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J Immunol 1999; 162:1423-1430;
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Induction of Specific T Cell Tolerance by Fas Ligand-Expressing Antigen-Presenting Cells

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Autocrine interaction of Fas and Fas ligand leads to apoptosis of activated T cells, a process that is critical for the maintenance of peripheral T cell tolerance. Paracrine interactions of Fas ligand with T cells also may play an important role in the maintenance of tolerance, as Fas ligand can create immune-privileged sites and prevent graft rejection by inducing apoptosis in T cells. We surmised that APCs that express Fas ligand might directly induce apoptosis of T cells during presentation of Ag to the T cells, thus inducing Ag-specific, systemic T cell tolerance. Here, we show that profound, specific T cell unresponsiveness to alloantigen was induced by treatment of H-2 b mice with H-2 b APCs that expressed Fas ligand and that profound T cell unresponsiveness specific for the H-Y Ag was induced by treatment of H-2 b/H-Y TCR transgenic female mice with H-2 b/H-Y APCs that expressed Fas ligand. The induction of this systemic T cell tolerance required the expression of Fas ligand on the APCs as well as the expression of Fas on the T cells. The tolerance was restricted to the Ag presented by the APCs. The rapid and profound clonal deletion of the Ag-specific, peripheral T cells mediated by the Fas ligand-expressing APCs contributed to the induction of tolerance. These findings demonstrate that Ag-specific T cell tolerance can be induced by APCs that express Fas ligand and suggest a novel function for APCs in the induction of T cell apoptosis. Furthermore, they indicate a novel immunointervention strategy for treatment of graft rejection and autoantigen-specific autoimmune diseases. The Journal of Immunology, 1999, 162: 1423–1430.

Antigen-presenting cells (APCs) play an important role both in the initiation of the T cell response and in the induction of T cell tolerance (1–3). Induction of complete T cell activation and proliferation requires the provision of two signals by the APCs. In the absence of the second signal, the so-called costimulatory signal, the T cells become anergic (4–7). APCs are also able to guide the CD4 T cell response, skewing it toward either a predominantly Th1 or Th2 response and influencing the development of several autoimmune diseases (8–13). The combination of the different Ag-presenting functions of the APCs determines the profile of the T cell response; thus, APCs can determine whether an immune response is immunogenic or tolerogenic (14).

The ability of APCs to influence the response to allografts is well established (15). It has long been recognized that the graft rejection reaction is most intense in tissues that contain lymphoreticular elements, but is relatively less intense in other tissues, such as muscle (16). Removal of APCs from graft tissue before transplantation greatly diminishes the characteristic rejection response (17, 18). Both purified MHC class I and class II alloantigens elicit only a weak response unless presented directly by viable donor APCs (19). Decreased expression of either MHC Ags or costimulatory molecules, such as B7, on donor APCs greatly increases the survival of allogeneic grafts (20, 21). Thus, direct Ag presentation by viable donor APCs plays a critical role in the initiation of graft rejection through the induction of a strong T cell response.

The importance of Fas-mediated apoptosis in the maintenance of T cell tolerance and the prevention of autoimmune disease has been demonstrated by the finding that mutations of the Fas or Fas ligand genes lead to autoimmune disease in lpr/lpr and gld/gld mice, respectively (22, 23). Both clonal deletion of peripheral T cells after Ag stimulation (24) and the maintenance of T cell tolerance to self-Ag and superantigen are defective in Fas-deficient lpr/lpr mice (25–27). Furthermore, correction of the Fas-mediated apoptosis defect in T cells by expression of a fas transgene prevents autoimmune disease in lpr/lpr mice and an age-related defect in T cell apoptosis in aged mice (28, 29). Fas is expressed on the cell surface and mediates apoptosis when ligated by Fas ligand or agonistic anti-Fas Ab (30–32). The induction of activation-induced cell death (AICD) occurs through an autocrine response involving Fas and Fas ligand expressed by the individual T cells (33–35), indicating the significance of Fas-mediated apoptosis in the maintenance of T cell tolerance.

Fas-mediated apoptosis of APCs contributes to down-regulation of the immune response. Activated T cells express elevated levels of Fas ligand and induce apoptosis of APCs (36). On the other hand, activated macrophages express Fas ligand and are able to induce apoptosis of the T cells (37). For example, the high level of expression of Fas ligand by HIV-infected macrophages has been implicated in the depletion of CD4-positive T cells in AIDS (38). It has been suggested that Fas ligand expression on dendritic cells plays a critical role in regulation of the T cell response (39, 40).

Received for publication June 8, 1998. Accepted for publication October 9, 1998.

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1 This work was supported in part by grants from the Arthritis Foundation, the Juvenile Diabetes Foundation, and the National Institutes of Health (AR44982) and by a grant from Sankyo Co. of Japan.

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3 Abbreviations used in this paper: AICD, activation-induced cell death; Mφ-FL, macrophages transfected with Fas ligand; Mφ-CV, macrophages transfected with the control vector; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.

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Fas-mediated apoptosis also plays a role in the maintenance of immunoprivileged sites. The immunoprivileged status of the testis and anterior chamber of the eye requires a high level of expression of Fas ligand in the parenchymal cells of these organs (41). In this situation, it has been suggested that the expression of Fas ligand by the parenchymal cells protects these tissues from destruction by T cells through induction of apoptosis of the T cells (42). As inoculation of virus into the anterior chamber of the eye leads to systemic T cell tolerance to the virus, the immune-privileged status of this site may involve induction of systemic T cell tolerance in addition to induction of local T cell tolerance (43). It has been proposed that the APCs that express Fas ligand together with the privileged Ag that are released from the immune-privileged sites mediate apoptosis of the peripheral T cells, thus inducing systemic T cell tolerance. Transplantation of allogeneic or xenogeneic tissues that do not express Fas ligand into the testis or the anterior chamber of the eye prevented rejection of these tissues (44–47). Direct evidence for the role of Fas ligand in the prevention of graft rejection has been provided by the finding that implantation of syngeneic muscle cells that express Fas ligand around allogeneic grafted islets leads to long term acceptance of the transplanted islets as well as local induction of T cell apoptosis by the Fas ligand-expressing cells around the graft (48). Maintenance of tolerance to the graft required the presence of expression of Fas ligand by the syngeneic muscle cells. This finding suggests a practical approach to the prevention of graft rejection in transplantation through manipulation of Fas ligand-mediated apoptosis. The function of Fas ligand in the grafts has been questioned, however; rather than conferring prolonged survival, it may induce an inflammatory response (49–51). Thus, the mechanisms underlying immune privilege have not been elucidated fully.

In the present study we sought to determine whether APCs that express Fas ligand induce systemic and Ag-specific T cell tolerance by mediating depletion of Ag-specific T cells. Our results demonstrate that treatment with allogeneic APCs that express Fas ligand induces a profound alloantigen-specific T cell unresponsiveness. Using H-2D\(^d\)/H-Y TCR transgenic mice, we show that this rapid and profound depletion of Ag-specific T cells contributes to the induction of systemic T cell tolerance.

Materials and Methods

Animals
MRL+/− mice, MRL-lpr/lpr mice, and C57BL/6-lpr/lpr mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility. C57BL/6 H-2D\(^d\)/H-Y-reactive TCR transgenic +/+ and lpr/lpr mice were generated as described previously (25).

Generation of macrophage cell line and transfection for Fas ligand expression
Macrophages were isolated from the peritoneal cavity of C57BL/6-lpr/lpr mice as described previously. Isolated macrophages were stimulated with LPS (100 ng/ml) for 24 h every 10 days. After three cycles the macrophages were grown in 10% FCS-RPMI 1640 in preparation for transfection. Macrophages (5 × 10\(^6\)/ml) were electroporated with purified pcDNAIII plasmid (10 μg) containing full-length murine Fas ligand cDNA at 960 mF, 250 mV using a gene purser (Bio-Rad, Hercules, CA). Transfected cells were cultured in 10-cm culture dishes for 48 h and selected with 0.5 mg/ml G418 for 4 wk.

Preparation of H-2D\(^d\)/H-Y cells expressing Fas ligand
Male or female C57BL/6-lpr/lpr, 8–12 wk of age, were injected i.p. with 1 ml of Pristane (Sigma, St. Louis, MO) to facilitate production of macrophages. The peritoneal macrophages were prepared as described previously (52). The isolated macrophages were transfected with a recombinant adenoviruses expression system (53).

Phenotypic analysis by flow cytometric analysis
Anti-CD3 (clone 145.2C11), anti-CD4 (clone GK 1.5), anti-CD8 (clone 53–47), anti-Mac-1 (clone M70), anti-Fas (clone Jo2), anti-IA\(^b\) (clone AP6–120.1), and anti-H-2D\(^d\) (clone 28–14–8) were purchased from PharMingen (San Diego, CA). The anti-D\(^d\)/H-Y TCR clone M33 was produced as described previously (25). Single cell suspensions of spleen cells were labeled with optimal concentrations of FITC-conjugated anti-CD8, PE-conjugated anti-CD3, (PharMingen, San Diego, CA), and biotin-conjugated M33 followed by Tandem-streptavidin (Southern Biotechnology Associates, Birmingham, AL). Viable cells (10,000/sample) were analyzed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA) equipped with logarithmic scales, and the data were processed using a Hewlett-Packard (Palo Alto, CA) computer. The number of cells in each population was determined by quadrant analysis of contour graphs. Phycoerythrin-conjugated anti-murine Fas ligand Ab (clone: MFL3) was purchased from PharMingen. The Fas ligand-transfected and control macrophages (10\(^6\)) were stained with 1/50 diluted anti-Fas ligand Ab on ice for 20 min. Ten thousand viable cells were analyzed.

\(^{51}\)Cr release assay for Fas ligand activity
A murine B lymphoma cell line (A20), which is very sensitive to Fas ligand-induced cytotoxicity, was used as the target cell in this cytotoxicity assay. A20 cells (5 × 10\(^5\)/ml) were incubated with 0.3 mCi of \(^{51}\)Cr sodium in complete medium (1 ml) for 45 min. After thorough washing, labeled A20 cells (1 × 10\(^6\)) were incubated with effector cells at different E/T cell ratios starting at 10:1 in 200 μl of complete medium for 12 h. The same number of labeled A20 cells were cultured in 200 μl of complete medium alone to determine spontaneous release and in complete medium with 0.01% SDS to determine maximum release. One hundred microliters of supernatant was collected and counted in a gamma counter. Specific release was calculated as follows: specific release (%) = (cpm of sample − cpm of minimum release)/cpm of maximum release × 100%.

Induction of allogeneic T cell tolerance by Fas ligand expressing macrophages
Female MRL+/+ and lpr/lpr mice, 4–6 wk of age, were injected i.p. with 2 × 10\(^7\) macrophages transfected with Fas ligand expression vector or macrophages with empty vector as a control. The injection was repeated every 3 days for six times. Three days after the last injection, mice were sacrificed for evaluation of tolerance induction.

Induction of H-2D\(^d\)/H-Y-specific T cell tolerance in TCR transgenic mice
Female H-2D\(^d\)/H-Y TCR transgenic +/+ and lpr/lpr mice, 4–6 wk of age, were injected once i.p. with 2–5 × 10\(^7\) adenovirus-transfected macrophages. T cell tolerance was analyzed on days 1–14 after the injection.

Analysis of the T cell proliferative response
To determine the allogeneic T cell response, T cells were purified from the spleen and peripheral lymph nodes of treated MRL+/+ and lpr/lpr mice using a T cell enrichment column. Purified T cells (5 × 10\(^6\)) were cultured in 96-well, round-bottom plates in a total volume of 200 μl with medium alone or with an equal number of gamma-irradiated (3300 rad) allogeneic spleen cells from C57BL/6 (H-2\(^b\)) or BALB/c (H-2\(^d\)) mice. For analysis of the H-2D\(^d\)/H-Y-specific T cell response, spleen T cells were purified from treated TCR transgenic mice as described above and were cultured in the presence of 50 U/ml of murine IL-2 (Genzyme, Cambridge, MA) for the indicated time with irradiated (3300 rad) syngeneic spleen cells obtained from C57BL/6 male or female mice. At the indicated time points, 1 μCi of \(^{3}H\) thymidine was added; the cells were harvested 16 h later, and incorporation of \(^{3}H\) thymidine was determined using a scintillation counter.

In situ terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) apoptosis staining
An apoptosis staining kit was purchased from Amersham (Arlington Heights, IL). Paraffin sections of the spleen were stained according to the manufacturer’s instructions.
FIGURE 1. Phenotypic analysis of a macrophage cell line for use as APCs. The peritoneal resident macrophages from C57BL/6-lpr/lpr mice were isolated and cultured as described in Materials and Methods. The macrophages were tested for expression of Fas (A), Mac-1 (B), F4/80 (C), IAα (D), H-2Dβ (E), and B7 (F) by flow cytometric analysis. Five thousand viable cells were analyzed by FACScan. The open histograms are controls for isotype Ab staining.

Results
Production and characterization of APCs that express Fas ligand
Peritoneal macrophages were used as APCs in the present study. Since most peritoneal macrophages express Fas and are susceptible to Fas ligand-induced apoptosis after activation, we derived a nontransformed peritoneal macrophage cell line from fas mutant C57BL/6-lpr/lpr mice. As expected, these macrophages did not express Fas (Fig. 1A), but did express most of the typical phenotypic markers of macrophages, including Mac-1 (Fig. 1B) and F4/80 (Fig. 1C). The macrophages expressed high levels of MHC class II IAα Ags (Fig. 1D), intermediate levels of MHC class I H-2Dβ Ags (Fig. 1E), and significant levels of the B7 costimulatory molecule, as detected by a CTLA4-Ig fusion protein (Fig. 1F).

As this cell line retains the characteristic phenotype of macrophages on long term culture, we chose it as the source of APCs for the experiments described here.

To generate APCs that express Fas ligand, the macrophage cell line was transfected with a eukaryotic expression vector (pcDNAIII) containing a full-length murine Fas ligand cDNA, using transfection with the empty vector as a control and selection with G418. The transfection and selection procedure did not alter the phenotypes of macrophages (data not shown). The macrophages were transfected with control vector (Mf-FL) or with an empty vector, using electroporation. Transfected macrophages were selected with 0.5 mg/ml G418 (Sigma). A, The Fas ligand activity of the selected macrophages was determined by mixing macrophages with 51Cr-labeled A20 cells at the indicated ratios, and after an 8-h incubation, the specific release was determined as described previously. B, Splenic T cells were purified from 4-wk-old MRL/Mpj+/+ and MRL/Mpj-lpr/lpr mice (The Jackson Laboratory) using a T cell enrichment column (R&D Systems, Minneapolis, MN). Purified T cells (5 x 105) were cultured with gamma-irradiated macrophages (5 x 105) in round-bottom, 96-well plates for 5 days, and proliferation was determined by adding 1 μCi of [3H]thymidine (Amersham) 16 h before harvest.

expressing macrophages requires Fas expression on the T cells and is specific for Fas-mediated apoptosis.

Induction of T cell unresponsiveness by alloageneic APCs that express Fas ligand
To determine whether APCs that express Fas ligand can induce alloantigen-specific T cell tolerance in vivo, Mf-FL or Mf-CV cells (H-2b) were administered through six i.p. injections given at 3-day intervals to 4-wk-old MRL+/+ and MRL/lpr/lpr (H-2k) mice. Three days after the last injection, splenic T cells from treated MRL mice were cocultured with gamma-irradiated total spleen cells from C57BL/6 (H-2b) mice. Treatment of MRL+/+ mice with H-2b macrophages that express Fas ligand considerably

FIGURE 2. Characterization of Fas ligand-expressing macrophages. The macrophages were transfected with a pcDNAIII expression vector (In-vitrogen, San Diego, CA) containing a full-length murine Fas ligand cDNA or with an empty vector, using electroporation. Transfected macrophages were selected with 0.5 mg/ml G418 (Sigma). A, The Fas ligand activity of the selected macrophages was determined by mixing macrophages with 51Cr-labeled A20 cells at the indicated ratios, and after an 8-h incubation, the specific release was determined as described previously. B, Splenic T cells were purified from 4-wk-old MRL/Mpj+/+ and MRL/Mpj-lpr/lpr mice (The Jackson Laboratory) using a T cell enrichment column (R&D Systems, Minneapolis, MN). Purified T cells (5 x 105) were cultured with gamma-irradiated macrophages (5 x 105) in round-bottom, 96-well plates for 5 days, and proliferation was determined by adding 1 μCi of [3H]thymidine (Amersham) 16 h before harvest.

FIGURE 3. T cell proliferative response of treated MRL+/+ and lpr/lpr mice to H-2b alloantigen. Four-week-old MRL+/+ (A) and lpr/lpr (B) mice were injected i.p. with 2 x 107 macrophages transfected with Fas ligand or control vector every 3 days for six times. On day 3 of the final injection, the splenic T cells were isolated from treated mice and cultured with 2 x 105 gamma-irradiated total spleen cells from C57BL/6+/+ mice. T cell proliferation was determined by incorporation of [3H]thymidine at the indicated time points. The error bars indicate the mean ± SEM for five mice analyzed separately in triplicate assays.
reduced the proliferative T cell response to H-2b alloantigen during 96 h of culture, whereas treatment with control macrophages had no effect (Fig. 3A). This result indicates that treatment with macrophages that express Fas ligand induces T cell unresponsiveness to the alloantigen. To determine whether expression of Fas is required for induction of T cell tolerance, MRL+/+ and lpr/lpr mice were treated similarly. In these mice, the T cell response to the H-2b Ag was not affected by treatment with Fas ligand-expressing macrophages (Fig. 3B), indicating that Fas expression is required for induction of T cell unresponsiveness.

T cell unresponsiveness is specific for the alloantigen presented by Fas ligand-expressing macrophages

To determine whether the T cell unresponsiveness induced by Fas ligand-expressing macrophages is alloantigen specific, T cells from MRL+/+ mice treated with Fas ligand-expressing H-2b macrophages were analyzed for their proliferative response to control alloantigens. H-2d, expressed on cells from BALB/c mice. The T cell response to H-2d in both MRL+/+ and MRL-lpr/lpr mice was unaffected by treatment with either Fas ligand-expressing H-2b or control macrophages (Fig. 4, A and B). These results indicate that the induced T cell unresponsiveness is specific for the alloantigens borne on the Fas ligand-expressing macrophages. The T cell proliferative response to cross-linking with anti-CD3 Ab was similar for T cells obtained from MRL+/+ mice treated with Fas ligand-expressing macrophages or control macrophages (data not shown), indicating that the treatment with Fas ligand-expressing macrophages does not result in nonspecific immunosuppression.

Induction of H-2D\(^b\)/H-Y-specific T cell tolerance by Fas ligand-expressing APCs in H-2D\(^b\)/H-Y TCR transgenic mice

To determine the mechanisms by which Fas ligand-expressing APCs induce systemic and Ag-specific T cell tolerance, we used TCR transgenic, H-2D\(^b\)/H-Y-reactive mice. In the female transgenic mice, the majority of peripheral CD8\(^+\) T cells bear the transgenic TCR and are reactive with the male H-Y Ag presented in the context of the H-2D\(^b\) Ag (52). Peritoneal monocytes isolated from male C57BL/6-lpr/lpr mice were used as the APCs. Because a conventional transfection technique failed to induce high levels of Fas ligand expression on the primary macrophages, a recombinant adenovirus that contains Fas ligand cDNA was used (53). High levels of Fas ligand expression in freshly isolated macrophages were obtained. Flow cytometric analysis showed that nearly 90% of the macrophages that were transfected with Fas ligand adenovirus expressed high levels of Fas ligand compared with those transfected with control adenovirus (Fig. 5A). The activity of Fas ligand was determined by a \(^{51}\)Cr release assay (Fig. 5B); the macrophages transfected with Fas ligand adenoviruses (M\(\beta\)-Ad/FL; closed circles) exhibited the highest Fas ligand activity among those obtained using a conventional method (M\(\beta\)-FL; open circles) and those transfected with the control viruses (M\(\beta\)-Ad/CV; closed squares) and those transfected with control adenoviruses (M\(\beta\)-Ad/FL; open circles). Thus, we were able to generate high Fas ligand expressing and H-2D\(^b\)/HY APCs using Fas ligand adenovirus transfection of macrophages obtained from C57BL/6 male mice.
H-2D<sup>b</sup>/?-Y TCR transgenic female mice were injected i.p. with Fas ligand-transfected macrophages that expressed H-2D<sup>b</sup>/?-Y Ag, and the T cell response to H-2D<sup>b</sup>/?-Y Ag was analyzed kinetically. TCR transgenic female B6<sup>+/+</sup> (Tg<sup>+/+</sup>) mice that received macrophages expressing Fas ligand and the H-2D<sup>b</sup>/?-Y Ag (Mφ-Ad/FL) exhibited a greatly decreased T cell proliferative response to the H-2D<sup>b</sup>/?-Y Ag (Fig. 6A, closed circles). Inhibition of the T cell response to the H-2D<sup>b</sup>/?-Y Ag was observed as early as day 1 and persisted at a low level on day 7 after treatment. In contrast, the transgenic female mice that received control macrophages (Mφ-Ad/CV) exhibited a gradual increase in the T cell response to the H-2D<sup>b</sup>/?-Y Ag, presumably due to a prostymulatory effect of the macrophage treatment. This result indicates that Fas ligand expression on H-2D<sup>b</sup>/?-Y-bearing macrophages is capable of inducing T cell unresponsiveness to the H-Y Ag, whereas the control macrophages prime the response. As described for the induction of allotientigen unresponsiveness, the expression of Fas Ag on the responding T cells is required, as treatment of TCR transgenic female B6<sup>1</sup> mice with Fas ligand-positive, H-2D<sup>b</sup>/H-Y-expressing macrophages did not affect the T cell proliferative response (Fig. 6B). Similar treatment with either Fas ligand-positive or -negative macrophages from female mice, which express H-2D<sup>b</sup>, but not the H-Y Ag, did not lead to a significant decrease in the T cell response (data not shown), indicating that H-Y Ag in the context of H-2D<sup>b</sup> also is required for the induction of T cell tolerance.

Rapid and profound clonal deletion of H-2D<sup>b</sup>/H-Y-reactive T cells induced by Fas ligand-expressing APCs

The ability of APCs that express Fas ligand to induce clonal deletion of Ag-specific T cells was tested directly in female, TCR transgenic mice. The use of these TCR transgenic mice allowed us to examine the clonal deletion of the H-2D<sup>b</sup>/H-Y-specific T cells by analyzing the numbers of M33<sup>+</sup>CD8<sup>+</sup> T cells in female, TCR transgenic mice (54). Tolerance induction was conducted as described above, and the numbers of H-2D<sup>b</sup>/H-Y-specific T cells in the spleen were determined by staining with an anti-TCR<sub>γδ</sub> clonotypic Ab (M33) and CD8, 7 days after treatment. In both untreated TCR transgenic B6<sup>+/+</sup> and B6<sup>lpr/lpr</sup> mice, approximately 18% of the splenic T cells were M33<sup>+</sup>CD8<sup>+</sup> (Fig. 7A). Treatment of TCR transgenic female B6<sup>+/+</sup> with Fas ligand-negative, H-2D<sup>b</sup>/H-Y macrophages (D<sup>b</sup>/HY Mφ-CV) only induced a slight reduction of the numbers of M33<sup>+</sup>CD8<sup>+</sup> T cells (15%) by day 7 after treatment. In contrast, the numbers of M33<sup>+</sup>CD8<sup>+</sup> T cells were significantly reduced (2.5%) in TCR transgenic female B6<sup>+/+</sup> mice that were treated with H-2D<sup>b</sup>/H-Y macrophages that expressed Fas ligand (D<sup>b</sup>/HY Mφ-FL). The depletion was specific for M33<sup>+</sup>CD8<sup>+</sup> T cells as M33<sup>+</sup>CD8<sup>-</sup> T cells were not affected. As similar treatment of TCR transgenic female lpr/lpr mice with either Fas ligand-negative or -positive macrophages did not affect the numbers of M33<sup>+</sup>CD8<sup>-</sup> T cells (20%), these results indicate that Fas ligand-expressing APCs induce clonal deletion of Ag-specific T cells through Fas/Fas ligand-mediated apoptosis. The H-Y Ag also was required for the induction of clonal deletion because the transgenic female mice treated with Fas ligand-expressing macrophages without the H-Y Ag did not exhibit a reduced number of M33<sup>+</sup>CD8<sup>+</sup> T cells.

Since the H-2D<sup>b</sup>/H-Y Ag alone could cause specific T cell clonal deletion through activation-induced T cell suicide, which also is mediated by Fas and Fas ligand, we examined the time course and efficiency of T cell deletion mediated by Fas ligand-expressing APCs and compared it to that mediated by APCs alone. Kinetic analysis of M33<sup>+</sup>CD8<sup>+</sup> T cells showed that depletion of M33<sup>+</sup>CD8<sup>+</sup> T cells in TCR transgenic B6<sup>+/+</sup> female mice occurred as early as day 3 and continued until day 14 after treatment with H-2D<sup>b</sup>/H-Y cells expressing Fas ligand as <3% of the T cells were M33<sup>+</sup>CD8<sup>+</sup> (Fig. 7B). M33<sup>+</sup>CD8<sup>+</sup> T cells in mice treated with Fas ligand-expressing macrophages remained at a low level for at least 30 days followed by a slow recovery (data not shown). In contrast, in TCR transgenic B6<sup>+/+</sup> mice treated with H-2D<sup>b</sup>/H-Y cells that did not express Fas ligand, the number of M33<sup>+</sup>CD8<sup>+</sup> T cells underwent a slow decrease after day 7 of treatment but never dropped below 10%. Treatment of transgenic-lpr/lpr mice with either Fas ligand-positive or -negative H-2D<sup>b</sup>/H-Y macrophages did not cause significant deletion of splenic M33<sup>+</sup>CD8<sup>+</sup> T cells at any time point (Fig. 7C), indicating that peripheral clonal deletion of H-2D<sup>b</sup>/H-Y reactive T cells mediated by AICD and Fas ligand-expressing macrophages is defective in lpr/lpr mice. Female H-2D<sup>b</sup> cells did not affect the numbers of M33<sup>+</sup>CD8<sup>+</sup> T cells in any group of mice (data not shown).

In summary, these results indicate that 1) Fas ligand-expressing H-2D<sup>b</sup>/H-Y, but not Fas ligand-negative, cells induce an early occurring and efficient deletion of M33<sup>+</sup>CD8<sup>+</sup> T cells, a process that is Fas dependent as it occurred in TCR transgenic B6<sup>+/+</sup> but not B6<sup>lpr/lpr</sup> mice; 2) the depletion of M33<sup>+</sup>CD8<sup>+</sup> T cells in TCR transgenic B6<sup>+/+</sup> mice requires the presence of the H-2D<sup>b</sup>/H-Y Ag as H-2D<sup>b</sup> cells from female mice, which lack the H-Y Ag, did not affect the numbers of M33<sup>+</sup>CD8<sup>+</sup> T cells.

FIGURE 7. Clonal deletion of H-2D<sup>b</sup>/H-Y-reactive T cells induced by Fas ligand-expressing H-2D<sup>b</sup>/H-Y macrophages. Four- to six-week-old female transgenic +/+ and lpr/lpr mice were injected i.p. once with 5 × 10<sup>6</sup> male B6 macrophages transfected with Fas ligand adenoviruses or control adenovirus. At the indicated time points, total splenic T cells were stained with M33, anti-CD3, and anti-CD8 Abs and analyzed by three-color flow cytometry. A, Representative flow cytometry profile of expression of M33 and CD8 on the gated CD3<sup>+</sup> splenic T cells. The experiment was performed on day 7 after treatment. Two-color contour plots of CD8 and M33 are shown, and the percentage of M33<sup>+</sup>CD8<sup>+</sup> T cells is indicated in the upper right quadrant. B, Kinetic analysis of the M33<sup>+</sup>CD8<sup>+</sup> splenic T cells as determined by three-color flow cytometry.
Our results indicate that Fas ligand-expressing APCs can induce T cell tolerance that is both systemic and Ag specific. This form of induction of T cell tolerance requires expression of Fas on the T cells as well as Fas ligand expression on the APCs, as Fas-deficient T cells from lpr/lpr mice did not undergo induction of tolerance, and Fas ligand-negative macrophages were unable to induce tolerance.

Fas-mediated apoptosis is a critical mechanism in the activation-induced suicide of T cells. In this process, autocrine interaction of Fas and Fas ligand occurs on the same T cell (33–35). Our results suggest that a paracrine process also plays an important role in Fas-mediated apoptosis in T cells. Apoptosis of T cells mediated by Fas ligand in a paracrine fashion has been shown previously to be critical for the maintenance of the immunoprivileged site (41–43, 48). High levels of Fas ligand expression on the surrounding immunoprivileged cells or tissues are able to induce apoptosis in T cells and prevent T cell attack. The mechanisms by which the T cell tolerance of the immunoprivileged tissues is induced and maintained remain unclear, however. Given the fact that local expression of high levels of Fas ligand can induce an inflammatory response, the role of Fas ligand in induction of local T cell tolerance has been challenged (49–51). A recent study suggests that the maintenance of immunoprivilege involves induction of systemic T cell tolerance (43). High levels of Fas ligand expression on some tumor cells have been proposed as a mechanism by which tumor cells escape from immune attack (57, 58). It has been reported that Fas ligand-expressing tumor cells inhibit the allogeneic Ab response (59). Our results provide direct evidence that Fas ligand-expressing APCs induce the depletion of responding T cells in the peripheral lymphoid organs, leading to systemic T cell tolerance that is specific for the Ags presented by APCs, and suggest a mechanistic basis for immunoprivilege in which Fas ligand-bearing cells released from immune-privileged tissues elicit systemic T cell tolerance.

Our findings indicate that T cell apoptosis induced by Fas ligand-expressing APCs is different from activation-induced T cell suicide. The former involves direct Ag presentation, occurs early, and is accomplished efficiently, whereas the latter occurs at a later point after Ag challenge, and the deletion is incomplete. It is not demonstrated by hematoxylin-eosin staining, from that observed in the spleens of mice that received control macrophages (Fig. 8, A and B). However, there was an increased number of apoptotic cells in the spleen of the transgenic mice that received Fas ligand-expressing macrophages but not in those that received control macrophages as demonstrated by in situ TUNEL staining (Fig. 8, C and D). Apoptotic cells in the spleen were clustered, presumably due to killing of the T cells by Fas ligand-expressing macrophages. Apoptosis induced by Fas ligand-expressing macrophages was also specific for Fas because apoptosis was not observed in the spleen of lpr/lpr mice (data not shown). Systemic administration of soluble Fas ligand or anti-Fas Ab can cause severe liver damage (32, 56); therefore, we also examined the livers of B6+/+ mice that received Fas ligand-expressing macrophages. No significant damage was observed by hematoxylin-eosin staining (Fig. 8, E and F). No significantly increased apoptosis in the liver was confirmed by Hoechst dye staining (55). These results indicate that Fas ligand-expressing macrophages primarily migrate into and reside in the spleen and do not elicit either an inflammatory response in the spleen or liver damage. Thus, the use of macrophages as carriers to deliver Fas ligand may be a safe strategy for Fas ligand-based therapy.

**Discussion**

It has been reported that local expression of high levels of Fas ligand results in neutrophil infiltration and tissue inflammation (49–51). To rule out the possibility that systemic administration of Fas ligand-expressing macrophages causes inflammation, the migration of Fas ligand-expressing macrophages after systemic administration was examined. Using green fluorescence protein as a tracer, >30% of injected macrophages were recovered from the spleen 48 h after injection, and migration was maintained at a constant level for at least 10 days (data not shown). In situ analysis showed that the Fas ligand-positive macrophages primarily migrated into the T cell zone of spleen, while no significant number of macrophages were observed in the liver (55). At 48 h after injection, the spleen of H-2Db/H-Y TCR transgenic female mice that received Fas ligand-expressing H-2Db/H-Y macrophages did not exhibit a significantly different inflammatory response, as demonstrated by hematoxylin-eosin staining, from that observed in the spleens of mice that received control macrophages (Fig. 8, A and B). However, there was an increased number of apoptotic cells in the spleen of the transgenic mice that received Fas ligand-expressing macrophages but not in those that received control macrophages as demonstrated by in situ TUNEL staining (Fig. 8, C and D). Apoptotic cells in the spleen were clustered, presumably due to killing of the T cells by Fas ligand-expressing macrophages. Apoptosis induced by Fas ligand-expressing macrophages was also specific for Fas because apoptosis was not observed in the spleen of lpr/lpr mice (data not shown). Systemic administration of soluble Fas ligand or anti-Fas Ab can cause severe liver damage (32, 56); therefore, we also examined the livers of B6+/+ mice that received Fas ligand-expressing macrophages. No significant damage was observed by hematoxylin-eosin staining (Fig. 8, E and F). No significantly increased apoptosis in the liver was confirmed by Hoechst dye staining (55). These results indicate that Fas ligand-expressing macrophages primarily migrate into and reside in the spleen and do not elicit either an inflammatory response in the spleen or liver damage. Thus, the use of macrophages as carriers to deliver Fas ligand may be a safe strategy for Fas ligand-based therapy.
clear whether apoptosis of T cells induced by Fas ligand-expressing APCs requires that the T cells be activated. Given the ability of naive T cells to undergo Fas ligand-mediated apoptosis (60), early activation of T cells may not be required for this form of apoptosis.

Induction of Ag-specific T cell tolerance by Fas ligand-expressing APCs suggests a novel role of APCs in modulation of the T cell response. Our results indicate that Fas ligand-expressing APCs induce an earlier and more profound clonal deletion of the Ag-reactive T cells than does activation-induced suicide of the T cells. This suggests that T cell tolerance can be induced by the APCs during an early stage of the T cell response. When naive T cells recognize the Ag presented by APCs, the fate of the T cells is determined by the APCs; the T cells either undergo complete activation if the APCs express appropriate costimulatory molecules, such as B7, undergo induction of anergy if the APCs do not express costimulatory molecules, or undergo apoptosis if the APCs express Fas ligand. The ability of APCs to present Ag to T cells in either an immunogenic or a tolerogenic fashion has been proposed to be a critical mechanism in regulation of the T cell response during early activation (14, 39, 40). Up-regulation of Fas ligand expression in macrophages also has been implicated as a mechanism by which T cells are depleted during HIV infection (38). Our previous study showed that activated macrophages express Fas ligand. The findings presented here imply the possibility of a novel apoptosis-inducing function of APCs in addition to their known functions in induction of activation and anergy of T cells. We propose that a normal population of APCs express Fas ligand and are continuously capable of presenting self Ags to new, potentially self-reactive T cells from the thymus, thereby inducing apoptosis and maintaining peripheral T cell tolerance to the self Ag. Indeed, it has been demonstrated that a subset of dendritic cells is capable of inducing apoptosis of CD4+ via Fas/Fas ligand interaction (61), suggesting that naturally existing APCs that express Fas ligand might play an important role in the maintenance of T cell tolerance.

In this study Ag-specific T cell tolerance induced by APCs that express Fas ligand was established in two experimental systems: allogeneic T cell tolerance and H-Y Ag-specific tolerance in TCR transgenic mice. This suggests that direct Ag presentation by donor APCs that express Fas ligand is required for induction of apoptosis in the Ag-responding T cells. Direct Ag presentation is a major component of allogeneic T cell activation and allograft rejection. APCs carrying allogeneic antigens released from the grafted organs and tissues are strongly immunogenic. As they migrate into the peripheral lymphoid organs of the recipients, a strong T cell response is elicited, and finally, these activated T cells attack the grafted tissue, resulting in rejection. The present study suggests a practical immunointervention strategy for the induction of systemic Ag-specific T cell tolerance by manipulating Fas ligand expression on the APCs. This implies that allogeneic T cell tolerance can be induced and maintained by removal of allogeneic-specific T cells in the recipients using Fas ligand-expressing donor APCs. A recent study demonstrated that the expression of Fas ligand in syngeneic muscle cells surrounding grafted allogeneic islets prevents rejection. The induction of tolerance to the graft required the presence of Fas ligand-expressing muscle cells, however, suggesting that the local protection of the graft by Fas ligand plays a major role. Our study extends these findings to induction of systemic T cell tolerance by transfusion of the donor APCs that express Fas ligand. It will be of interest to determine whether induction of systemic T cell tolerance to the alloantigen is sufficient for prevention of allogeneic graft rejection.

Although our study demonstrates that the Ag presented by Fas ligand-expressing macrophages is required for the induction of Ag-specific T cell tolerance, one may argue that Fas ligand-expressing macrophages might also be able to eliminate activated T cells in a non-Ag-specific fashion. Using a murine experimental autoimmune encephalitis model, we found that only those Fas ligand-expressing macrophages loaded with the autoantigen peptides exhibited the most potent induction of T cell tolerance and inhibition of autoimmune disease, while Fas ligand-expressing macrophages alone were much less potent (H. Zhang et al., unpublished observation). These results suggest that the Ag-specific interaction between the responding T cells and Fas ligand-expressing APCs is a primary mechanism leading to the induction of T cell tolerance.

The interaction between T cells and APCs plays a major role in the initiation of an autoimmune response. Inappropriate Ag presentation by certain MHC molecules has been shown to be a key step toward activation of autoreactive T cells and the development of autoimmune disease. The manner in which Ag is presented by APCs determines the cytokine profile of the T cell response, which may be involved in the pathogenesis of autoimmune disease. The rapid elimination of the Ag-responding T cells by Fas ligand-expressing APCs, suggests that this strategy would be applicable to the treatment of those autoimmune diseases that are T cell dependent and of known autoantigen specificity. One can anticipate that the APCs carrying autoantigen and Fas ligand may facilitate elimination of the autoantigen-specific T cells by enhancing Fas-mediated apoptosis in the autoreactive T cells.

Acknowledgments

We thank Dr. Fiona Hunter for careful review of the manuscript, and Mrs. Judy White for excellent secretary support.

References


