlying the FK contributions, FK might be a potentially powerful agent for use during organ transplantation.

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Figure legends

Figure 1. Alteration of serum levels of AST, ALT, and Total bilirubin (T.Bil) after rat OLT. The levels of AST and T.Bil in the allo-FK group tended to be lower than the allo group throughout after OLT. There was a significant difference on day 7 between the allo group and the allo-FK group in ALT levels after OLT. Data are plotted as the means±SD. *Statistically significant differences between the two groups ($P < 0.05$, Mann-Whitney U test).

Figure 2. The grafted liver after OLT (H and E staining; magnification, X200). Acute rejection was observed to be more severely advanced in the allo group than in the other two groups. In the allo-FK group, the grade of acute rejection was lower than that of the allo group on day 3, 5, and 7. In the syn group, there were no cases with acute rejection.

Figure 3. Apoptotic cells identified in the allo-FK, the allo, and the syn group using TUNEL staining of allografted livers on postoperative days (POD) 1, 5, and 7 (magnification, X200). The number of TUNEL positive lymphocytes on POD 5 in the

allo-FK group was greater than that in the allo group after liver transplantation. TUNEL positive hepatocytes were detected mainly in the periportal areas in both the allo-FK and the allo group and their number in the allo group was greater than that in the allo-FK group. The number of TUNELpositive hepatocytes in the syn group was smaller than in the other groups after OLT.

Figure 4. The apoptotic index (AI) in allografted liver transplantation. The AI was calculated as the number of TUNEL positive lymphocytes per 1000 lymphocytes (a) and hepatocytes (b) likewise in the allo-FK group (closed columns), and the allo group (open columns). Data are plotted as the means±SD. *Statistically significant differences between the two groups ($P < 0.05$, Mann-Whitney U test).

Figure 5. A Western blot analysis of Fas, Fas-ligand, Bcl-2, and Bax in allografted liver. The expression of Fas in the allo group was more intense than that in the allo-FK group on day 1 and 3 after OLT. The expression of Bcl-2 in the allo group was less intense than that in the allo-FK group throughout posttransplantation. There was no significant difference in the expression of Fas-ligand and Bax between the two

groups. Protein samples (10 ug) from allografted livers were separated by SDS-PAGE (10%–20%gradient gels) and subjected to an immunoblot analysis as described.

Figure 6. Immunohistochemical staining of Bcl-2 in representative grafted liver sections on postoperative days (POD) 1 and 5 (magnification, X200). The immunoreactivity of Bcl-2 in the allo-FK group was stronger than that in the allo group after liver transplantation on POD 1, 3, and 5. Bcl-2 positive hepatocytes in the allo-FK group were more frequently detected than those in the allo group mainly in pericaval area (zone 3), in comparison to the periportal area (zone 1). In normal LEW rats, Bcl-2 positive hepatocytes were not found in the pericaval area (lower left); **zone 2; ***zone 3.

Figure 7. Scheme of the multipotent functions of FK506 in allogeneic rat liver transplantation. It is possible that FK has hepatoprotective effects not only by the suppression of Fas-Antigen (Fas) on hepatocytes in the periportal areas, and the up-regulation of Bcl-2 on hepatocytes in the pericaval areas but by the direct promotion of the pro-apoptotic genes in infiltrating T lymphocytes, and the indirect induction of apoptosis in activated T lymphocytes through the deprivation of several cytokines.

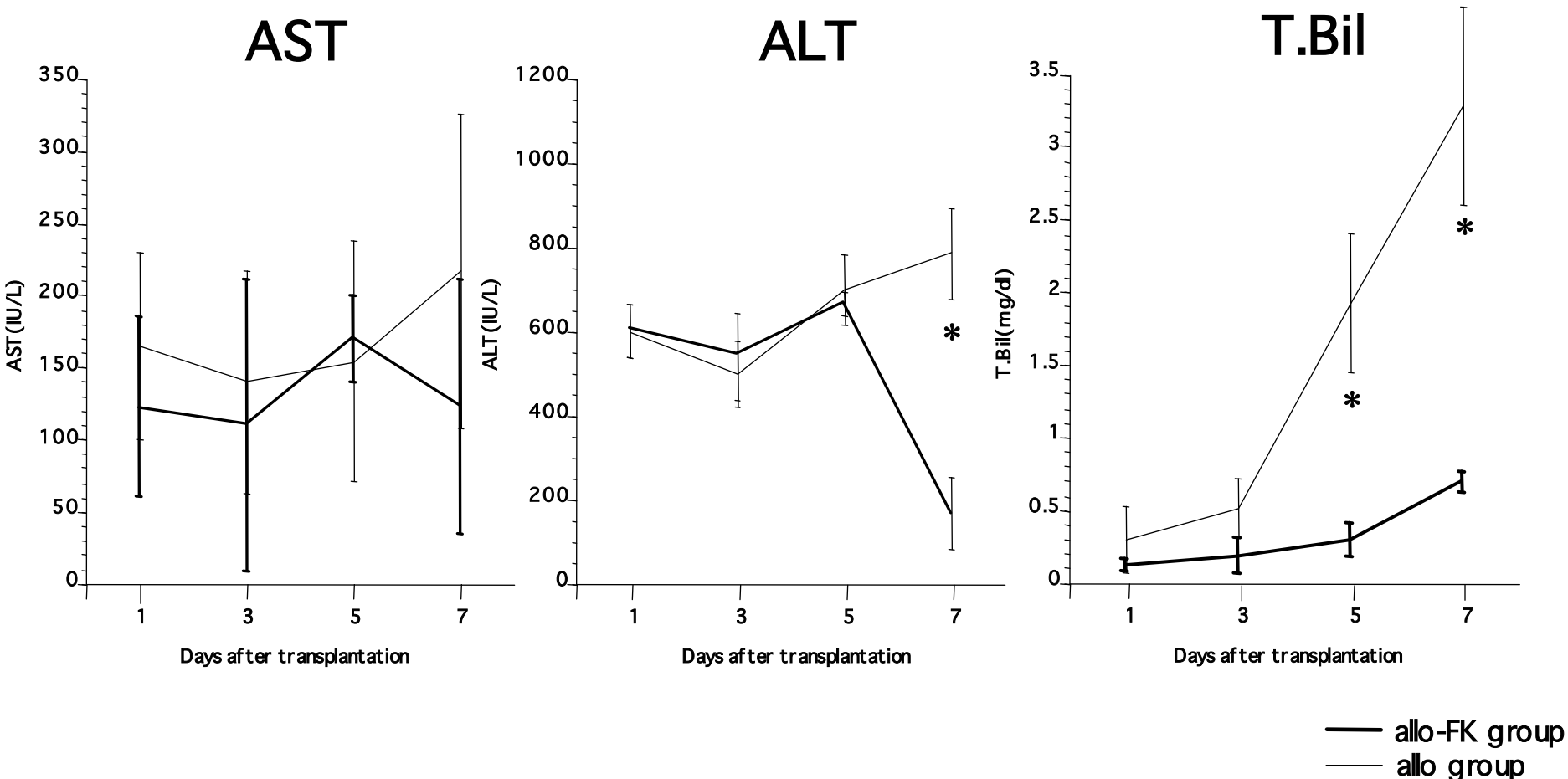

AST
AST(IU/L)
350
300
250
200
150
100
50
0
1
3
5
7
Days after transplantation
ALT
ALT(IU/L)
1200
1000
800
600
400
200
0
*
T.Bil
T.Bil(mg/dl)
3.5
3
2.5
2
1.5
1
0.5
0
*
*
allo-FK group
allo group

Figure 1.

Figure 2.

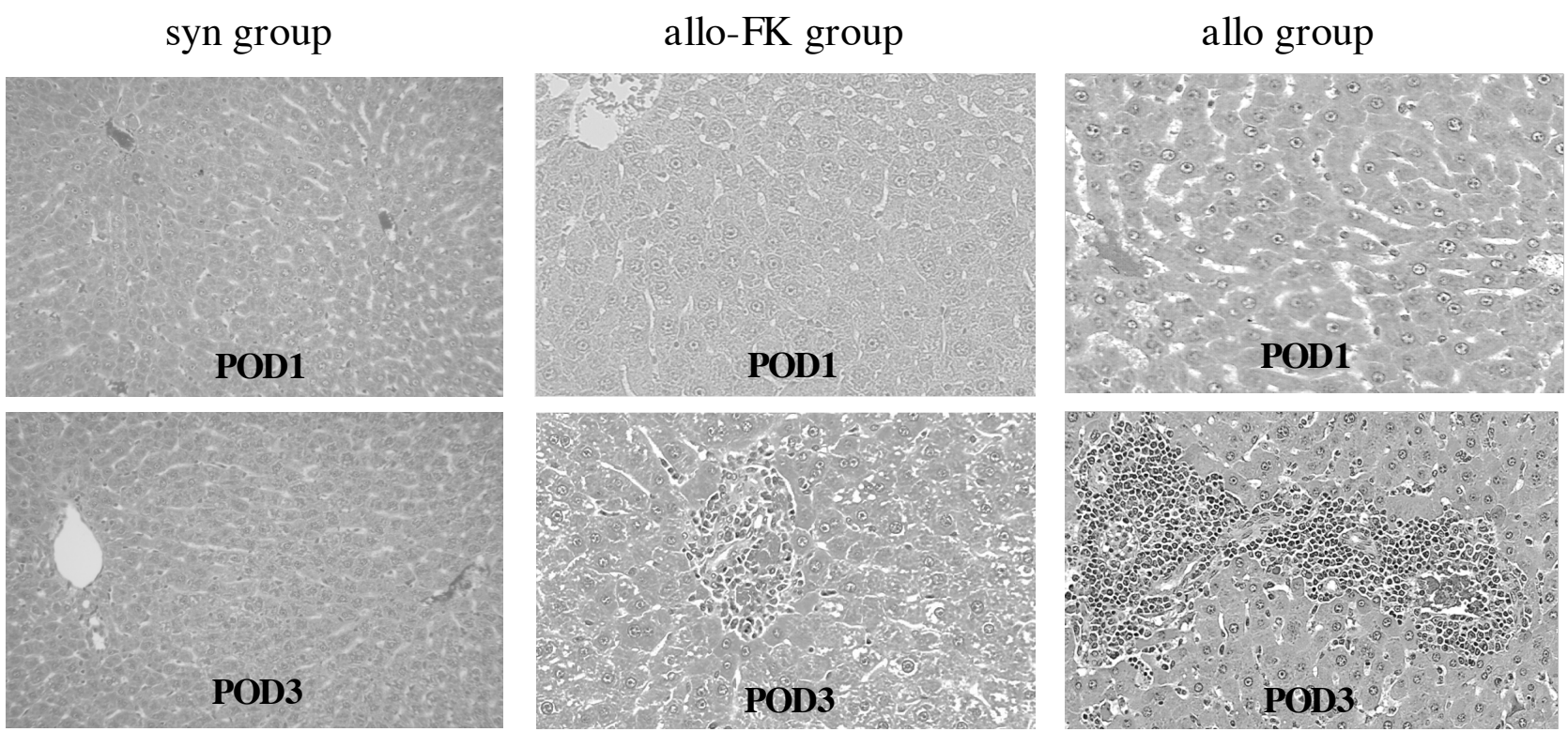
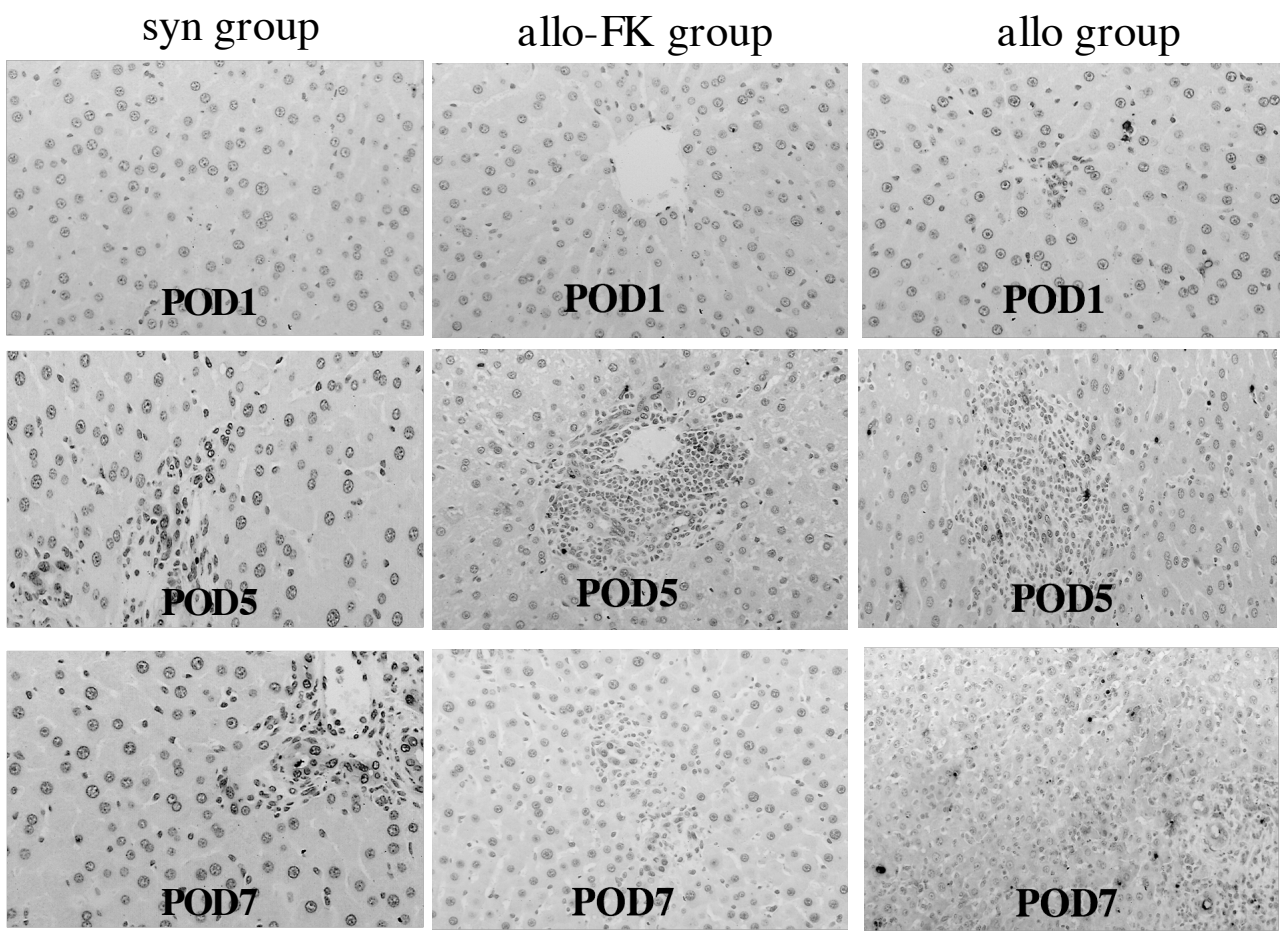

syn group
allo-FK group
allo group
POD1
POD1
POD1
POD3
POD3
POD3

Figure 3.


syn group
allo-FK group
allo group
POD1
POD1
POD1
POD5
POD5
POD5
POD7
POD7
POD7

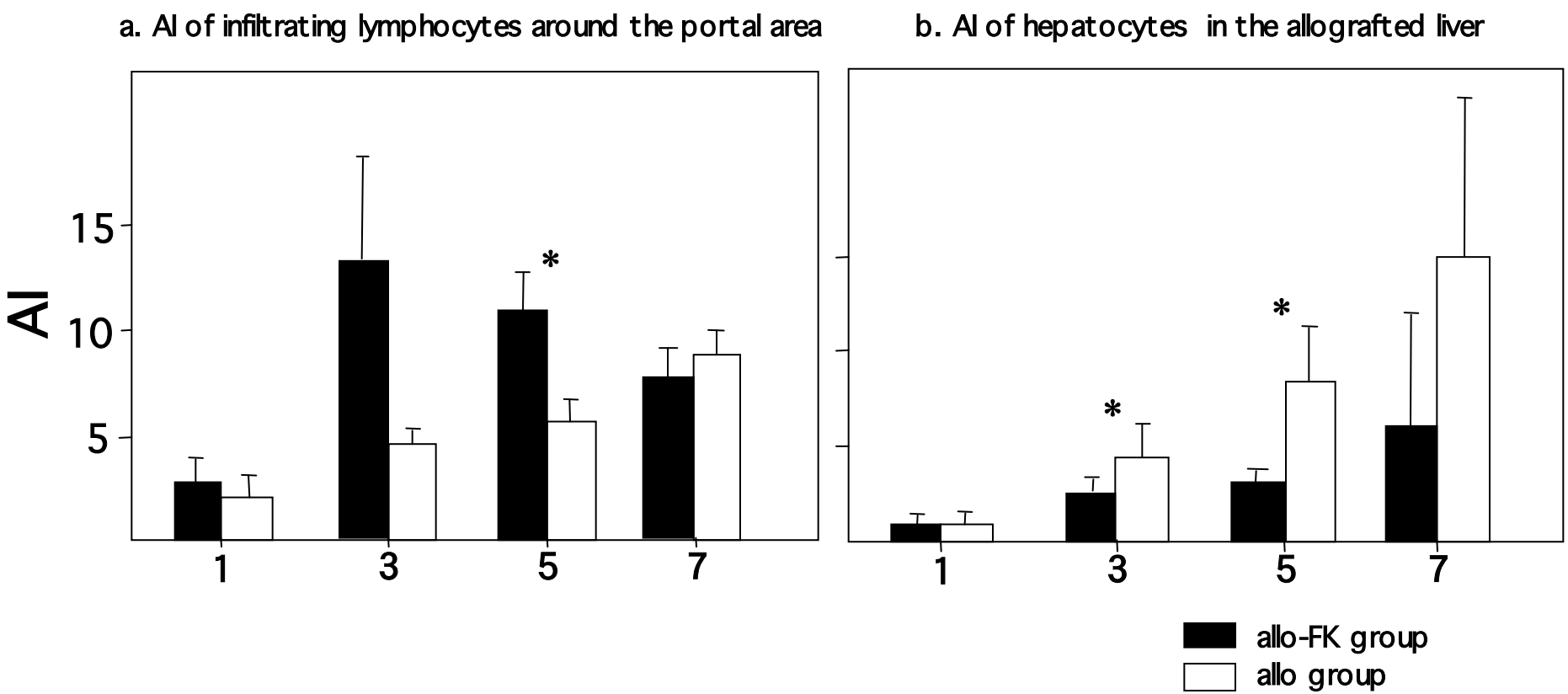
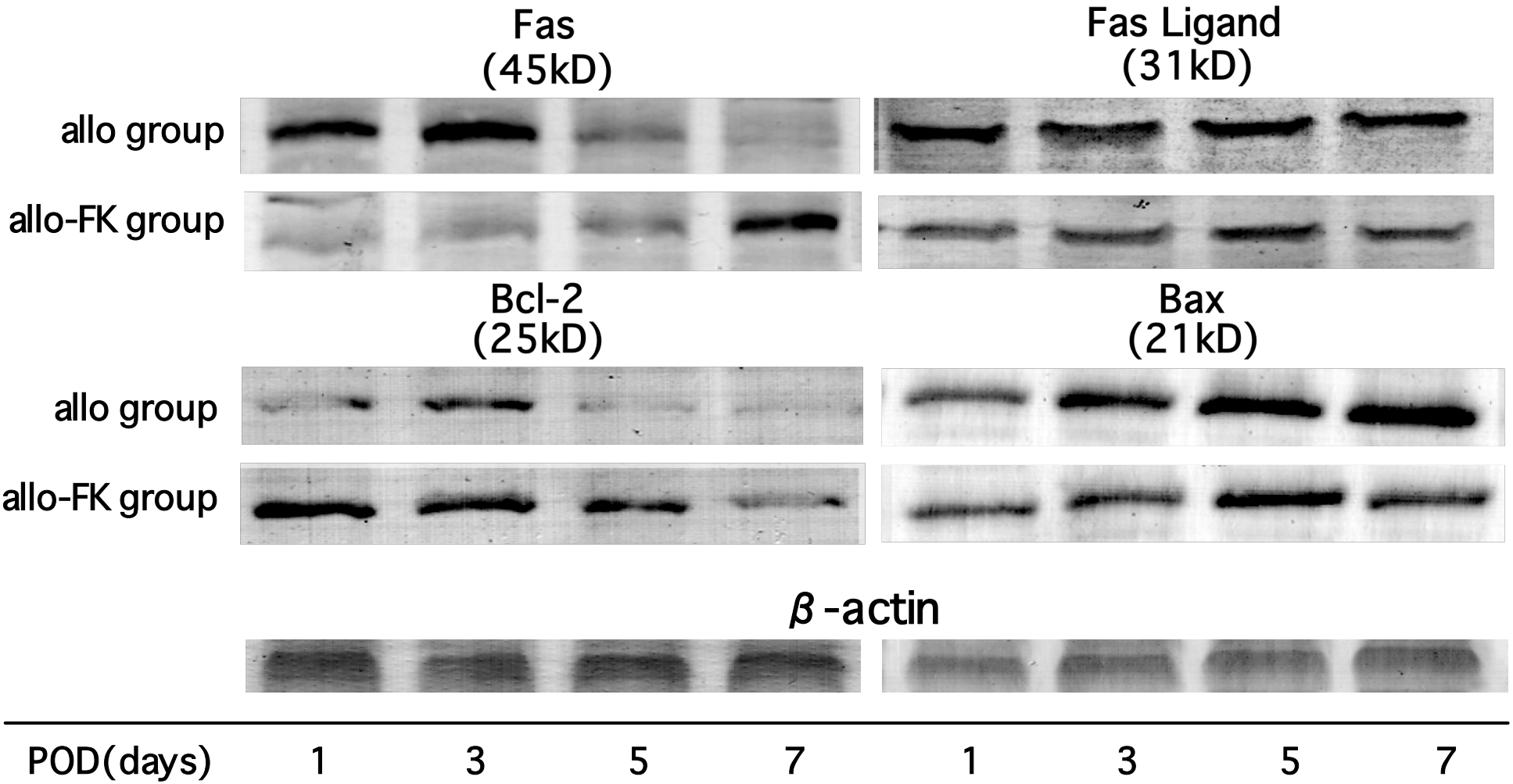

a. AI of infiltrating lymphocytes around the portal area
b. AI of hepatocytes in the allografted liver
AI
15
10
5
1
3
5
7
*
allo-FK group
allo group
Days after transplantation

Figure 4.

Figure 5.


Fas
(45kD)
Fas Ligand
(31kD)
allo group
allo-FK group
Bcl-2
(25kD)
Bax
(21kD)
allo group
allo-FK group
β-actin
POD(days) 1 3 5 7 1 3 5 7

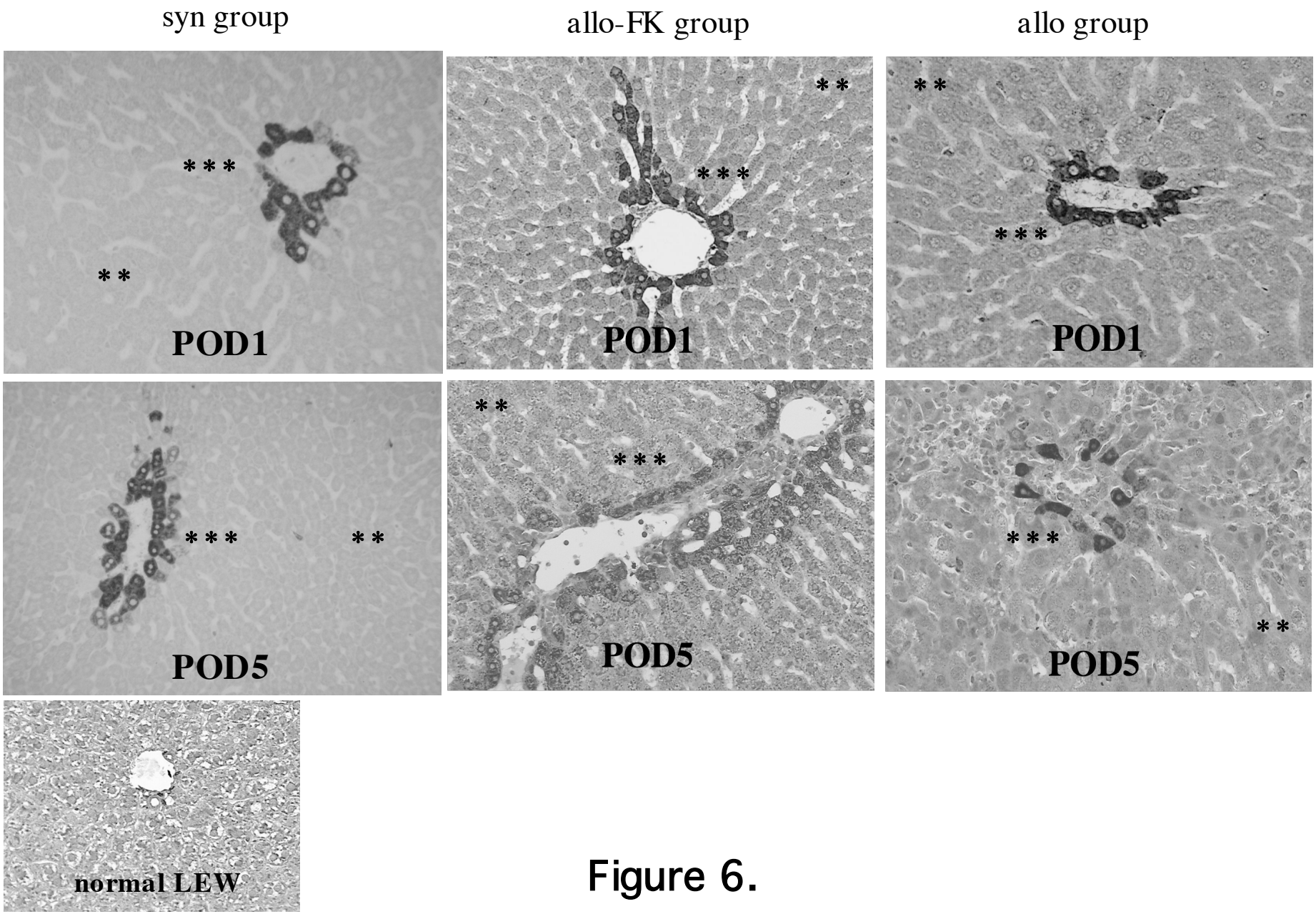

syn group
allo-FK group
allo group
POD1
POD1
POD1
POD5
POD5
POD5
normal LEW

Figure 6.

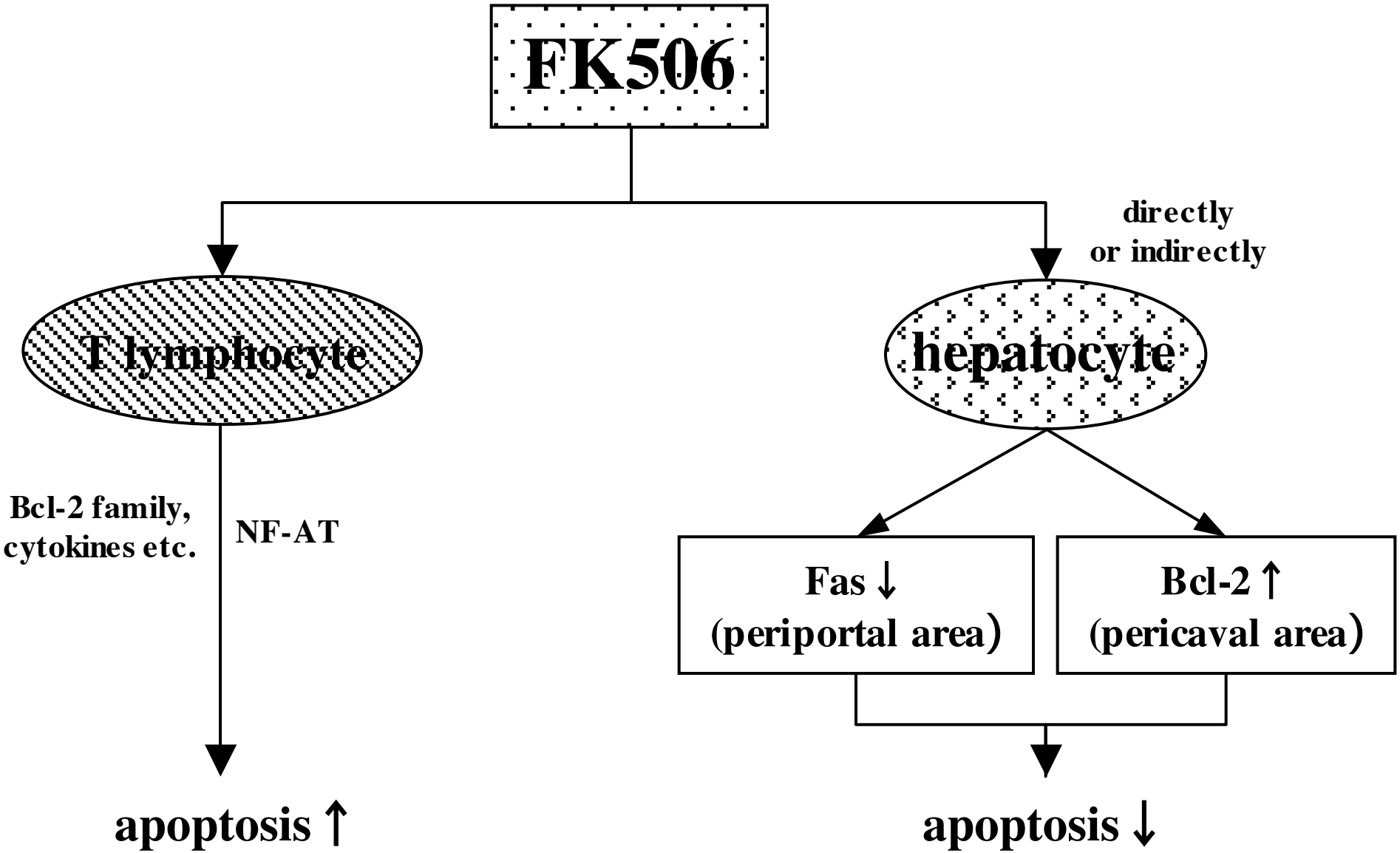

FK506
directly
or indirectly
T lymphocyte
hepatocyte
Bcl-2 family,
cytokines etc.
NF-AT
Fas ↓
(periportal area)
Bcl-2 ↑
(pericaval area)
apoptosis ↑
apoptosis ↓

Figure 7.