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Memory T cells respond in several functionally different ways from naive T cells and thus function as efficient effector cells. In this study we showed that primed T cells were more resistant to Fas-mediated activation-induced cell death (AICD) than naive T cells using OVA-specific TCR transgenic DO10 mice and Fas-deficient DO10 lpr/lpr mice. We found that apoptosis was efficiently induced in activated naive T cells at 48 and 72 h after Ag restimulation (OVA peptide; 0.3 and 3 μM), whereas apoptosis was not significantly increased in activated primed T cells at 24–72 h after Ag restimulation. We further showed that the resistance to AICD in primed T cells was due to the decreased sensitivity to apoptosis induced by Fas-mediated signals, but TCR-mediated signaling equally activated both naive and primed T cells to induce Fas and Fas ligand expressions. Furthermore, we demonstrated that primed T cells expressed higher levels of Fas-associated death domain-like IL-1β-converting enzyme inhibitory protein (FLIP), an inhibitor of Fas-mediated apoptosis, at 24–48 h after Ag restimulation than naive T cells. In addition, Bcl-2 expression was equally observed between activated naive and primed T cells after Ag restimulation. Thus, these results indicate that naive T cells are sensitive to Fas-mediated AICD and are easily deleted by Ag restimulation, while primed/memory T cells express higher levels of FLIP after Ag restimulation, are resistant to Fas-mediated AICD, and thus function as efficient effector cells for a longer period.

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Immunological memory can be defined as the faster and stronger response of an animal that follows re-exposure to the same Ag (1–4). Memory T cells express a different pattern of cell surface markers, and they respond in several functionally different ways from naive T cells (1–4). Murine memory T cells are CD44high and CD62L (MEL-14)low, while naive T cells express low levels of CD44 and high levels of CD62L (5). Memory T cells secrete a wider range of cytokines than do naive T cells. Naive T cells produce only IL-2, whereas memory T cells can be polarized to secrete particular restricted patterns of cytokines (6). Thus, they can be specialized to perform unique functions specified by the patterns of cytokines they secrete. The requirements for the activation of memory T cells for proliferation and cytokine production are not quite as strict as those of naive T cells, and less costimulation and less Ags are required for optimum responses (7, 8).

Upon TCR stimulation with Ag, resting T cells are activated to proliferate and produce cytokines. When activated T cells are re-stimulated with Ag through TCR, those T cells undergo apoptosis termed activation-induced cell death (AICD) (9, 10). The susceptibility to AICD develops during the activation of T cells; resting T cells that are resistant to TCR-mediated apoptosis become sensitive to the death by TCR ligation (11–14). AICD is thus thought to be an important mechanism to control clone size and to terminate immune responses (9, 10). AICD also plays important roles in the establishment of peripheral tolerance. AICD has recently been shown to be mediated predominantly by Fas/Fas ligand (FasL) interaction (15–18), in which activated T cells express FasL, which interacts with Fas on their surface, resulting in activating caspases and inducing apoptosis of these cells (19). However, it is still unknown whether the susceptibility of memory T cells to AICD differs from that of naive T cells.

To elucidate this issue, we studied apoptosis of activated naive and primed T cells from OVA-specific TCR transgenic DO10 mice and Fas-deficient DO10 lpr/lpr mice after in vitro Ag restimulation. We further investigated the sensitivity to apoptosis induced by Fas-mediated signals and the expression levels of FLICE inhibitory protein (FLIP) (20, 21), an inhibitor of Fas-mediated apoptosis, and of Bcl-2 (22, 23). Our results indicate that naive T cells are sensitive to Fas-mediated AICD and are easily deleted by Ag restimulation, while primed/memory T cells express higher levels of FLIP after Ag restimulation, are resistant to Fas-mediated AICD, and thus function as efficient effector cells for a longer period.

Materials and Methods

Mice

DO10 TCR transgenic mice (BALB/c background; H-2ab), which express a transgenic TCRαβ specific for OVA peptide 323–339 (OVA peptide)/I-Ak complex (24), were provided by Dr. D. Loh (Washington University, Seattle, WA) and were maintained in our university animal facility. Fas-deficient mice bearing the transgenic DO10 TCR and H-2d (DO10 lpr/lpr mice) were generated by crossing DO10 TCR transgenic mice with MRL-lpr/lpr mice for more than six generations. All mice were housed under specific pathogen-free conditions and used at the age of 6–8 wk.

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Generation of memory T cells

Mice were immunized i.p. twice with 10 μg of OVA (Sigma, St. Louis, MO) in 4 mg of aluminum hydroxide at a 2-wk interval. Two weeks after the second immunization, splenic CD4+ T cells were prepared from the mice and used as primed T cells for experiments. Splenic CD4+ T cells from unimmunized mice were used as naive T cells for experiments.

Flow cytometry

Splenocytes (1 × 10^6) before and after culture were stained with fluorescein- or biotin-conjugated Abs in PBS containing 1% FCS for 30 min at 4°C. The following FITC-, PE-, or biotin-conjugated Abs were used: CD4 (YTS191.1), CD8α (YTS169.4), CD25 (PC61.5; Caltag, South San Francisco, CA), CD69 (H1.2F3), CD44 (IM7), CD62L (MEL-14), and Fas (Jo2; PharMingen, San Diego, CA). DO10 transgenic TCR clonotype mAb KJ1-26 (24) (a gift from Dr. D. Loh) was purified from the hybridoma supernatant by a protein G affinity column. Cells stained with biotinylated mAb were then incubated with streptavidin-PE or -Tricolor (Caltag). Stained cells were resuspended in PBS containing 1% FCS and analyzed by FACScan (Becton Dickinson, Mountain View, CA) using LYSIS II software.

Induction of AICD by in vitro Ag restimulation

Splenic CD4+ T cells were prepared as described previously (25). Briefly, splenocytes were passed over nylon columns and then incubated with anti-CD8 mAb (53.6.7; PharMingen) followed by addition of magnetic beads (Advanced Magnetics, Cambridge, MA) coupled with goat anti-mouse IgG Ab (Cappel, West Chester, PA). Splenic CD4+ T cells (2 × 10^7/ml) from primed or unprimed mice were stimulated with OVA peptide (0.3 μM) and APC (30 gray-irradiated normal BALB/c splenocytes; H-2d) on nylon columns. Cells were cultured at 37°C for 24–96 h after restimulation with Ag. Cells were harvested, and viable and apoptotic KJ1-26+ CD4+ T cells were counted by flow cytometry.

Detection of apoptosis

Apoptotic cells among KJ1-26+ CD4+ T cells were detected by the TUNEL method. Cells were stained with KJ1-26 Ab and fixed with PBS containing 4% paraformaldehyde for 30 min at 4°C. The cells were then washed with PBS, permeabilized with 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min at 4°C, and washed twice with PBS. DNA strand breaks by apoptosis in the cells were labeled by the fluorescein-labeled TUNEL reaction mix for 60 min at 37°C using an in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s recommendations. As a negative control, cells were incubated without TdT. Cells were then washed with PBS and analyzed by flow cytometry.

Detection of FasL and FLIP mRNA by RT-PCR

FasL and FLIP mRNA expression was detected by PCR amplification of cDNA from splenocytes using specific primers. Briefly, total RNA was prepared from splenocytes by the method of acid guanidinium thiocyanate/phenol/chloroform extraction using Isogen solution (Nippon Gene, Tokyo, Japan). The first-strand cDNA was then synthesized from 0.1–1 μg of total RNA in 20 μl of reaction buffer containing oligo(dT) primer using avian myeloblastosis virus reverse transcriptase (1st Strand cDNA Synthesis Kit, Boehringer Mannheim). The reaction mixture was incubated at 25°C for 10 min and then at 42°C for 60 min.

PCR amplification of the cDNA was performed with Taq DNA polymerase (Boehringer Mannheim) in the presence of specific primers for FasL (5′-TATTCTCTTGGGCTCAGATG-3′ and 5′-TCTGGTGGCTCTGGAAG-3′), FLIP (5′-CTAGTGATGATGTTGGGAGGC-3′ and 5′-GAGCTGAGCTGAGCTGATG-3′), or HPRT (5′-GCTGGATACAGGGCAGCTTGGTGTT-3′ and 5′-GATTCACACTGCGCTTCATCTTGGC-3′). The denaturing step was performed at 95°C for 1.5 min, the annealing step at 60°C for 1 min, and the extension step at 72°C for 1 min for 30 cycles on a DNA thermal cycler (Perkin-Elmer, Norwalk, CT). The PCR-amplified sample was run on 1.5% agarose gel and visualized using ethidium bromide. FasL and FLIP mRNA expression was measured by a densitometry and expressed as the amount relative to that of HPRT.

In vitro Ag restimulation induces apoptosis of activated naive T cells

To investigate the difference in the susceptibility to AICD in naive and primed T cells, we examined the apoptosis of OVA-specific T cells induced by in vitro Ag restimulation between activated naive

![FIGURE 1. CD44 and MEL-14 expression on splenic Ag-specific CD4+ T cells from primed and unprimed DO10 mice. DO10 TCR transgenic mice were immunized i.p. with 10 μg of OVA in aluminum hydroxide at a 2-wk interval. Two weeks after the second immunization, splenocytes were prepared from primed (solid line histograms) and unprimed (dotted line histograms) mice, and CD44+ or MEL-14+ populations among KJ1-26+ CD4+ T cells were analyzed by flow cytometry. The data shown are representative of six experiments.](http://www.jimmunol.org/)
and primed T cells. Splenic CD4+ T cells from primed and unprimed DO10 mice were stimulated with OVA peptide (0.3 μM) plus APC (irradiated normal BALB/c splenocytes) at 37°C for 48 h and restimulated with OVA peptide (0.3 and 3 μM) plus APC for 24–72 h. The time of Ag restimulation, OVA-specific T cells (K J1-26+ CD4+ T cells) had been activated by Ag, because those cells were large, expressed CD25, and produced IL-2 (data not shown). At 24–72 h after restimulation with Ag, apoptotic cells among K J1-26+ CD4+ T cells were detected by the TUNEL method and analyzed by flow cytometry. The data shown are representative of five experiments.

Apoptosis was efficiently induced in activated naive T cells at 48 and 72 h after Ag restimulation (OVA peptide; 0.3 and 3 μM; 21.4 ± 3.5% at 3 μM at 72 h; n = 10 mice; p < 0.001; Figs. 2 and 3). On the other hand, apoptosis was not significantly increased in activated primed T cells at 24–72 h after Ag restimulation (6.9 ± 1.4% at 3 μM at 72 h; Figs. 2 and 3). Because AICD has been shown to be mediated largely by the Fas/FasL interaction (15–18), we determined whether the Fas/FasL system is involved in AICD of activated naive T cells induced by Ag restimulation. In contrast to naive and primed T cells from DO10 mice, apoptosis was not significantly induced in either activated naive or primed T cells from Fas-deficient DO10 lpr/lpr mice at 72 h after Ag restimulation (n = 5 mice in each group; Fig. 4). These results indicate that primed T cells are more resistant to Fas-mediated AICD than naive T cells.

**Fas and FasL expression on activated naive and primed T cells**

To investigate the mechanism(s) for the resistance to Fas-mediated AICD in primed T cells, we first examined the expression of Fas and FasL on activated naive and primed T cells after Ag restimulation. As shown in Fig. 5, when the T cells were activated by OVA peptide (0.3 μM) for 48 h and restimulated by OVA peptide (3 μM) for 48 h, the levels of Fas expression were not significantly different in activated naive and primed T cells before or after Ag restimulation.

FasL mRNA was not significantly detected in either resting naive or primed T cells (Fig. 6). When the T cells were activated by OVA peptide (0.3 μM) for 48 h and restimulated by OVA peptide (3 μM) for 24–48 h, both activated naive and primed T cells similarly expressed FasL mRNA at high levels for the first 24–48 h (Fig. 6). Because two forms of FasL have been reported (26, 27), soluble and membrane-bound FasL, we performed flow cytometry analysis to detect membrane-bound FasL, an apoptosis-inducing form, on activated T cells at 24 and 48 h after Ag restimulation. We found that the levels of FasL expression were similar in activated naive and primed T cells (Fig. 6). When the T cells were activated by OVA peptide (0.3 μM) for 48 h and restimulated with OVA peptide (0.3 μM) for 24–72 h as described in Fig. 2. After the culture, apoptotic cells among K J1-26+ CD4+ T cells were detected by the TUNEL method and analyzed by flow cytometry. Data are the mean ± SD for 10 mice in each group. Asterisks indicate a significant difference from the mean value of corresponding responses of activated naive T cells: *, p < 0.005; **, p < 0.001.

![FIGURE 2. Apoptosis of activated naive and primed T cells by in vitro Ag restimulation. Splenic CD4+ T cells (2 × 10⁶/ml) from primed and unprimed DO10 mice were stimulated with OVA peptide (0.3 μM) plus APC (irradiated normal BALB/c splenocytes; 5 × 10⁵/ml) at 37°C for 48 h. Cells were then resuspended at 2 × 10⁶/ml and restimulated with or without 3 μM OVA peptide plus APC (5 × 10⁵/ml). At 72 h after restimulation with Ag, apoptotic cells among K J1-26+ CD4+ T cells were detected by the TUNEL method and analyzed by flow cytometry. The data shown are representative of five experiments.](http://www.jimmunol.org/)
Activated primed T cells are more resistant to Fas-induced apoptosis than activated naive T cells

Because TCR-mediated signaling equally activated both naive and primed T cells to induce Fas and FasL expressions, we next examined the susceptibility to Fas-induced apoptosis between activated naive and primed T cells using anti-Fas Ab. Apoptosis of activated naive and primed T cells was induced by the stimulation with anti-Fas mAb (Jo2; 0.003–1 μg/ml) for 24 h. As shown in Fig. 7, activated primed T cells were ~10-fold more resistant to anti-Fas mAb-induced apoptosis than activated naive T cells. Apoptosis of activated naive T cells was induced by anti-Fas mAb at a minimal dose of 0.003 μg/ml and was increased by higher doses of anti-Fas mAb in a concentration-dependent manner (Fig. 7). In contrast, apoptosis of activated primed T cells was induced by 0.01 μg/ml of anti-Fas mAb and was increased by higher concentrations of anti-Fas mAb (0.03–1 μg/ml; Fig. 7), but the apoptosis of activated primed T cells induced by anti-Fas mAb was significantly lower than that of activated naive T cells at any concentrations examined (0.003–1 μg/ml; n = 6 mice at each concentration; p < 0.005–0.01).

Expression of Bcl-2 and FLIP in activated naive and primed T cells

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To determine whether the activation of antiapoptotic molecules is different between activated naive and primed T cells, we examined the expression of the antiapoptotic molecule Bcl-2 (22, 23) assayed by intracellular staining and FLIP (20, 21) detected by RT-PCR using specific primers. Bcl-2 was equally expressed in activated naive and primed T cells and was further increased at 48 h after the restimulation with OVA peptide (3 μM) in both naive and primed T cells, but the levels of Bcl-2 expression were not significantly different in activated naive and primed T cells before or after Ag restimulation (Fig. 8).

In contrast to Bcl-2 expression, FLIP expression was significantly long lasting at high levels in activated primed T cells after Ag restimulation than that in activated naive T cells (Fig. 9). FLIP mRNA was not significantly expressed in either resting naive or primed T cells (Fig. 9). When the T cells were activated by OVA peptide (0.3 μM) for 48 h (at 0 h after Ag restimulation), FLIP mRNA was expressed at highest levels equally in the activated naive and primed T cells (Fig. 9). However, after Ag restimulation, FLIP expression was rapidly decreased in activated naive T cells, while FLIP expression remained at significantly higher levels in activated primed T cells at 24–48 h after Ag restimulation (Fig. 9).
The sensitivity of cells to Fas-mediated apoptosis is controlled by multiple intracellular antiapoptotic molecules that counteract apoptotic signals (21). FLIP, a homologue of caspase-8 (FLICE) that has death effector domain (DED), but not proteolytic, activity, binds to Fas-associated death domain (FADD) and caspase-8/10 via DED-DED interaction and blocks FADD/caspase-8 interaction and the resultant apoptosis (20, 21). Therefore, our observations that primed T cells show the sustained expression of FLIP after Ag restimulation in correlation with the decreased sensitivity to Fas-mediated apoptosis strongly suggest that the higher levels of FLIP expression render activated primed T cells resistant to Fas-mediated AICD.

The levels of FLIP expression are also kinetically linked with Fas-mediated AICD in naive T cells. FasL expression was strongly induced in activated naive T cells at 24–48 h after Ag restimulation (Fig. 6). FLIP expression was transiently induced at 0 h and drastically decreased after 24 h of Ag restimulation in activated naive T cells (Fig. 9). Subsequently, Fas-mediated AICD was induced in activated naive T cells at 48–72 h after Ag restimulation (Fig. 3). Our findings are consistent with a previous report by Imler et al. (20) showing that FLIP is expressed during the early stage of T cell activation (on day 1 after stimulation) in Con A-activated naive T cells, but disappears when T cells become susceptible to FasL-mediated apoptosis (on day 3).

Bcl-2 has been shown to prevent cells from apoptosis by radiation, withdrawal of growth factors (30), and Fas (31). However, Bcl-2 expression was equally observed between activated naive and primed T cells after Ag restimulation (Fig. 8), indicating that Bcl-2 expression is not responsible for the difference in susceptibility to AICD between activated naive and memory T cells. This

Discussion

In this study we showed that primed T cells were more resistant to Fas-mediated AICD than naive T cells, as indicated by the apoptosis of activated naive and primed T cells from DO10 mice was stimulated with OVA peptide (0.3 μM) for 48 h and restimulated with OVA peptide (3 μM) for 48 h. Cells were stained with K J1-26 mAb, stained intracellularly with anti-Bcl-2 mAb (solid line histograms) or hamster IgG as a negative control (dotted line histograms), and analyzed by flow cytometry. The data shown are representative of three experiments.

There have been several mechanisms reported by which activated T cells undergo apoptosis (28). When activated T cells are restimulated with Ag through TCR, those T cells undergo apoptosis predominantly by Fas/FasL interaction, in which activated T cells express FasL, which interacts with Fas on their surface resulting in apoptosis of these cells (15–18). TNF-α produced by activated T cells also induces apoptosis of the activated T cells through the activation of caspase cascades in a way similar to that of Fas/FasL interaction (29). Furthermore, withdrawal of growth factors such as IL-2 also causes the death of activated T cells (30). The apoptosis of activated T cells induced by in vitro Ag restimulation in our system was mediated by the Fas/FasL system, but not by other mechanisms, because apoptosis of activated naive or primed T cells was not induced by Ag restimulation in Fas-deficient DO10 lpr/lpr mice (Fig. 4), but was readily induced in DO10+/−/− mice. In addition, we found that the addition of exogenous IL-2 into the culture had no effect on apoptosis produced by Ag restimulation (unpublished observations).

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finding is consistent with a previous report that overexpression of Bcl-2 did not protect T cells from Fas-mediated apoptosis but resisted the x-ray irradiation-induced apoptosis (32).

In addition to FLIP and Bcl-2, other antiapoptotic molecules have been reported. Bcl-x, a member of the Bcl-2 family (33), has been shown to be up-regulated in activated T cells (34). The inhibitor of the apoptosis protein (IAP) family, cellular IAP-1 and IAP-2 (35), X-linked IAP (36) and survivin (37), has also recently been identified to inhibit caspase-3, an essential molecule for Fas-mediated apoptosis (38). Survivin has also been shown to be induced in activated T cells (39). Therefore, further studies are needed to examine whether Bcl-x and IAPs might be involved in the resistance of primed T cells to Fas-mediated AICD.

AICD has been considered to play two roles in vivo: clonal downsizing after Ag elimination and clonal deletion of autoreactive T cells in the periphery (9, 10, 40). Overexpanded T cells are removed by AICD to terminate immune responses effectively. AICD is also an important mechanism of self tolerance. Our findings of the resistance of memory T cells to Fas-mediated AICD imply that memory T cells could escape AICD in the presence of antigenic stimuli such as micro-organisms and could proliferate and differentiate to effector cells and thus efficiently eliminate those micro-organisms. Conversely, in autoimmune diseases, autoreactive memory T cells could be hardly deleted by AICD, and the persistence of activated autoreactive T cells could cause the progression of the disease. Thus, modulation of antiapoptotic molecules such as FLIP, which can render memory T cells susceptible to Fas-mediated AICD, would be a new strategy for treatment of autoimmune diseases.

In conclusion, we have shown that naive T cells are sensitive to Fas-mediated AICD and are easily deleted by Ag restimulation, while primed/memory T cells express higher levels of FLIP after Ag restimulation, are resistant to Fas-mediated AICD, and thus function as efficient effector cells for a longer period. It is suggested that this characteristic of Ag-activated memory T cells would be beneficial to protect the infections of micro-organisms, whereas the same character of autoreactive T cells would be harmful to cause autoimmune diseases.

Acknowledgments
We thank Dr. D. Loh for providing DO10 TCR transgenic mice and KJ1-26 mAb.

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