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*J Immunol* 2005; 175:4783-4788; doi: 10.4049/jimmunol.175.7.4783

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Essential Roles of the Fas-Associated Death Domain in Autoimmune Encephalomyelitis

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The Fas-associated death domain (FADD) protein mediates apoptosis by coupling death receptors with the caspase cascade. Paradoxically, it also promotes cell mitosis through its C-terminal region. Apoptosis and mitosis are opposing processes that can have radically different consequences. To determine which of the FADD effects prevails in T cell-mediated autoimmune diseases, we studied myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE) using mice that express a dominant-negative FADD (FADD-DN) transgene in the T cell lineage. We found that FADD blockade in T cells prevented the development of autoimmune encephalomyelitis and inhibited both Th1 and Th2 type responses. Myelin oligodendrocyte glycoprotein-specific T cell proliferation was also dramatically reduced in FADD-DN mice despite the resistance of T cells to activation-induced cell death. These results indicate that although FADD expressed by T cells is involved in regulating both mitosis and apoptosis, its effect on mitosis prevails in EAE, and that strategies inhibiting FADD functions in T cells could be effective in preventing the disease. The Journal of Immunology, 2005, 175: 4783–4788.

Experimental autoimmune (or allergic) encephalomyelitis (EAE) is an animal model for human multiple sclerosis. The disease can be induced in susceptible strains of animals by immunization with myelin Ags such as myelin basic protein, proteolipid protein, or myelin oligodendrocyte glycoprotein (MOG). Myelin-specific T cells are both initiators and effectors of autoimmune encephalomyelitis. Myelin-specific T cells recruit and activate cells of the innate immune system to initiate autoimmune inflammation and to cause central nervous tissue destruction. Blocking the functions of myelin-specific T cells blocks EAE (1). Yet, myelin-specific precursor T cells are present in all healthy individuals at low frequencies, and are normally not pathogenic. Development of autoimmune encephalomyelitis requires that the myelin-specific precursor T cells become activated, and differentiate into Th1-type effector cells. Understanding the mechanisms whereby the fates of myelin-specific T cells are regulated is therefore crucial for the development of specific strategies to prevent or treat autoimmune encephalomyelitis.

The Fas-associated death domain (FADD) protein, also known as MORT1, plays crucial roles in regulating T cell death and survival. On the one hand, it mediates apoptosis by coupling death receptors (DRs) (such as Fas, TNFRI, and TRAIL receptors) with the caspase cascade (2). On the other hand, it is also capable of promoting cell cycle progression through its C-terminal region (3). Germline disruption of the FADD gene led to embryonic lethality indicating that it plays crucial roles in embryonic development (4, 5). Selective blockade of FADD function in T cells by introducing a dominant-negative FADD mutant revealed that it was required for both apoptosis and mitosis of T cells (6, 7). However, FADD does not appear to be essential for the development of T cells as thymic selections are relatively normal in mice carrying dominant-negative FADD mutants or a FADD gene mutation in T cells (6–8).

Apoptosis and mitosis are two radically different processes that can have distinct consequences. In EAE, apoptosis of T cells can prevent the disease or lead to disease recovery. In contrast, mitosis of the same cell could promote EAE or prevent disease recovery. The paradoxical roles of FADD in T cells raise a fundamental question: does FADD expressed in T cells promote or prevent disease? To address this question, we studied EAE in mice that carry a dominant-negative FADD mutant in T cells (6). As reported previously, mice carrying FADD mutants in T cells developed normally and did not suffer from any spontaneous autoimmune or inflammatory diseases (6). We report here that although FADD is involved in promoting both apoptosis and mitosis of T cells in these mice, its net effect is the promotion of the development of the T cell-mediated disease. Therefore, FADD-mediated mitosis but not apoptosis of T cells prevails in EAE. Additionally, we also found that FADD is required for the generation of effector Th1 and Th2 cells in vivo.

Materials and Methods

Animals

C57BL/6 mice carrying a dominant-negative FADD (FADD-DN) transgene under the control of the T cell-specific early Ick promoter were generated as previously described by Newton et al. (6). They were crossed with normal C57BL/6 mice (The Jackson Laboratory) to generate FADD-DN transgenic and normal control mice used in this study. Mice were screened for FADD-DN transgene by PCR. FADD-DN mice were also crossed with MOG-specific TCR transgenic C57BL/6 mice described by Bettelli et al. (9) to generate double transgenic mice. The MOG-specific transgenic TCR was identified by flow cytometry using anti-mouse TCR Va3.2-FITC, (BD Pharmingen) and anti-mouse TCR Vb11-PE (Caltag Laboratories). All mice were housed in the University of Pennsylvania Laboratories.
Animal Care Facilities under pathogen-free conditions and all procedures were preapproved by the Institutional Animal Care and Use Committee.

**Induction and clinical evaluation of EAE**

For the induction of EAE, mice received 1) a s.c. injection on flanks of 300 μg of MOG38–50 peptide in 0.1 ml of PBS emulsified in an equal volume of CFA containing 4 mg/ml Mycobacterium tuberculosis H37RA (Difco), and 2) an i.v. injection of 100 ng of pertussis toxin in 0.2 ml of PBS. A second injection of pertussis toxin (100 ng/mouse) was given 48 h later. Mice were examined daily for signs of EAE and scored as follows: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, moribund; 5, dead.

**AgS and ELISA**

Mouse MOG38–50 peptide (GWYRSPFSRVVHL) was synthesized using F-moc solid phase methods and purified through HPLC by Invitrogen Life Technologies. Pertussis toxin was purchased from List Biological Laboratories. The following reagents were purchased from BD Pharmingen: purified rat anti-mouse IL-2, IL-4, IL-6, and IFN-γ mAb; recombinant mouse IL-2, IL-4, IL-6, and IFN-γ. Quantitative ELISA for cytokines was performed using paired mAbs specific for corresponding cytokines per manufacturer’s recommendations.

**CFSE labeling of cells**

Splenocytes were washed twice in PBS and incubated for 5 min at room temperature in PBS containing 2.5 μM CFSE (Molecular Probes). The labeling reaction was stopped by the addition of PBS to a final concentration of 10%. Cells were washed and resuspended in 10% FBS for in vitro culture or in PBS for in vivo injection.

**Cell culture**

For cytokine assays, splenocytes were cultured at 1.5 × 10^6 cells/well in 0.2 ml of DMEM with 10% FBS in the presence of different concentrations of MOG38–50 peptide, or 1 μg/ml Con A. Culture supernatants were collected 40 h later, and cytokine concentrations were determined by ELISA. For proliferation assays, 0.5 × 10^6 cells/well were used. [3H]Thymidine was added to the culture at 48 h, and cells were harvested 16 h later. Radioactivity was determined using a flatbed counter (Wallac). For CFSE-labeled cultures, cells were incubated at 1 × 10^6/ml in the presence of plate-bound anti-CD3 (1 μg/ml) (clone 145-2C11; American Type Culture Collection) and soluble anti-CD28 mAbs (1 μg/ml; BD Pharmingen).

**Activation-induced cell death (AICD)**

Splenocytes were isolated and cultured for 4 days at 2 × 10^6 cells/well in 0.75 ml of complete DMEM medium in the presence of plate-bound anti-

**Results**

**FADD blockade in T cells prevents the development of EAE**

To study the roles of FADD in the development of EAE, we immunized transgenic mice expressing a dominant interfering mutant of FADD (FADD-DN) and control C57BL/6 (B6) mice with MOG38–50 peptide, and monitored the disease by both physical examination and histochemistry. Fig. 1 illustrates typical disease development and clinical features of MOG-induced EAE. As shown in Table I, consistent with these clinical findings, histochemical examination of the CNS tissues revealed dramatic differences in the two groups. In the control B6 group, multiple inflammatory foci were observed in the spinal cord and brain. H&E staining revealed various inflammatory cell types in the infiltrates (Fig. 2). These include cells with morphological characteristics of lymphocytes, granulocytes, macrophages, microglial cells as well as astrocytes. In contrast, no lesions were detected in the CNS of most FADD-DN mice that showed no symptoms of EAE. In those FADD-DN mice that did develop EAE, similar inflammatory infiltrates were observed. The
degree of cell death in the CNS as determined by TUNEL correlated well with the degree of inflammation (Fig. 2C). A large number of apoptotic cells was detected in control but not FADD-DN mice. Taken together, these results indicate that FADD plays an important role in the pathogenesis of EAE.

Activation and differentiation of autoreactive T cells are blocked in FADD-DN transgenic mice

Resistance to EAE in FADD-DN mice can be either due to the inability of myelin-specific T cells to differentiate into effector T

FIGURE 2. Histopathological and apoptotic profiles of CNS. Mice were immunized with MOG38–50 peptide as described in Fig. 1 and sacrificed 31 days later. Brain and spinal cord were analyzed by histochemistry as described in Materials and Methods. A, A spinal cord section of a FADD-DN mouse with a disease score of 0. B, A spinal cord section of a B6 mouse with a disease score of 4. Original magnification, ×200. C, The degree of apoptosis in the spinal cord. Apoptotic cells were detected by TUNEL as described in Materials and Methods. Data presented are means and SEM of apoptotic cells per spinal cord section (n = 16 for the control group; n = 20 for the FADD-DN group); p < 0.001 as determined by ANOVA.

FIGURE 3. T cell proliferation and cytokine production in vitro. Mice were treated as in Fig. 1 and sacrificed 31 days after immunization. Splenocytes were cultured and tested as described in Materials and Methods. Results are shown as mean ± SD from a total of eight mice with four mice per group. The differences between the two groups are statistically significant as determined by ANOVA for all the parameters presented (p < 0.01). The experiments were repeated twice with similar results. □, FADD-DN mice; ■, control mice. A, Cytokines. B, Thymidine incorporation presented as cpm.
cells in the periphery, or due to the inability of differentiated effector T cells to induce demyelinating inflammation in the CNS, or both. To address this, we first examined whether activation and differentiation of myelin-specific T cells were normal in FADD-DN animals. Therefore, splenocytes were collected from both control and FADD-DN mice 31 days after immunization, and tested in vitro for their cytokine production and proliferation in response to MOG\(38–50\) peptide. As shown in Fig. 3, splenocytes of control animals proliferated vigorously in response to MOG peptide and produced significant amounts of both Th1 (IL-2 and IFN-\(\gamma\)) and Th2 (IL-4 and IL-6) type cytokines. By contrast, splenocytes from FADD-DN animals produced significantly less cytokines, and proliferated poorly in response to MOG stimulation (Fig. 3). The effect of FADD blockade on T cells is not MOG-specific because T cell responses to mitogen Con A were also significantly reduced in the FADD-DN group (Fig. 3). Furthermore, when purified T cells from nonimmunized animals were stimulated with anti-CD3 and anti-CD28 Abs, significant reduction in cytokine responses was also detected in the FADD-DN group (Fig. 4). Taken together, these results indicate that both activation and differentiation of T cells are blocked by the FADD-DN transgene.

FADD blockade prevents both mitosis and apoptosis of CD4\(^+\) and CD8\(^+\) T lymphocytes

FADD has been implicated in mediating both cell cycle progression and DR-induced apoptosis, which is responsible for AICD of T lymphocytes. To determine whether the transgenic expression of FADD-DN affects T cell mitosis, splenocytes were isolated from control B6 and FADD-DN mice, labeled with CFSE and activated in vitro with anti-CD3 mAb and anti-CD28 mAb for 4 days. The percentage of CD4\(^+\) and CD8\(^+\) T cells that have undergone at least one cycle of cell division was determined by flow cytometry. As shown in Fig. 5A, >30% of CD4\(^+\) and CD8\(^+\) cells underwent mitosis in the control group. This was significantly reduced in the FADD-DN group. To determine whether apoptosis was also affected by FADD blockade, live cells were purified and restimulated with anti-CD3 mAb and the rate of apoptosis was determined by flow cytometry. As shown in Fig. 5B, AICD of CD4\(^+\) and CD8\(^+\) T cells, as measured by the percent of annexin V-positive cells, was significantly inhibited in the FADD-DN group. Similar results were obtained when cell death was determined by propidium iodide staining (not shown). These results indicate that

FIGURE 4. Inhibition of cytokine production by FADD-DN transgene. CD4\(^+\) T cells were isolated from the spleen of FADD-DN C57BL/6 mice and their littermate controls (\(n = 5\)) using anti-CD4 mAb-coupled microbeads (Miltenyi Biotec). Cells were cultured at 0.5 \(\times\) 10\(^6\)/ml in complete DMEM for 6 days in the presence of 10% FBS, 50 ng/ml IL-2, plate-bound anti-CD3 mAb (1 \(\mu\)g/ml), and soluble anti-CD28 mAb (20 \(\mu\)g/ml). Live cells were then isolated and cultured at 0.5 \(\times\) 10\(^6\)/ml in the presence of 10% FBS, plate-bound anti-CD3 mAb and soluble anti-CD28 mAb. Supernatants were collected at 24, 48, and 72 h, and cytokine concentrations determined by ELISA. The differences between control and FADD-DN groups are statistically significant as determined by ANOVA (\(p < 0.01\)).

FIGURE 5. Inhibition of mitosis and apoptosis by FADD-DN transgene. Splenocytes were isolated from 4- to 5-wk-old FADD-DN C57BL/6 mice and their littermate controls (\(n = 5\)), labeled with or without CFSE, and cultured in DMEM for 4 days in the presence of 10% FBS, plate-bound anti-CD3 mAb and anti-CD28 mAb (27). A. For mitosis analysis, CFSE-labeled cells were stained with anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry. The percent of dividing cells \(=\) (the number of CD4\(^+\) or CD8\(^+\) cells that have undergone cell division/total number of CD4\(^+\) or CD8\(^+\) cells) \(\times\) 100. B. For apoptosis studies, IL-2 was added to the culture at the end of day 3 to a final concentration of 20 ng/ml. Live cells were purified through a Ficoll gradient and cultured with or without plate-bound anti-CD3 mAb for an additional 24 h. The percentage of apoptotic cells was determined by flow cytometry based on annexin V staining as described in Materials and Methods. The spontaneous apoptotic rates in cultures containing no anti-CD3 mAb were 5.74 and 9.04% for CD4\(^+\) and CD8\(^+\) T cells of B6 mice, 17.9 and 10.7% for CD4\(^+\) and CD8\(^+\) T cells of FADD-DN mice, respectively. The activation-induced apoptotic rates presented were calculated by subtracting the spontaneous apoptotic rates from the apoptotic rates of cultures containing anti-CD3 mAb. The differences between control and FADD-DN groups for both A and B are statistically significant as determined by ANOVA (\(p < 0.01\)). The experiments were repeated twice with similar results.
FADD mediates both mitosis and AICD in CD4+ and CD8+ T cells in vitro.

**FADD blockade reduces the frequency of MOG-specific T cells in vivo**

The dual role of FADD in mitosis and apoptosis raises the question of whether it helps to increase or decrease the size of the Ag-specific T cell pool during an immune or autoimmune response. To address this issue, we introduced the FADD-DN transgene into MOG-specific TCR transgenic mice and studied the frequency of MOG-specific T cells in both naive and immunized animals. We found that the frequencies of MOG-specific transgenic T cells in the total CD4+ T cell pool were comparable in naive control and FADD-DN mice (data not shown). However, when the same number of TCR transgenic T cells was transferred into mice that were subsequently immunized with MOG peptide, the frequency of FADD-DN MOG-specific T cells in the spleen was significantly reduced as compared with that of the control group (Fig. 6A). Similar reduction was observed in the lymph nodes (not shown) and in the total number of MOG-specific T cells per lymphoid organ (Fig. 6B). These results indicate that while FADD may be required for both mitosis and apoptosis, the net effect of FADD during an autoimmune response in vivo is to increase the frequency of autoreactive T cells.

**Discussion**

The possibility of promoting both apoptosis and mitosis by the same molecule is a recurrent theme in apoptosis research and provides an exciting opportunity to uncover the molecular pathways that regulate cell death and survival. FADD is one of such molecules that promote both apoptosis and mitosis. In this study, we examined what this paradigm entails in a system in which apoptosis and mitosis have radically different consequences. We found that FADD expressed by T cells can regulate both their apoptosis and mitosis, but the effect on mitosis prevails in vivo. The net effect of FADD blockade in T cells is the prevention of the development of pathogenic T cells and the autoimmune disease induced by them. Thus, FADD serves as an enhancer of autoimmune encephalomyelitis.

FADD is crucial for the functions of DRs, which include Fas (CD95), TNFRI, DRs 3, 4, and 5 (13, 14). Interestingly, most of these receptors are involved in regulating both lymphocyte mitosis and apoptosis, and therefore, are important for immune homeostasis and self-tolerance. Thus, DR ligation can activate the FADD-caspases pathway, leading to DNA fragmentation and cell death. In contrast, the same receptors can also promote cell survival through activating NF-xB or the c-Jun pathway of signal transduction (15, 16). This appears to be mediated through TNFR-associated factor-2 and/or receptor interacting protein as recently demonstrated by several laboratories (16, 17). Using gene microarray technology, Kumar-Sinha et al. (17) recently showed that DRs were able to activate a large number of genes in tumor cells. These include NF-xB-dependent genes such as cIAP2, A20, and E-selectin (17). Whether FADD mediates these activating events needs now to be investigated. Fas has been reported to both inhibit and promote autoimmune inflammation. Mutations of genes encoding Fas or Fas ligand (FasL) lead to lymphocytic proliferation and autoimmune inflammatory diseases in both humans and mice (18). Under these conditions, T cells of presumably autoimmune origin accumulate in extremely large numbers and exhibit a peculiar phenotype, i.e., CD4+CD8+ B220+ or CD4+CD8+ B220+. In the late stages of the disease these aberrant cells become functionally inactive or anergic. Although these observations have led to the recognition that Fas is essential for maintaining self-tolerance, presumably by deleting autoreactive cells through AICD, other studies suggest that Fas/FasL interaction can also contribute to autoimmune inflammation. Kang et al. (19), Giordano et al. (20), and Chervonsky et al. (21) reported that Fas/FasL interaction may contribute to the pathogenesis of autoimmune thyroiditis and diabetes. Similarly, Waldner et al. (22) and Sabelko et al. (23, 24) reported that EAE was diminished and apoptosis was inhibited in mice carrying the lpr (Fas) or gld (FasL) mutation, suggesting that Fas/FasL interaction may play an active role in the pathogenesis of EAE. In EAE, FasL is expressed by microglial cells and neurons as well as activated T cells infiltrating the CNS. By adoptive cell transfer, Sabelko-Downes et al. (24) recently showed that Fas expressed by recipient mice but not T cells, is involved in the pathogenesis of EAE. Although Fas may promote EAE by directly killing neural cells, it can also inhibit EAE by deleting inflammatory cells. Miller and colleagues (25) reported that EAE was significantly enhanced in Fas-deficient SJL lpr/lpr mice, which displayed significantly increased mean peak clinical scores, reduced

![FIGURE 6. Reduced frequency of FADD-DN MOG-specific TCR transgenic cells in vivo. Groups of irradiated (5 Gy) C57BL/6 mice (n = 5) were injected i.v. with 1.2 × 106 MOG-specific TCR transgenic T cells that do or do not carry the FADD-DN transgene (9). Twenty-four hours later, mice were immunized with MOG peptide in CFA as described in Materials and Methods. All mice were sacrificed on day 6 and the frequencies of transgenic T cells in lymphoid organs were determined by flow cytometry as described in Materials and Methods. A. The frequency of MOG-specific TCR transgenic T cells expressed as a percentage of total splenocytes. B. Total number of MOG-specific TCR transgenic T cells in the spleen of recipient mice. The differences between the two groups are statistically significant as determined by ANOVA (p < 0.05).](http://www.jimmunol.org/)
remission rates, and increased mortality when compared with their SJL +/1pr littermates. Similarly, we found that spontaneous EAE was dramatically exacerbated in myelin-specific TCR transgenic mice carrying Fas or FasL gene mutation (26). The exacerbation of EAE was evidenced primarily by an increase in disease incidence and a decrease in spontaneous disease recovery (26). Results reported here indicate that FADD-mediated mitosis, but not apoptosis of myelin-specific T cells, is crucial for the development of EAE. This is consistent with reports that FADD blockade in T cells significantly inhibited cell cycle progression (6, 7). However, it is unclear whether the mitotic effect of FADD requires the participation of DRs. Further studies are needed to address this issue. Regardless of the exact mechanisms involved, our demonstration that FADD blockade in T cells prevented EAE and inhibited Th1- and Th2-type autoimmune responses indicates that FADD is essential for the development of the autoimmune disease and strategies targeting FADD could be effective in preventing or treating the disease.

Acknowledgments
We thank Dr. Andreas Strasser (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) for providing the FADD-DN mice and Dr. Vijay Kuchroo (Harvard Medical School, Boston, MA) for the MOG-specific TCR transgenic mice.

Disclosures
The authors have no financial conflict of interest.

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