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Induction of Allograft Tolerance in the Absence of Fas-Mediated Apoptosis

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Using certain immunosuppressive regimens, IL-2 knockout (KO) mice, in contrast to wild-type (wt) controls, are resistant to the induction of allograft tolerance. The mechanism by which IL-2 regulates allograft tolerance is uncertain. As IL-2 KO mice have a profound defect in Fas-mediated apoptosis, we hypothesized that Fas-mediated apoptosis of alloreactive T cells may be critical in the acquisition of allograft tolerance. To definitively study the role of Fas in the induction of transplantation tolerance, we used Fas mutant B6.MRL- lpr mice as allograft recipients of islet and vascularized cardiac transplants. Alloantigen-stimulated proliferation and apoptosis of Fas-deficient cells were also studied in vivo. Fas mutant B6.MRL- lpr mice rapidly rejected fully MHC-mismatched DBA/2 (H-2d) islet allografts and vascularized cardiac allografts with a tempo that is comparable to wt control mice. Both wt and B6.MRL- lpr mice transplanted with fully MHC-mismatched islet allografts or cardiac allografts can be readily tolerated by either rapamycin or combined costimulation blockade (CTLA-4Ig plus anti-CD40L mAb). Despite the profound defect of Fas-mediated apoptosis, Fas-deficient T cells can still undergo apoptotic cell death in vivo in response to alloantigen stimulation. Our study suggests that: 1) Fas is not necessarily essential for allograft tolerance, and 2) Fas-mediated apoptosis is not central to the IL-2-dependent acquisition of allograft tolerance. The Journal of Immunology, 1999, 163: 2500–2507.

Tolerance to self Ags is established primarily in the thymus by deletion of autoreactive T cells through negative selection (1, 2). Similarly, tolerance to fully MHC-mismatched allografts and even xenografts can be achieved by creation of mixed hematopoietic chimerism, where deletion of donor-specific alloreactive T cells in the thymus is a principle mechanism through which tolerance is induced (3). Thus, it is generally believed that deletional mechanisms leading to allograft tolerance provide the most robust form of tolerance in transplantation. Is a deletional process involved in the establishment of peripheral allograft tolerance in the absence of immune system ablation and reconstitution? The answer remains enigmatic.

Recently, we have used rapamycin and others used costimulation blockade treatment to demonstrate that IL-2 is not required for rejection (4). In fact, IL-2 can be required for tolerance induction (5, 6). The precise mechanisms by which IL-2 regulates tolerance induction remain unknown. T cells in wild-type (wt) and IL-2 knockout (KO) mice express comparable levels of CTLA-4 upon activation (our unpublished observations), which has been shown to be critical for induction of peripheral tolerance (7). In stark contrast to wt mice, IL-2 KO mice exhibit a profound defect in Fas-mediated apoptosis, despite normal expression of Fas upon T cell activation (8). As IL-2 is absolutely required for priming activated T cells to undergo Fas-mediated apoptosis (9), it has been suggested that the failure of tolerance induction in IL-2 KO mice may be due to impaired Fas-mediated deletion of activated alloreactive T cells. This hypothesis is reinforced by the finding that cotransplantation of syngeneic myoblasts genetically engineered to express Fas ligand with islets allograft leads to long-term allograft survival, and T cell apoptosis induced by the Fas ligand-expressing myoblasts is the principle mechanism of long-term engraftment (10). Moreover, systemic administration of macrophages genetically constructed to express high levels of Fas ligand into allogeneic recipients promotes rapid apoptosis of alloreactive T cells, leading to Ag-specific T cell tolerance (11). George et al. (12) recently demonstrated, in a bone marrow-induced skin allograft tolerance model, that: 1) expression of Fas ligand by donor bone marrow cells is critical for tolerance induction; and 2) apoptosis of alloreactive T cells is essential in the establishment of tolerant status in this model.

To definitively study whether Fas expression is an absolute requirement for the induction and maintenance of IL-2-dependent allograft tolerance that occurs with rapamycin or costimulation treatment, we studied the allograft response in Fas mutant B6.MRL- lpr mice as transplant recipients. We also investigated the alloantigen-driven T cell proliferation and activation-induced cell death (AICD) in vivo. We found that: 1) allograft tolerance can be induced in the absence of Fas expression, 2) alloantigen-activated and Fas-deficient T cells can still undergo apoptotic cell death in vivo, and 3) Fas-mediated apoptosis is not central to IL-2-dependent acquisition of allograft tolerance.
Table I.  Survival of DBA/2 islet allografts in wt control and Fas mutant MRL-lpr recipient mice a

<table>
<thead>
<tr>
<th>Donors</th>
<th>Recipients</th>
<th>n</th>
<th>Treatment</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2</td>
<td>wt control</td>
<td>5</td>
<td>None</td>
<td>10, 13, 13, 18</td>
</tr>
<tr>
<td>DBA/2</td>
<td>wt control</td>
<td>4</td>
<td>Rapamycin</td>
<td>&gt;100, &gt;100, &gt;100, &gt;100</td>
</tr>
<tr>
<td>DBA/2</td>
<td>wt control</td>
<td>6</td>
<td>CTLA-4Ig + MR1</td>
<td>&gt;100, &gt;100, &gt;100, &gt;100, &gt;100</td>
</tr>
<tr>
<td>DBA/2</td>
<td>B6.MRL-lpr</td>
<td>7</td>
<td>None</td>
<td>9, 9, 9, 11, 11, 13, 14</td>
</tr>
<tr>
<td>DBA/2</td>
<td>B6.MRL-lpr</td>
<td>6</td>
<td>Rapamycin</td>
<td>15, 32, &gt;100, &gt;100, &gt;100, &gt;100</td>
</tr>
<tr>
<td>DBA/2</td>
<td>B6.MRL-lpr</td>
<td>3</td>
<td>CTLA-4Ig + MR1</td>
<td>&gt;100, &gt;100, &gt;100</td>
</tr>
</tbody>
</table>

a Crude islets (300–400) from DBA/2 donors were transplanted under the kidney capsule into diabetic wt and B6.MRL-lpr recipients. Some recipient mice were treated with rapamycin (0.2 mg/kg/day for 14 days) or combined CTLA-4Ig (0.25 mg i.p. on posttransplant day 2) and anti-CD40L mAb (0.2 mg i.p. on posttransplant days 0, 2, and 4).

Materials and Methods

Animals

Eight- to ten-week-old male DBA/2 (H-2b) mice, B6.MRL−/− wt (H-2b), and Fas mutant B6.MRL-lpr/lpr mice (H-2b) were obtained from the Jackson Laboratory (Bar Harbor, ME).

Islet cell transplantation

Islet transplantation was performed as described previously (13). Briefly, pancreatic islets were isolated from donor DBA/2 (H-2b) mice via collagenase digestion and subsequent ficoll gradient centrifugation method. Crude islets (300–400) were transplanted under the renal capsule into wt mice (H-2b) and Fas mutant B6.MRL-lpr/lpr mice (H-2b) rendered diabetic by a single i.p. injection of streptozotocin (225 mg/kg, Sigma, St. Louis, MO). Allograft function was monitored by sequential blood glucose measurements. Primary graft function was defined as blood glucose levels under 100 mg/dl on day 3 posttransplantation, and graft rejection was defined as a rise in blood glucose levels exceeding 300 mg/dl following a period of primary graft function.

Vascularized heterotopic cardiac transplantation

Heterotopic cardiac transplantation was performed (14). Briefly, cardiac grafts from DBA/2 donors were prepared by ligating the pulmonary veins and the superior vena cava. The grafts were harvested by dividing and excising the aorta and the pulmonary artery. The cardiac grafts were then transplanted into recipient mice by suturing donor aorta and donor pulmonary artery end to side to the recipient’s abdominal aorta and vena cava, respectively. Graft function was monitored every other day following transplantation by transabdominal palpation and scored on a scale of 1 to 100, based on the strength and the rate of impulses. Graft rejection was defined by a complete cessation of palpable beat and confirmed by direct visualization upon laparotomy.

Reagents and tolerizing protocols

Rapamycin was kindly provided by Dr. Suren Sehgal (Wyeth-Ayerst, Princeton, NJ) and prepared in carboxymethylcellulose (Sigma), as recommended by the company. Murine CTLA-4Ig was constructed and expressed in our laboratory as previously described (15). A B cell hybridoma producing MR1 (IgG2a) hamster anti-mouse CD40L mAb was obtained from American Type Culture Collection (Manassas, VA). The hybridoma cells were grown in serum free UltraCulture medium (BioWhittaker, Walkersville, MD). MR1 mAb was purified using protein G columns (Sigma).

Treatment with rapamycin consisted of 0.2 mg/kg/day i.p. for the first 3 days after transplantation, followed by every other day for 14 days. Combined costimulation blockade treatment consisted of CTLA-4Ig at 0.25 mg i.p. on posttransplant day 2 and anti-CD40L mAb at 0.2 mg i.p. on posttransplant days 0, 2, and 4.

Analysis of Fas expression

Spleenic leukocytes were prepared from wt control mice and Fas mutant B6/MRL-lpr mice. In some experiments, cells were activated with anti-CD3 (2 μg/ml, 145-2C11; PharMingen, San Diego, CA) in vitro for 3 days. Cells were stained with a PE-conjugated anti-mouse Fas Ab (Jo-2, PharMingen) on ice for 20 min, followed by washing the cells twice in PBS/0.5% BSA. An isotype-matched hamster IgG2a (PharMingen) was used in the staining as a control. Cells were then analyzed with FACSORT (Becton Dickinson, Mountain View, CA). Results are presented in histograms.

Analysis of AICD

AICD in vitro was analyzed by annexin V staining (16). Briefly, splenic leukocytes (2 × 10^7/ml) prepared from wt and Fas mutant B6/MRL-lpr mice were activated with anti-CD3 (2 μg/ml, 145-2C11; PharMingen) in vitro for 3 days. Viable T cell blasts were isolated using Lympholyte-M medium (Cedarlane, Ontario, Canada) and then cultured on anti-Fas (Jo-2; PharMingen)-coated plates for 8 h. Cells cultured on control hamster IgG-coated plates were used as a control. Cells were harvested, stained with PE-conjugated annexin V (PharMingen) on ice for 15 min, and analyzed by FACSort (Becton Dickinson).

Tracking of 5-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled lymphocytes in vivo

Spleens and lymph nodes from wt and B6.MRL-lpr mice were harvested and single cell suspension prepared in HBSS by gently pressing the tissue through a fine metal mesh. RBC were lysed by hypotonic shock. Lymphocytes were washed and resuspended in serum-free HBSS at 1 × 10^7/ml for labeling with a tracking fluorochrome CFSE (CFDASE; Molecular Probes, Portland, OR), as previously described (17, 18). Briefly, CFDASE was dissolved in DMEM, and an aliquot was added into the cell suspension at a final concentration of 5 μm. Cells were incubated at room temperature for 6 min, and the labeling was terminated by the addition of one-tenth of the volume of FCS. Cells were then washed twice in cold RPMI 1640 medium supplemented with 10% FCS and resuspended in HBSS for i.v. injection.

CFDASE is a lipophilic chemical that can passively diffuse into the plasma membrane of living cells, where it is converted to a green fluorochrome CFSE by esterase hydrolysis. CFSE forms stable complex with cellular proteins and cannot diffuse out of the cells. As cells divide, CFSE segregates equally between two daughter cells. Thus, the fluorescent intensity of CFSE halves consecutively with each cell division, allowing precise monitoring of the mitotic activity of cells in vivo (17, 18).

DBA/2 mice were lethally irradiated (1000 rad) with a GammaCell (Ontario, Canada) irradiator immediately before i.v. injection of CFSE-labeled lymphocytes. Each mouse then received 4–6 × 10^7 labeled cells suspended in 0.2 ml of HBSS (containing 20% FCS and 50 μg/ml gentamycin) i.v.

![FIGURE 1](http://www.jimmunol.org/) Induction of allograft tolerance in rapamycin-treated B6.MRL-lpr recipients. Nephrectomy of the islet graft-bearing kidney in three B6.MRL-lpr recipients 100 days after transplantation led to a sharp rise of blood glucose levels. Each of the three nephrectomized recipient mice accepted a second DBA/2 islet allograft without any immunosuppression.
via the penile vein. Mice were sacrificed 2–3 days after i.v. injection of labeled lymphocytes, spleens were harvested from host mice, and a single cell suspension was prepared. The cellular pattern of proliferation in vivo, as reflected by distinct CFSE profile, was analyzed by flow cytometry. Data were collected and analyzed by gating onto the CFSE-positive cells.

Analysis of cell divisions and apoptosis of alloreactive T cells in vivo

CFSE-labeled cells (4–6 × 10⁷/mouse) from wt and B6.MRL-lpr mice were injected i.v. into lethally irradiated DBA/2 hosts. Splenic leukocytes from the host mice were prepared 2 days later. Cells were stained with a biotinylated anti-mouse CD4 mAb (GK1.5; PharMingen) on ice for 20 min, followed by staining with streptavidin-CyChrome (PharMingen) and PE-conjugated annexin V on ice for 15 min. Cells were washed twice in annexin labeling buffer. Proliferation and apoptotic cell death of CD4⁺ T cells in each distinct cell division were analyzed by flow cytometry. Data were collected and analyzed by gating onto CD4⁻CFSE⁺ cells.

Results

To study the allograft response in the absence of Fas expression, we transplanted fully MHC-mismatched DBA/2 (H-2d) islet allografts into Fas mutant B6.MRL-lpr (H-2b) mice and compared the rejection with that of wt control (H-2b) recipients. As shown in Table I, wt control mice rejected DBA/2 islet allografts with a mean survival time (MST) of 13 days (n = 5), and Fas mutant B6.MRL-lpr mice rejected the DBA/2 islet allografts with an MST of 11 days (n = 7). Histological examination of rejecting islet allografts in B6.MRL-lpr mice on posttransplant day 8 revealed dense mononuclear infiltration, which was essentially identical to that observed in wt control recipients (data not shown). Thus, expression of Fas by transplant recipients is not required for the execution of allograft rejection.

To probe the amenability of Fas mutant B6.MRL-lpr mice to IL-2-dependent allograft tolerance, we treated allograft recipients with rapamycin (0.2 mg/kg/day) starting on the day of transplantation for 14 days. Treatment with rapamycin, a potent blocker of growth factor signals required for optimal T cell proliferation (19), does not lead to allograft tolerance in IL-2 KO mice (5). In keeping with our previous report (20), rapamycin is remarkably effective in producing long-term allograft survival, and all rapamycin-treated wt control mice experienced permanent engraftment of DBA/2 islets (MST > 100 days, n = 4). Interestingly, treatment of B6.MRL-lpr recipients with rapamycin also induced long-term islet allograft survival (MST > 100 days, n = 6) (Table I).

Nephrectomy of the left kidney (the islet allografts were placed under the renal capsule) in three B6.MRL-lpr recipients 100 days after transplantation led to a sharp rise in the blood glucose levels, demonstrating that euglycemia was maintained by the islet allografts. Each of the three nephrectomized mice accepted a second DBA/2 islet allograft without any immunosuppression (Fig. 1), indicating a state of stable allograft tolerance.

To determine whether the susceptibility to tolerance induction in Fas mutant B6.MRL-lpr mice is unique to rapamycin, we treated recipient mice with combined CTLA-4Ig and anti-CD40L mAb, a powerful tolerizing therapy, as previously reported (20, 21). As shown in Table I, combined CTLA-4Ig and anti-CD40L treatment

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Expression of Fas by splenic leukocytes from wt control mice and Fas mutant B6.MRL-lpr mice analyzed by FACS. A, Resting splenic leukocytes. B, Anti-CD3-activated splenic leukocytes.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Fas-mediated apoptosis of activated T cells in vitro. Splenic leukocytes from wt and Fas mutant B6.MRL-lpr mice were stimulated with anti-CD3 (2C11, 2 μg/ml) for 3 days. Activated T cells were isolated and further cultured on anti-Fas-coated plates (Jo-2, 2 μg/ml) for 8 h. Apoptotic cell death was analyzed by annexin V staining. Cells cultured on control hamster IgG were included as a control.

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Table II. Survival of DBA/2 cardiac allografts in control and Fas mutant B6.MRL-lpr recipient mice

<table>
<thead>
<tr>
<th>Donors</th>
<th>Recipients</th>
<th>n</th>
<th>Treatment</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2J</td>
<td>wt control</td>
<td>3</td>
<td>None</td>
<td>11, 14, 14</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>B6.MRL-lpr</td>
<td>3</td>
<td>None</td>
<td>8, 16, 16</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>B6.MRL-lpr</td>
<td>5</td>
<td>Rapamycin</td>
<td>61, &gt;100, &gt;100, &gt;100, &gt;100</td>
</tr>
</tbody>
</table>

a Some cardiac allograft recipients were treated with rapamycin at 0.2 mg/kg/day for 14 days.
produced permanent islet allograft survival in both wt and Fas mutant B6.MRL-lpr mice (MST > 100 days, n = 3–6).

To prove that tolerance induction in Fas mutant B6.MRL-lpr mice is not restricted to the islet allografts, vascularized DBA/2 (H-2b) cardiac allografts were transplanted into Fas mutant B6.MRL-lpr mice (H-2b) and treated with rapamycin. As shown in Table II, untreated B6.MRL-lpr mice rejected the DBA/2 cardiac allografts with a MST of 13 days (n = 3) with no marked difference, compared with wt control mice (MST = 13 days, n = 3). Similar to our finding in the islet model, treatment with rapamycin produced long-term cardiac allograft survival in both wt and Fas mutant B6.MRL-lpr recipient mice (MST > 100 days, n = 3–5).

To confirm that Fas is truly defective in the B6.MRL-lpr mice used in the present study, we first stained for Fas expression on resting or anti-CD3-activated splenocytes. In contrast to cells from wt control mice, resting and anti-CD3-activated splenocytes from Fas mutant B6.MRL-lpr mice did not express Fas (Fig. 2). Furthermore, anti-CD3-activated T cells from B6.MRL-lpr mice failed to undergo apoptotic cell death when cultured on anti-Fas coated plates. In contrast, apoptotic cell death was readily induced with the anti-Fas mAb in activated T cells from wt mice (Fig. 3).

Clearly, Fas-mediated apoptosis is neither required for rejection nor required for induction of allograft tolerance occurring with rapamycin or costimulation blockade treatment. To probe the possible mechanism of Fas-independent allograft tolerance, we administered high doses of IL-2/Fc (5 μg i.p. daily for 10 days), a long-lived IL-2 and IgGFC fusion protein (τ1/2 > 24 h, as compared with few min for native IL-2) (22), into 3 Fas mutant lpr recipients 100 days after cardiac transplantation. This course of IL-2/Fc can successfully abolish tolerance induction with costimulation blockade (22). As shown in Fig. 4, systemic IL-2/Fc failed to provoke cardiac allograft rejection, suggesting that the tolerant status is unlikely to be maintained by IL-2-sensitive T cell anergy.

As peripheral T cell deletion can occur in Fas mutant mice and activated T cells from lpr mice can undergo apoptotic cell death upon cross-linking of their TCR in vitro with anti-CD3 (23), we wondered if AICD occurs in B6.MRL-lpr recipient mice in response to alloantigen stimulation. To probe this possibility, we first assessed the proliferation capacity of donor-specific alloreactive cells in rapamycin-tolerized B6.MRL-lpr recipient mice and compared that with untreated B6.MRL-lpr controls. Splenocytes from tolerized B6.MRL-lpr mice (120 days after islet transplantation) and from control mice were labeled with CFSE and injected into irradiated DBA/2 hosts. As cells that proliferate to donor alloantigen in vivo can be precisely followed through analysis of their CFSE profiles, the magnitude and tempo of cell proliferation in tolerized allograft recipients and in untreated control mice can be directly compared (17, 18). This analysis provides a relative index of Ag-induced T cell activation under defined experimental conditions. As shown in Fig. 5, CFSE-labeled lymphocytes from control B6.MRL-lpr mice proliferated vigorously in irradiated DBA/2 hosts. Seven discrete generations of cell proliferation were observed 2 days after cell transfer. By day 3, the majority of the alloreactive cells have divided seven to eight times. In contrast, lymphocytes from rapamycin-tolerized lpr mice showed minimal proliferation in vivo in response to the donor alloantigens, indicating a decreased frequency of proliferating alloreactive cells in response to the donor alloantigens in vivo in tolerized recipients.

Does a Fas-independent pathway of AICD exist in vivo? To precisely follow apoptosis of Fas-deficient lymphocytes in vivo in response to alloantigen stimulation, we stained CFSE-labeled CD4+ B6.MRL-lpr T cells with PE-conjugated annexin V after recovery from DBA/2 hosts. Annexin V binds to cell membrane-associated phosphorylserine, which is restricted to the interior side of the cell membrane in living cells and is exposed to the exterior side of cell membrane in the early stages of apoptotic cell

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**FIGURE 4.** IL-2/Fc failed to provoke cardiac allograft rejection in rapamycin-tolerized Fas mutant lpr recipients. IL-2/Fc was given i.p. at 5 μg/ daily for 10 days starting on posttransplant day 100.

**FIGURE 5.** Analysis of donor-specific alloreactive lymphocytes in vivo in tolerized B6.MRL-lpr recipient mice. Splenocytes from control B6.MRL-lpr mice and rapamycin-tolerized B6.MRL-lpr mice were labeled with CFSE and injected into irradiated DBA/2 hosts. Cell proliferation was analyzed by the fluorescent profiles of CFSE-labeled cells.
death. As shown in Fig. 6, seven distinct generations of CFSE-labeled CD4\(^+\) T cells were identified 2 days after i.v. injection of B6.MRL-\(lpr\) cells into irradiated DBA/2 hosts. Each generation of T cells was then gated and analyzed for apoptotic cell death. Interestingly, the percentage of annexin V-positive cells increased with each cell division. In the undivided mother cell generation, 12% of the cells were annexin V-positive. In the sixth generation, however, 47% of the cells were positive for annexin V staining. Analysis of CFSE-labeled CD4\(^+\) T cells from wt control mice showed a similar pattern of annexin V staining profile (Fig. 7). This finding suggests that T cells become increasingly susceptible to apoptotic cell death with each wave of proliferation, despite the lack of Fas expression.

### Discussion

The execution of AICD via Fas is of fundamental importance in the homeostasis of the immune system, especially in the periphery. Indeed, functional defect of Fas-mediated apoptosis in the presence of certain background genes leads to lymphoproliferative disorders and autoimmunity as manifested in Fas mutant \(lpr\) and Fas
ligand mutant *gld* mice (24). In certain models, Fas-triggered apoptosis of activated T cells has been suggested to be a key mechanism in the induction of allograft tolerance (10, 25). Our interest in the possibility that Fas-mediated apoptosis may play a central role in the acquisition of peripheral allograft tolerance was kindled by the inability of our lab using rapamycin (5) and others using costimulation blockade (6) to achieve allograft tolerance in IL-2 KO mice. IL-2 KO mice have a gross defect in Fas-mediated apoptosis and others have emphasized the importance of Fas in allograft tolerance in certain models (10, 25). Is Fas-triggered apoptosis of alloreactive T cells a prerequisite for the acquisition of peripheral allograft tolerance? In the present study, we have demonstrated, by using two distinct transplantation models (nonvascularized islet allografts and vascularized cardiac allografts), that Fas-mediated apoptosis is not essential in the acquisition of transplantation tolerance, as permanent engraftment of fully MHC-mismatched islet cell allografts and vascularized cardiac allografts can be readily induced in the Fas mutant B6.MRL- *lpr* mice by two different tolerizing protocols (rapamycin and combined costimulation blockade). The tolerability of Fas mutant mice to allografts is remarkably similar to wt control mice (Tables I and II). Fas is truly defective in B6.MRL- *lpr* recipient mice used in the present study, as lymphocytes from these mice do not express Fas, regardless of the activation status of the cells, and activated T cells from

**FIGURE 7.** Proliferation and apoptosis of wt T cells in vivo in response to alloantigen. Splenic lymphocytes from wt control mice were labeled with CFSE and injected i.v. into irradiated DBA/2 hosts. Two days later, spleens from DBA/2 hosts were harvested and single cell suspension prepared. Cells were then stained with Cy-Chrome-anti-CD4 and PE-annexin V. Proliferation and apoptosis of CFSE-labeled CD4*+* T cells among distinct generations were analyzed by flow cytometry. Representative data of three experiments are shown.
lpr mice failed to undergo Fas-triggered apoptosis in vitro (Figs. 2, 3). In addition, Fas expression by recipient mice is not required for robust T cell activation and the execution of acute allograft rejection. The Fas/Fas ligand interaction is one of the pathways in the execution of CTL functions; however, the absence of Fas has no apparent effect on the tempo of acute allograft rejection.

Since the elucidation of a critical role of Fas/Fas ligand in conferring the immune privilege in the anterior chamber of the eyes and the testis (25, 26), there has been great interest in constructing Fas ligand-expressing allografts as a mean to promote allograft tolerance by killing graft-infiltrating Fas-positive T cells. Clearly, high levels of Fas ligand expression by the parenchymal cells of the testis and anterior chamber of the eyes are required for conferring the immune privileged status of these organs (25, 26). Elegant studies using Fas mutant and Fas ligand mutant mice have also demonstrated that transplantation of allogeneic, or even xenogeneic, tissues that express high levels of Fas ligand into the testis or the anterior chamber of the eye prevented rejection of these tissues, while tissues from Fas ligand mutant mice were rapidly rejected (25). While Fas/Fas ligand interaction is clearly critical in the tolerant status in some situations, our study suggests that expression of Fas by recipient lymphocytes is not a prerequisite for the induction and maintenance of peripheral allograft tolerance. Yet, Fas/Fas ligand interactions play a minimal role in preventing allograft rejection outside of the immune-privileged sites (27). Apparently, the unique local microenvironment of immune-privileged sites, aside from the expression of Fas ligand, must also play an active role in conferring the tolerant status. Indeed, induction of Ag-specific tolerance in the privileged sites is often associated with production of high levels of immunoregulatory cytokines, such as TGF-β (28). Thus, the mechanisms underlying immune privilege and the role of Fas/Fas ligand in the induction of tolerant status other than the privileged sites warrant further study.

Another key finding of our study is that the Fas-mediated apoptosis is not central to the IL-2-dependent mechanisms governing the acquisition of allograft tolerance. Interestingly, the phenotype of Fas mutant B6.MRL-lpr mice and IL-2 KO mice share several common attributes. Both strains of mice develop lymphoproliferative disorders, autoimmunity, and accumulation of T cells with activated phenotype in the periphery (24, 29). The critical role of IL-2 for the functional integrity of Fas is further underscored by the finding that Fas-triggered apoptosis of activated T cells requires active IL-2 priming (9). Although the precise mechanism by which IL-2 regulates AICD remains to be firmly defined, a recent study suggested that IL-2 can down-regulate the expression of cellular Fas-associated death domain-like IL-1-converting enzyme (FLICE) inhibitory protein (FLIP) (30). Structurally, FLIP has two death effector domains (DED) and a caspase 8-like domain (31). FLIP can dimerize with Fas-associated death domain protein (FADD) and/or caspase 8 via DED-DED interaction, which prevents the recruitment of death effector caspases downstream of FADD (31). Thus, in the absence of IL-2, FLIP is believed to be expressed at high levels, and therefore, FADD cannot recruit key caspases to execute AICD upon ligand binding (30, 31). Despite the intimate association of IL-2 with the functional integrity of Fas, it is fascinating that allograft tolerance can be readily induced in Fas mutant B6.MRL-lpr mice, whereas IL-2 KO mice are totally resistant to the induction of allograft tolerance (5, 6). This finding raises intriguing questions about the precise nature of IL-2-dependent processes that govern the acquisition of peripheral allograft tolerance.

We still favor the possibility that IL-2-regulated apoptotic events may be required for the acquisition of peripheral allograft tolerance. It has been shown that delivery of IL-2 into Fas mutant lpr mice through a recombinant viral vector can induce deletion of autoreactive T cells and remission from autoimmunity (32). In the present study, we also clearly show that Fas-deficient lymphocytes can still undergo AICD in vivo in response to alloantigen stimulation, and this type of AICD appears to be cell cycle-dependent (Fig. 6). IL-2-regulated AICD, a critical component of immune regulation, is not confined to the Fas pathway. The Fas/Fas ligand interaction is a part, but certainly not the entirety, of this network. For example, IL-2 KO mice have a defect in superantigen-triggered AICD that is Fas-independent (our unpublished observation). Although defects in Fas or Fas ligand are permissive for autoimmunity, most Fas- or Fas ligand-defective mouse strains do not suffer from overt autoimmunity. Our study further illustrates the limitations of Fas/Fas ligand-triggered apoptosis in determining peripheral allograft tolerance, as defective expression of Fas did not lead to a precipitous decline in AICD or inability to tolerance induction. Clearly, IL-2 influences multiple aspects of T cell apoptosis. The apoptotic pathways are extremely redundant and complex, at least five distinct death receptors have been identified (Fas, TNF receptor type I, DR3, DR4, and DR5), and each can mediate apoptotic cell death (33). Alternatively, IL-2 may be required for the generation of regulatory cells that are essential to contain the alloreactivity to the allografts. In the absence of IL-2 during a critical stage of immune activation, a key regulatory process may not be developed by allograft recipients, and therefore, acquisition of allograft tolerance may prove to be extremely difficult. These possibilities are being actively pursued in the lab.

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