

## Crucial Fas–Fas ligand interaction in spontaneous acceptance of hepatic allografts in mice

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### SUMMARY

The Fas/Fas ligand (FasL) system plays important roles in the immune system, including host immunoregulation and cytotoxicity. In this study, we investigated the involvement of Fas–FasL interactions in spontaneous acceptance of hepatic allografts in murine orthotopic liver transplantation. Liver transplantation between the C57BL/6 (B6, H-2<sup>b</sup>) donor and the MRL/Mp (MRL, H-2<sup>k</sup>) recipient was performed in various combinations of donor and recipient mice with wild type (+/+), Fas-mutant (*lpr*) or FasL-mutant (*gld*) genotypes. The prolongation and spontaneous acceptance of the fully allogeneic grafts in recipients was not observed in either MRL-*lpr* recipients with B6<sup>+/+</sup> livers or MRL<sup>+/+</sup> recipients with B6-*gld* livers. Moreover, the serum alanine aminotransferase (ALT) levels and the degree of cell infiltration into hepatic allografts on day 7 after transplantation were inversely correlated with the recipient survival time (in days). The donor-specific cytotoxic T-lymphocyte (CTL) activities of the graft-infiltrating cells (GICs) from MRL-*gld* recipients with B6<sup>+/+</sup> livers were much lower than those from MRL<sup>+/+</sup> or -*lpr* recipients on days 5 and 10 after transplantation. However, the CTL activities of the GICs from MRL<sup>+/+</sup> and -*gld* recipients predominately disappeared by day 15 after transplantation. Furthermore, the anti-donor CTL activities induced in MRL<sup>+/+</sup> recipients were ascribed to CD8<sup>+</sup> cells, and were not mediated by Fas–FasL interactions. These results strongly suggest that the Fas/FasL system plays a critical role for recipient immunoregulation, enabling recipients in accepting hepatic allografts by deletion of the donor-specific T cells, but not for CTL/target cell interaction in MRL<sup>+/+</sup> recipients.

### INTRODUCTION

Engagement of Fas by Fas ligand (FasL) results in the apoptotic death of Fas-bearing cells.<sup>1–3</sup> The Fas antigen expressed on hepatocytes plays an important role in liver

diseases, including viral hepatitis, fulminant hepatitis and alcoholic cirrhosis.<sup>4–6</sup> In a murine model, administration of an anti-Fas monoclonal antibody (mAb) (Jo2) induces lethal liver damage caused by hepatocyte apoptosis.<sup>7</sup> T-cell-mediated cytotoxicity is one of the most critical and fundamental effector mechanisms of allogeneic target cell death. Previous studies have delineated that cytotoxicity is mediated through several major molecular pathways, principally the perforin/granzyme-mediated and/or the Fas–FasL-mediated pathways.<sup>8–10</sup> However, their precise roles in hepatic allograft rejection have not yet been determined.

Interestingly, it has been shown that a transplanted, fully allogeneic liver is often spontaneously accepted across a major histocompatibility barrier in mice.<sup>11</sup> Murine liver allografts revealed that prominent apoptotic cells were dispersed throughout the non-parenchymal cell population, implying that T-cell deletion may be responsible for spontaneous liver allograft acceptance.<sup>12</sup> The underlying

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Abbreviations: ALT, alanine aminotransferase; Con A, concanavalin A; c.p.m., counts per minute; E:T, effector to target (ratio); FasL, Fas ligand; FITC, fluorescein isothiocyanate; GICs, graft-infiltrating cells; H & E, haematoxylin & eosin; mAb, monoclonal antibody; PE, phycoerythrin; SEM, standard error of the mean; TUNEL, terminal deoxynucleotidyl-transferase-catalysed deoxyuridine triphosphate-digoxigenine nick-end labelling.

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mechanism remains to be elucidated in detail. On the other hand, in the case of rat liver transplantation, hepatic allografts can be spontaneously accepted only in limited combinations (e.g. DA→PVG), although the recipients showed transient rejection after transplantation.<sup>13</sup> In the rat hepatic allografts, the expression of FasL gradually switched from graft-infiltrating cells (GIC) to hepatocytes when the rejection was naturally overcome and tolerance was induced.<sup>14</sup> Therefore, Fas/FasL pathways may contribute to the control of the immune response, but there is no direct evidence to demonstrate the idea that the Fas/FasL system is involved in the induction of hepatic allograft tolerance. It has also been reported that Fas–FasL interaction mediates liver injury during rat hepatic allograft rejection.<sup>15</sup>

Only in mice are mutations of Fas (*lpr*) or FasL (*gld*) available. These mutated mice develop massive lymphadenopathy and are susceptible to autoimmune disease as a consequence of disrupted T-cell homeostasis.<sup>16</sup> The activation-induced cell death of mature T cells does not occur in *lpr* or *gld* mice *in vivo*.<sup>17</sup> In the present study, we used these mutant mice to clarify whether the Fas–FasL system is involved in recipient immune regulation through activation-induced cell death and spontaneous acceptance/rejection of fully allogeneic hepatic grafts in mice. We also examined whether FasL expression by allograft parenchymal cells or residual donor-derived non-parenchymal cells, such as Kupffer cells, downregulates local immune responses, as observed at the immunologically privileged sites such as in the testis and eye.<sup>4,18–20</sup>

## MATERIALS AND METHODS

### Animals

Male MRL/Mp (MRL, H-2<sup>k</sup>)-wild type (+/+), *lpr/lpr* (*lpr*), *gld/gld* (*gld*) and C57BL/6 (B6, H-2<sup>b</sup>)-*lpr* mice were purchased from SLC (Shizuoka, Japan). Male B6<sup>+/+</sup> and BALB/c (H-2<sup>d</sup>) mice were bred and maintained in the Institute of Experimental Animals, Kyushu University (Fukuoka, Japan). Male B6-*gld* mice were purchased from Jackson Laboratory (Bar Harbor, ME). In this study, we used young animals, 5–10 weeks of age, in order to prevent age-dependent dysregulation of the immune system in *lpr* and *gld* mutant mice. All mutant mice used in the present study had apparently normal phenotypes (no lymphadenopathy). All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

### Orthotopic liver transplantation

B6 donors into MRL (+/+, *lpr*, *gld*) recipients were used. The operations were performed under ether anaesthesia. Livers were transplanted orthotopically, according to Qian *et al.*, with minor modifications.<sup>21</sup> This transplantation procedure resulted in indefinite survival for more than 90% of the recipient mice that received syngeneic hepatic grafts. Neither immunosuppressive agents nor antibiotics were used. We used the indicated number of mice in each group for measurement of graft survival. For the

measurement of alanine aminotransferase (ALT), histopathological analyses and cytotoxic assays, other recipient mice were used.

### ALT assay

ALT levels were measured, using a standard clinical automatic analyser (Hitachi, Tokyo, Japan), in blood samples collected 7 days after grafting. The data from samples with apparent biliary complications were omitted in this study.

### Histopathological analyses of GICs and apoptotic cells

Liver samples were fixed in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin, sections were taken and these sections were stained with haematoxylin and eosin (H & E). GICs in the portal areas of liver grafts were detected by H & E staining. Apoptotic bodies were detected by terminal deoxynucleotidyl-transferase-catalysed deoxyuridine triphosphate-digoxigenine nick-end labelling (TUNEL), performed using the *in situ* cell death detection kit (Oncor, Gaithersburg, MD), according to the manufacturer's instructions. Active caspase-3<sup>+</sup> cells were detected with purified monoclonal rabbit anti-active caspase-3 (PharMingen, San Diego, CA), used according to the manufacturer's instructions. The sections were also counterstained with haematoxylin. The number of TUNEL<sup>+</sup> and active caspase-3<sup>+</sup> cells per high-power field was counted by microscopy (magnification ×40).

### Preparation of GICs

After the mice were killed, hepatic allografts were surgically removed following *in situ* perfusion with saline and then disrupted using two glass slides. GICs were isolated by centrifugation, using a Percoll gradient (Sigma Chemical Co., Louis, MO). Cells at the 1.086 and 1.055 Percoll interface were recovered and then washed twice with saline. The GIC suspensions in each group were pooled and the number of viable nucleated cells was counted using Trypan Blue dye exclusion. CD4-depleted or CD8-depleted GICs were prepared immunomagnetically using magnetic beads (DynaL, Oslo, Norway) and anti-CD4 (clone H129.19) or anti-CD8 (clone 53-6.7) mAb (PharMingen), according to the manufacturer's instructions.

### Flow cytometric analyses

GICs isolated from liver allografts were analysed. After blocking non-specific antibody binding with a mAb specific for the mouse Fc receptor (clone 2.4 G2), the expressions of various cell-surface antigens were analysed by two-colour flow cytometry using a fluorescence-activated cell sorter (FACSCalibur; Becton-Dickinson, Mountain View, CA). The following mAbs were used in this study: fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-H-2K<sup>b</sup> (Meiji Institute of Health Science, Tokyo, Japan), anti H-2K<sup>k</sup> (Meiji Institute of Health Science), anti-CD3 (clone 145-2C11; PharMingen), anti-CD4 (clone H129.19; PharMingen) and anti-CD8 (clone 53-6.7; PharMingen). The data were acquired and processed using CELLQuest software (Becton-Dickinson).

### Cytotoxic assays

Freshly isolated GICs obtained from the B6 hepatic allografts in the MRL recipients were used as effectors. Concanavalin A (Con A)-stimulated spleen cells were used as target cells. Effector cells were incubated with  $1 \times 10^4$   $^{51}\text{Cr}$ -labelled target cells at various effector:target (E:T) ratios in 0.2 ml of complete RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY) in a 96-well round-bottomed microplate at  $37^\circ$  in humidified air with 5%  $\text{CO}_2$ . After a 4-hr incubation period, every culture supernatant was collected and the radioactivity was measured using an Autowell gamma counter (Aloka, Tokyo, Japan). The % cytotoxicity of specific  $^{51}\text{Cr}$  release was calculated using the following formula:

$$\% \text{ Specific lysis} = 100 \times \left[ \frac{\text{c.p.m. of experimental } ^{51}\text{Cr release} - \text{c.p.m. of spontaneous } ^{51}\text{Cr release}}{\text{c.p.m. of maximum } ^{51}\text{Cr release} - \text{c.p.m. of spontaneous } ^{51}\text{Cr release}} \right]$$

Radioactivities of maximum and spontaneous  $^{51}\text{Cr}$  release [in counts per minute (c.p.m.)] were obtained by incubating target cells with 1% Triton-X-100 and with medium alone, respectively. All samples were performed in triplicate.

### Statistical analyses

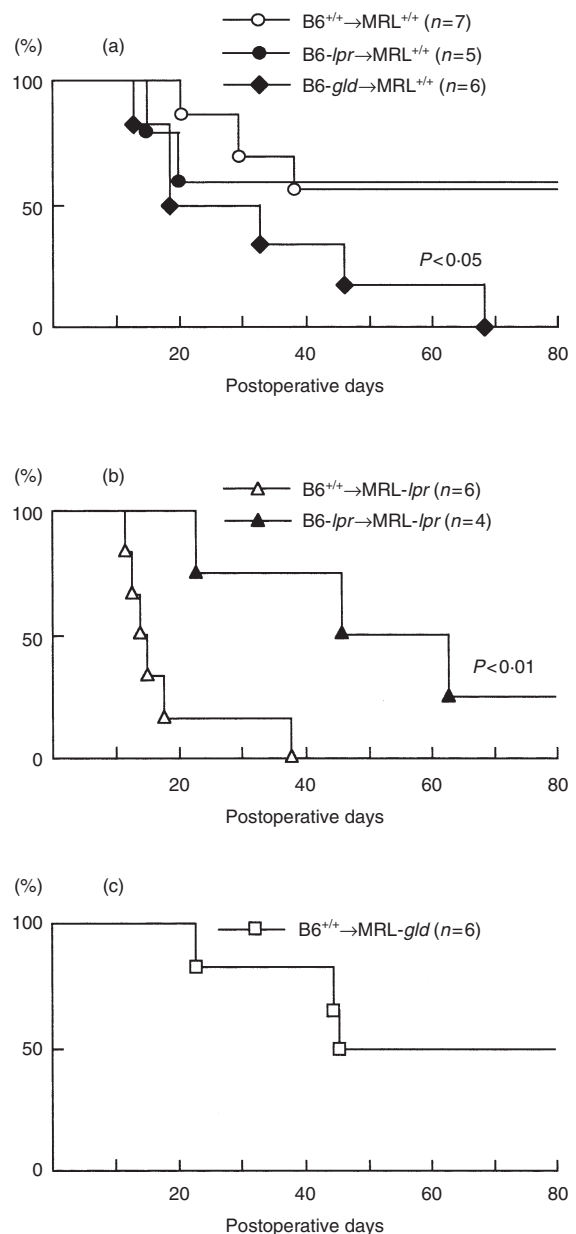
All the data, except survival duration (in days), were presented as means  $\pm$  standard error of the mean (SEM). They were first analysed for the homogeneity of variance with the *F*-test. The Student's *t*-test was used if the group variance appeared homogeneous, and Welch's *t*-test was used if the variance was not homogeneous. The numbers of days that the grafts survived were compared using Kaplan-Meier analysis and the log-rank test. A *P*-value of less than 0.05 was considered statistically significant.

## RESULTS

### Survival of recipient mice after liver transplantation

In order to determine the contribution of the Fas-FasL system to recipient survival, B6-derived hepatic allografts (+/+, *-lpr* or *-gld*) were transplanted to MRL recipients (+/+, *-lpr* or *-gld*) in various combinations. The recipient's survival was checked daily after liver transplantation. As shown in Fig. 1(a), in the case of MRL<sup>+/+</sup> recipients grafted with B6<sup>+/+</sup> donor livers, the recipients experienced relatively long-term survival (median survival >80 days) and four of the seven recipients survived for longer than 80 days. The median survival time of MRL<sup>+/+</sup> recipients grafted with B6<sup>+/+</sup> donor livers (>80 days) was comparable to that of MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> grafts. In contrast, MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> grafts died significantly earlier (median survival 32 days) than MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> grafts (*P* < 0.05). MRL<sup>+/+</sup> recipients grafted with B6<sup>+/+</sup> donor livers appeared systemically ill (weight loss and mild lethargy) and died shortly after transplantation (median survival 14.5 days),

whereas MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> grafts generally appeared well and survived for longer (median survival >54 days) (*P* < 0.01), as shown in Fig. 1(b). MRL<sup>+/+</sup> recipients grafted with B6<sup>+/+</sup> donor livers experienced long-term survival (median survival >63 days), as observed in MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> grafts (Fig. 1c). Thus, the prolongation and spontaneous acceptance of the fully allogeneic grafts in recipients was not observed in the case



**Figure 1.** Survival of recipient mice after allogeneic liver transplantation. Hepatic allografts from B6<sup>+/+</sup>, *-lpr* and *-gld* donor mice were orthotopically transplanted into MRL<sup>+/+</sup> (a), *-lpr* (b) or *-gld* (c) recipients. Survival of mice was observed daily after transplantation.

of either B6<sup>+/+</sup> grafting into MRL-*lpr* recipients or B6-*gld* grafting into MRL<sup>+/+</sup> recipients. These results strongly suggest that in MRL<sup>+/+</sup> recipients, hepatic allograft destruction is not mediated by the Fas–FasL system. Specifically, FasL expression in the grafted liver is relevant to the acceptability of hepatic allograft. By contrast, this research demonstrates that in MRL-*lpr* recipients, hepatic allograft destruction is partially mediated by the Fas–FasL system.

#### Serum ALT levels in recipients with liver transplants

In order to evaluate animals for possible liver failure after transplantation, serum levels of the cytosolic enzyme, ALT, were determined in recipients on day 7 after transplantation, when no recipient mice had died but some appeared ill. ALT levels were also examined on days 5, 10 and 15 post-transplantation. On day 5, the rejection process was not fully evident, with only a slight increase in the ALT level (data not shown). On days 10 and 15, ALT levels varied too widely (even in the same group) for comparison of the differences between given groups (data not shown). There were salient differences in serum ALT levels on day 7 after transplantation among some combination groups (Table 1). The mean serum ALT level of MRL<sup>+/+</sup> recipients grafted with B6-*lpr* donor livers was comparable to that of MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> grafts, although both serum ALT levels were approximately sixfold higher than the baseline MRL<sup>+/+</sup> level. By contrast, the serum ALT level of MRL<sup>+/+</sup> recipients with B6-*gld* grafts increased significantly, compared with that of MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> grafts ( $P < 0.05$ ). A dramatic increase in serum ALT level was observed in MRL-*lpr* recipients with B6<sup>+/+</sup> grafts, but not in those with B6-*lpr* grafts ( $P < 0.05$ ). The mean serum ALT level of MRL-*gld* recipients with B6<sup>+/+</sup> or -*gld* grafts was comparable to the non-treated or naive MRL level (baseline). These results revealed that serum ALT levels correlated highly with the recipient survival, equivalent to hepatic allograft rejection.

**Table 1.** Serum alanine aminotransferase (ALT) levels on day 7 after liver transplantation

Combination	ALT (U/l)
Naive MRL <sup>+/+</sup>	49 ± 3
B6 <sup>+/+</sup> into MRL <sup>+/+</sup>	309 ± 52*
B6- <i>lpr</i> into MRL <sup>+/+</sup>	309 ± 64
B6- <i>gld</i> into MRL <sup>+/+</sup>	657 ± 151*
B6 <sup>+/+</sup> into MRL- <i>lpr</i>	1412 ± 271†
B6- <i>lpr</i> into MRL- <i>lpr</i>	388 ± 248†
B6 <sup>+/+</sup> into MRL- <i>gld</i>	69 ± 9
B6- <i>gld</i> into MRL- <i>gld</i>	60 ± 13

Data represent average ± standard error of the mean (SEM) values obtained from three to five recipient animals.

\*Significant difference ( $P < 0.05$ ) between B6<sup>+/+</sup> into MRL<sup>+/+</sup> and B6-*gld* into MRL<sup>+/+</sup>.

†Significant difference ( $P < 0.05$ ) between B6<sup>+/+</sup> into MRL-*lpr* and B6-*lpr* into MRL-*lpr*.

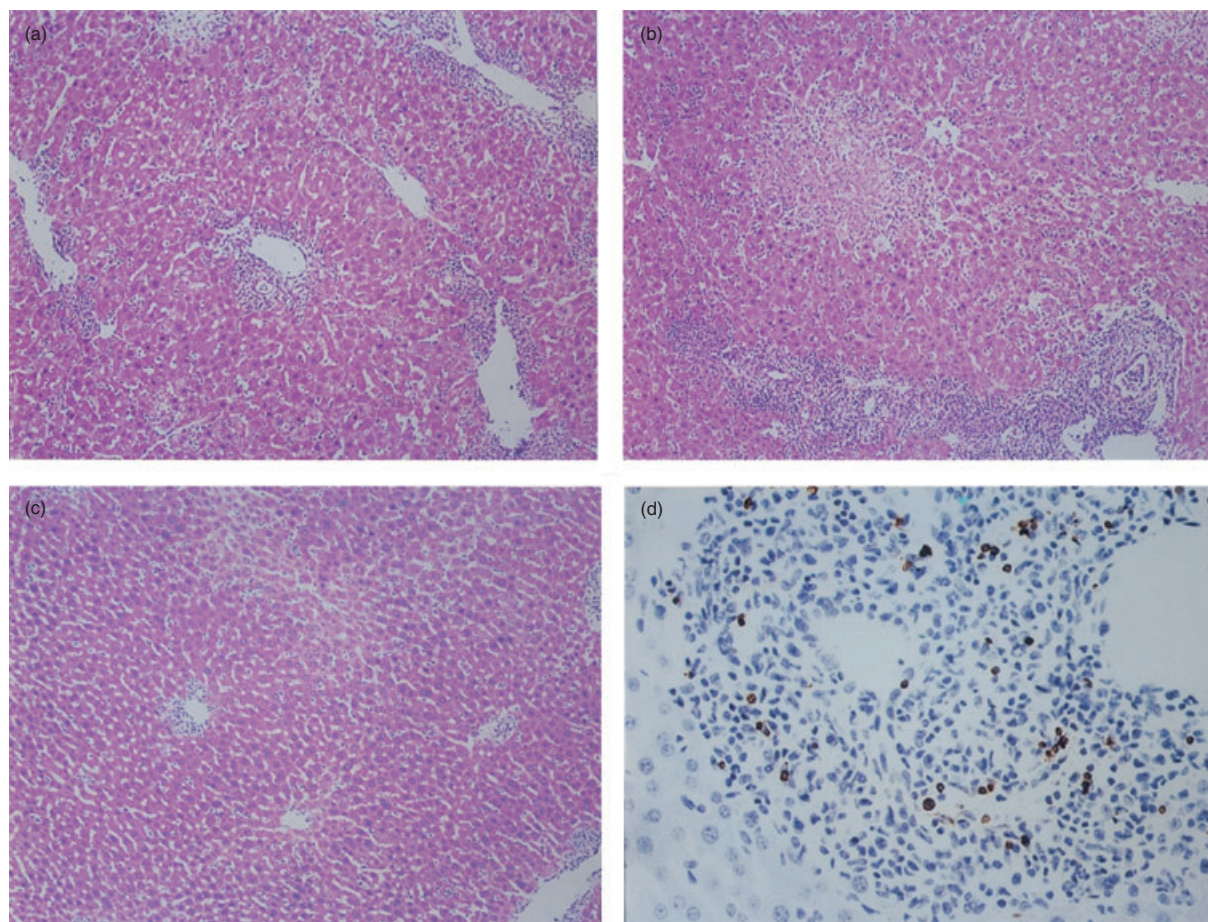
#### Histopathological studies

We next examined histopathological differences in the donor livers among the combination groups on day 7 after transplantation. Mild-to-moderate infiltration of mononuclear cells was observed in B6<sup>+/+</sup> donor hepatic allografts in MRL<sup>+/+</sup> recipients (Fig. 2a). The infiltration was also observed in B6-*gld* donor hepatic allografts in MRL<sup>+/+</sup> recipients (data not shown). B6<sup>+/+</sup> donor hepatic allografts in MRL-*lpr* recipients showed multifocal areas with parenchymal necrosis and massive infiltration of GICs (Fig. 2b), while their severities were ameliorated in B6-*lpr* donor hepatic allografts (data not shown). On the other hand, B6<sup>+/+</sup>, as well as B6-*gld*, donor hepatic allografts in MRL-*gld* recipients, showed a significantly milder infiltration compared with B6<sup>+/+</sup> donor hepatic allografts in MRL<sup>+/+</sup> or -*lpr* recipients (Fig. 2c).

In order to determine whether the Fas–FasL system is a major mechanism of apoptotic cell death in rejecting hepatic allografts, we compared the number of apoptotic cells within rejecting hepatic allografts in MRL<sup>+/+</sup>, -*lpr* and -*gld* recipients on day 7 after transplantation using the TUNEL method. B6<sup>+/+</sup>, as well as -*gld*, donor hepatic allografts in MRL<sup>+/+</sup> recipients showed that few parenchymal cells (data not shown) and GICs were TUNEL<sup>+</sup> (Table 2). FasL expression in the grafted liver did not result in an increase in TUNEL<sup>+</sup> GICs. B6<sup>+/+</sup> donor hepatic allografts in MRL-*lpr* recipients showed an increased number of TUNEL<sup>+</sup> parenchymal cells in the necrotic areas (data not shown) and a relatively increased number of TUNEL<sup>+</sup> GICs beyond expectation. By contrast B6<sup>+/+</sup>, as well as B6-*gld*, donor hepatic allografts in MRL-*gld* recipients had a significantly increased number of TUNEL<sup>+</sup> GICs as compared to that in MRL<sup>+/+</sup> recipients (Fig. 2d). These results strongly suggest that Fas expression on the GICs is irrelevant to the number of TUNEL<sup>+</sup> GICs. To confirm these results in detail regarding apoptosis, we examined active caspase-3<sup>+</sup> GICs. Active caspase-3 (CPP32) is required for the induction of apoptosis by certain effectors, such as Fas.<sup>22,23</sup> The results of active caspase-3 immunohistochemical examination were similar to that of TUNEL examination (Table 2).

#### Cytotoxic T lymphocyte (CTL) activities of freshly isolated graft-infiltrating cells

MRL recipients grafted with B6<sup>+/+</sup> donor livers were killed on days 5, 10 or 15 after transplantation. Immediately after killing the mice, GICs were isolated as described in the Materials and methods. As reported previously,<sup>12</sup> the hepatic allografts were rapidly infiltrated by recipient-derived cells after transplantation. More than 95% of GICs were H-2<sup>k</sup>-positive (recipient-derived) through the observation period. The proportion of CD4<sup>+</sup> CD8<sup>+</sup> cells in GICs were very similar among MRL<sup>+/+</sup>, -*lpr* and -*gld* recipient mice. Approximately 20% of GICs were CD4<sup>+</sup> and 33% of GICs were CD8<sup>+</sup>. Few double-negative T cells (CD3<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup>) were found, even in MRL-*lpr* and -*gld* recipients (data not shown). As shown in Fig. 3, high CTL activities of GICs against donors, but not against a third party (BALB/c, H-2<sup>d</sup>), were observed in both



**Figure 2.** Analysis of graft-infiltrating cells (GICs) and apoptotic cells on B6<sup>+/+</sup> donor hepatic allografts in MRL recipient mice. Sections were prepared from B6<sup>+/+</sup> donor livers in MRL<sup>+/+</sup> (a), *-lpr* (b) and *-gld* (c and d) recipients on day 7 after transplantation. The sections were stained with haematoxylin & eosin (a–c), and apoptotic cells in portal areas of liver grafts were detected by terminal deoxynucleotidyl-transferase-catalysed deoxyuridine triphosphate-digoxigenine nick-end labelling (TUNEL) staining (d). Representative photographs are shown. Original magnification: (a–c),  $\times 10$ ; (d),  $\times 40$ .

**Table 2.** The number of terminal deoxynucleotidyl-transferase-catalysed deoxyuridine triphosphate-digoxigenine nick-end labelling (TUNEL)-positive and active caspase 3-positive graft-infiltrating cells (GICs) on day 7 after liver transplantation

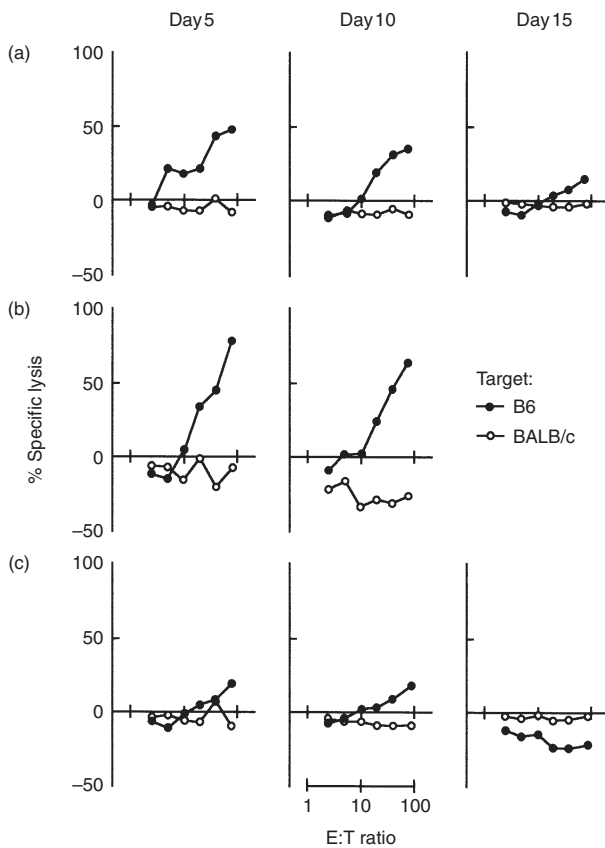
Combination	TUNEL <sup>+</sup> GICs	Active caspase 3 <sup>+</sup> GICs
B6 <sup>+/+</sup> into MRL <sup>+/+</sup>	7.0 $\pm$ 0.7*	4.4 $\pm$ 1.2†
B6- <i>gld</i> into MRL <sup>+/+</sup>	6.6 $\pm$ 1.4	4.2 $\pm$ 0.4
B6 <sup>+/+</sup> into MRL- <i>lpr</i>	9.8 $\pm$ 1.5	6.4 $\pm$ 0.4
B6 <sup>+/+</sup> into MRL- <i>gld</i>	15.6 $\pm$ 2.9*	9.2 $\pm$ 0.7†
B6- <i>gld</i> into MRL- <i>gld</i>	14.2 $\pm$ 1.9	8.6 $\pm$ 1.4

Data represent average  $\pm$  standard error of the mean (SEM) values obtained from five high-power fields of each slide. Similar results were obtained in two other independent experiments.

\*Significant difference ( $P < 0.05$ ) between B6<sup>+/+</sup> into MRL<sup>+/+</sup> and B6<sup>+/+</sup> into MRL-*gld*.

†Significant difference ( $P < 0.01$ ) between B6<sup>+/+</sup> into MRL<sup>+/+</sup> and B6<sup>+/+</sup> into MRL-*gld*.

MRL<sup>+/+</sup> and *-lpr* recipients grafted with B6<sup>+/+</sup> donor livers on days 5 and 10 after transplantation. However, a major part of the CTL activities of GICs from the MRL<sup>+/+</sup> recipients disappeared by day 15 after transplantation. By contrast, anti-donor CTL activities of GICs from MRL-*gld* recipients with B6<sup>+/+</sup> livers were much lower than those observed in MRL<sup>+/+</sup> and *-lpr* recipients on days 5 and 10 after transplantation, and they were undetectable on day 15 after transplantation. Although only two data were obtained for CTL activities in *lpr* recipients on day 15 (when *lpr* recipient animals were critically ill), the CTL activity persisted as compared to MRL<sup>+/+</sup> recipients (data not shown). We next examined whether these CTL activities are dependent on the Fas/FasL system. As shown in Fig. 4(a), the anti-donor CTL activities of GICs from MRL<sup>+/+</sup> recipients were independent of the Fas/FasL system, because CTL activities were not depressed against B6-*lpr* target cells. It was also revealed that CD4<sup>+</sup> cell-depleted,

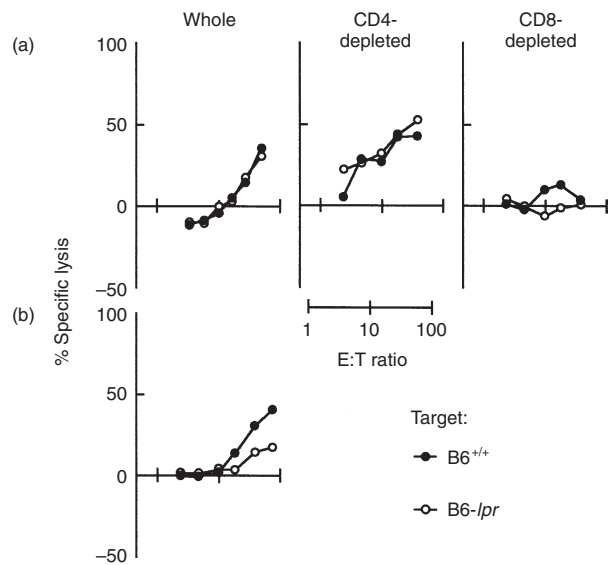


**Figure 3.** Cytotoxic T-lymphocyte (CTL) activities of graft-infiltrating cells (GICs) in  $B6^{+/+}$  hepatic allografts in MRL recipient mice. GICs were recovered from  $B6^{+/+}$  donor livers in MRL $^{+/+}$  (a),  $-lpr$  (b) and  $-gld$  (c) recipients on days 5, 10 and 15 after transplantation. A short-term 4-hr  $^{51}\text{Cr}$ -release assay was performed to determine the cytotoxicities of GICs against B6 and BALB/c splenic concanavalin A (Con A) blast cells. Data represent average values from triplicate wells. Similar results were obtained in two other independent experiments. E:T ratio, effector-to-target ratio.

but not  $\text{CD8}^+$  cell-depleted, GICs had the CTL activities in MRL $^{+/+}$  recipients. By contrast, Fas–FasL-dependent CTL activities were apparent in MRL- $-lpr$  recipients, because CTL activities were depressed against B6- $-lpr$  target cells, as shown in Fig. 4(b).

## DISCUSSION

Perforin/granzyme- and Fas/FasL-based mechanisms may account for all T-cell-mediated cytotoxicity in short-term *in vitro* assays.<sup>8</sup> In normal mice, *in vivo* injection of anti-Fas mAb leads to massive apoptosis in the liver, with death of the animal following within a few hours as a result of fulminant hepatitis because hepatocytes, which express Fas antigen, are highly sensitive to Fas-mediated apoptosis.<sup>7</sup> In one aspect of the present study, we examined whether Fas–FasL interaction (CTL/target liver cell interaction) is a major pathway of hepatic allograft destruction. Previous studies have indicated that Fas–FasL interactions may



**Figure 4.** Cytotoxic T-lymphocyte (CTL) activities, 5 days post-transplantation, of graft-infiltrating cells (GICs) in  $B6^{+/+}$  hepatic allografts in MRL $^{+/+}$  (a) and  $-lpr$  (b) recipients, grafted with  $B6^{+/+}$  donor livers. A short-term 4-hr  $^{51}\text{Cr}$ -release assay was performed to determine the cytotoxicities of whole, CD4-depleted and CD8-depleted GICs against  $B6^{+/+}$  (Fas $^+$ ) and  $-lpr$  (Fas $^-$ ) splenic concanavalin A (Con A) blast cells. Data represent average values from triplicate wells. Similar results were obtained in two other independent experiments.

not be essential mediators of T-cell-mediated allograft damage in murine cardiac transplantation.<sup>24–26</sup> However, as reported many times previously, Fas and FasL are expressed in various cells, such as hepatocytes, Kupffer cells and lymphocytes, which are involved in hepatic allograft rejection. We also examined these expressions in hepatic allografts using FACS analysis and immunohistochemistry (data not shown). Fas and FasL were also expressed in so many different types of cells in our examination that elucidating the Fas and FasL involvement in the hepatic allograft was difficult. However, it is conceivable that both Fas and FasL are involved in hepatic allograft rejection. Therefore, we studied the involvement of Fas and FasL in hepatic allograft rejection, using various donor–recipient combinations of wild type ( $+/+$ ),  $-lpr$  and  $-gld$  mice.

In MRL $^{+/+}$  recipients, Fas–FasL interactions are not absolutely required for hepatic allograft destruction, as evidenced by the lack of apparent improvement of recipient survival and ALT data by grafting B6- $-lpr$  livers and by generation of Fas/FasL-independent donor-specific cytotoxicities of GICs. These results strongly suggest that the perforin/granzyme pathway is one of the major pathways of T-cell-mediated cytotoxicity in murine hepatic allograft rejection in the case of MRL $^{+/+}$  recipients, and only  $\text{CD8}^+$  T cells are responsible for the donor-specific cytotoxicity. Although apoptosis has been reported as a mode of hepatocyte death in allograft rejection,<sup>27</sup> the Fas/FasL system hardly affected this apoptotic process of hepatocytes in MRL $^{+/+}$  recipients, as shown in the present study.

By contrast, in the MRL-*lpr* recipients, an involvement of the Fas/FasL system in severe hepatic allograft destruction was apparent because it was ameliorated by grafting B6-*lpr* livers into the recipients. In fact, *lpr* mouse-derived T cells scarcely express Fas antigen, but overexpress FasL antigen.<sup>28</sup> Additionally, Russel *et al.*<sup>17</sup> have reported that *in vivo* activation-induced cell death of mature T cells does not occur in *lpr* or *gld* mice. The emergence of Fas/FasL-mediated cytotoxicity in the MRL-*lpr* recipient is probably the result of repeated antigenic stimulation of T cells that cannot undergo apoptosis through the Fas-mediated pathway. However, the number of TUNEL-positive GICs increased in the MRL-*lpr* recipients more significantly than anticipated, as compared with that of the MRL<sup>+/+</sup> recipients. Desbarats *et al.*<sup>29</sup> demonstrated that FasL itself can transduce signals leading to cell-cycle arrest and cell death in CD4<sup>+</sup> T cells. Thus, interactions between FasL-bearing recipient GICs and Fas expressed in hepatic allografts may increase the number of TUNEL<sup>+</sup> GICs. It is also possible that other death signals play important roles in the apoptotic process of GICs in *lpr* recipients.

Because, according to previous reports,<sup>16</sup> *in vivo* activation-induced cell death of mature T cells may not occur in *gld* recipients, there may be few apoptotic T cells with the capacity of anti-donor cytotoxicity (which normally results in severe rejection). However, in the MRL-*gld* recipients, the increased number of apoptotic GICs was associated with a milder infiltration of cells into the hepatic allografts, lower serum ALT levels and lower anti-donor CTL activities on postoperative day 15, in comparison to those observed in the MRL<sup>+/+</sup> or -*lpr* recipients. Wagener *et al.*<sup>30</sup> also demonstrated that heart allografts can be accepted without T-cell apoptosis mediated by the Fas-FasL interactions using *gld* recipients. One possible explanation for the abrogation of CTL function in *gld* recipients might be a hyporesponsiveness of T cells from *gld* mice towards the alloantigens. We examined *in vitro* CTL induction using spleen cells from *gld* mice, but the induced CTL activities were comparable to those from the wild-type mice (data not shown). The results indicate that the abrogation of CTL function in the *gld* recipients is not a result of their hyporesponsiveness to the donor alloantigens. Therefore, a more plausible explanation is that the lower serum ALT levels and the abrogation of CTL function in MRL-*gld* recipients are attributable to the massive apoptosis of GICs, as shown in Table 2 and Fig. 2(d). Moreover, the increased number of GICs may not be a result of the interaction between Fas-expressing GICs and FasL-bearing cells in the graft because the number of apoptotic GICs was not significantly decreased in the B6-*gld* liver allografts (Table 2). The precise mechanism that accounts for these results is controversial. However, it has been reported that the Fas-FasL interaction resulted in the release of proinflammatory cytokines such as interleukin (IL)-1 $\beta$ .<sup>31</sup> FasL transgenic mice with FasL expression in pancreatic  $\beta$  cells under the control of the insulin promoter underwent destruction of the  $\beta$  cells and consequent diabetes as a result of massive neutrophil recruitment. This neutrophilic response may be responsible for the direct

action of FasL itself or may be mediated by a neutrophilic cytokine, IL-8.<sup>32</sup> The lack of Fas-FasL engagement and the resultant release of proinflammatory cytokines may lead to accelerated apoptosis and restricted infiltration of GICs.

FasL is a highly efficient mediator of apoptosis in activated T lymphocytes, thus creating an immunoprivileged site, much like the anterior chamber of the eye and Sertoli cells of testis.<sup>19,20</sup> Testis grafts, derived from mice that can express functional FasL, survived indefinitely when transplanted under the kidney capsule of allogeneic animals, whereas testis grafts derived from mutant *gld* mice were rejected.<sup>20</sup> Li *et al.*<sup>33</sup> have reported prolonged survival of rat liver allografts transfected with FasL-expressing plasmids. FasL mRNA was found within hepatocytes in an inflammatory liver disease.<sup>4</sup> The interaction between FasL on hepatic allografts and Fas on GICs may provide a mechanism that underlies apoptosis of GICs. We next examined whether FasL expression by allograft parenchymal cells<sup>4</sup> or residual donor-derived non-parenchymal cells, such as Kupffer cells,<sup>18</sup> creates an immunologically privileged status such as testis and eye, comparing the data from B6<sup>+/+</sup> donor livers with that of B6-*gld* donor livers. In the present study, MRL<sup>+/+</sup> recipients grafted with B6-*gld* hepatic allografts died significantly earlier than MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> hepatic allografts. Additionally, serum ALT levels of MRL<sup>+/+</sup> recipients with B6-*gld* donor livers significantly increased as compared with those of MRL<sup>+/+</sup> recipients with B6<sup>+/+</sup> donor livers. However, the histological differences on day 7 post-transplantation were not apparent between B6<sup>+/+</sup> and -*gld* donor hepatic allografts in MRL<sup>+/+</sup> recipients. The involvement of antibody, complement, cytokines, etc., may result in these discrepancies. Moreover, the number of TUNEL<sup>+</sup> GICs in B6<sup>+/+</sup> hepatic allografts in MRL<sup>+/+</sup> recipients was comparable to that in B6-*gld* hepatic allografts in MRL<sup>+/+</sup> recipients. These results indicate that FasL expression in hepatic allografts *per se* cannot create an immunoprivileged site. On the other hand, George *et al.*<sup>34</sup> demonstrated that expression of functional FasL on donor bone marrow cells can induce tolerance induction. Hepatic allografts have a relatively large number of passenger leucocytes<sup>35</sup> and probably contain pluripotent stem cells, such as bone marrow.<sup>36</sup> It is feasible that FasL-bearing passenger leucocytes or stem cells play an important role in the acceptability of hepatic allografts. Despite our study, the mechanism by which the donor-reactive T cells were deleted remains controversial. No lymphatic vessels could be reconstructed within the grafted livers in this system. However, the possibility that the donor APCs migrate to the lymphoid tissues and, thereafter, that the apoptosis of the recipient T cells is induced, cannot be excluded.

Conclusively, the Fas-FasL system plays a critical role in the spontaneous acceptability of hepatic allografts by deleting donor-specific CTLs, but not for CTL-target cell interactions in MRL<sup>+/+</sup> recipients. Thus, Fas-FasL interactions are involved in the early phase of tolerance induction in murine liver transplantation. The question remains as to why hepatic allografts are readily accepted in mice compared with other animals.

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