

Constitutive Phosphorylation Mutation in Fas-associated Death Domain (FADD) Results in Early Cell Cycle Defects*

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Fas-associated death domain (FADD) is an adaptor molecule for the death receptor subfamily of the tumor necrosis factor receptor superfamily, but it is also required for cell proliferation. Cell cycle-specific regulation of FADD phosphorylation plays an important role in FADD proliferative function since mice with a mutant form of FADD mimicking constitutive phosphorylation at serine 191 (FADD-D) exhibit defective T cell proliferation. Here we characterized these mice in detail and found that T cell development in 2–4-week-old mice is relatively normal, although mature FADD-D T cells manifest defective G₀ and G₁ to S transition with abnormalities in regulation of p130, p27 degradation, retinoblastoma protein phosphorylation, and CDK2 kinase activity. These downstream defects are further associated with the failure to up-regulate the forkhead box M1 cell cycle transcription factor, FoxM1. FADD-D protein is also mislocalized during cell cycle progression. Thus, regulation of FADD phosphorylation is crucial for proper cell cycle entry.

“Proliferation apoptosis couplers” are a proposed class of molecules that regulate the balance between cell life and death (1). These bifunctional couplers can function in both the proliferative and the apoptotic pathways, and in recent years, accumulating data have suggested that the adaptor molecule, Fas-associated death domain (FADD),² may be one such coupler. FADD, which contains a death domain (DD) and a death effector domain, was initially identified as a crucial molecule for Fas-induced apoptosis (2, 3). Fas belongs to the DD-containing death receptor subfamily of the tumor necrosis factor receptor superfamily. Upon ligation of Fas by Fas ligand, FADD binds Fas through homotypic DD interactions (2, 4, 5). FADD then recruits the death effector domain-containing effector protease, procaspase-8, initiating caspase-8 activation, which eventually leads to apoptosis (6, 7). In addition to Fas, FADD was shown to be a necessary adaptor molecule for all known death

receptors (8–10). Although the role of FADD in apoptosis has been well characterized, the precise role of FADD in proliferation remains a mystery. FADD has proved to be important for T cell, and more recently, B cell proliferation. T cells from T cell-specific FADD knock-out mice, FADD^{-/-} → RAG-1^{-/-} chimeric mice, and transgenic mice expressing a human FADD dominant negative (FADD-DN) transgene, all exhibit defects in proliferation in response to T cell activation (8, 11–14). Recent work demonstrates that the role of FADD in proliferation is mostly through regulation of the cell cycle machinery, although it is not clear whether FADD operates at the G₂/M or G₁/S transition of the cell cycle. FADD-null T cells exhibit intact membrane-proximal T cell activation events but display dysregulated expression of many cell cycle proteins before and after T cell activation (15). Mouse and human FADD proteins are phosphorylated on equivalent serine residues (serine 191 in mouse and serine 194 in human) that reside within the C-terminal tail, a region outside the DD and death effector domain apoptotic domains (16, 17). Phosphorylation of FADD is induced by T cell activation starting at the G₁ phase of the cell cycle and continues throughout the cell cycle (18). In cell lines, human FADD has been shown to be phosphorylated in a G₂/M phase-specific manner by casein kinase Iα (16, 19). Our laboratory has previously shown that mice bearing a Ser-to-Asp mutation at serine 191 in FADD [FADD(S191D) or FADD-D], which mimics constitutive phosphorylation, exhibit proliferative but not apoptotic T cell defects (17). The exact molecular mechanisms of defects in FADD-D T cells, however, have not been investigated. In this regard, we utilized the FADD-D mice to parse out the molecular mechanisms by which FADD is involved in the cell cycle regulation in T cells. In contrast to the cell line data, we show that FADD is phosphorylated within hours of mature T cell activation, correlating with entry into cell cycle. FADD-D T cell development is relatively normal in 2–4-week-old mice. However, their mature T cells have an impaired ability to proliferate in response to activation due to specific defects at the G₁/S transition. These defects can be traced to a failure of FADD-D T cells to express FoxM1 protein. FoxM1 is a cell cycle-associated transcription factor that is important for the G₁/S transition, as well as mitosis. During the G₁/S transition, FoxM1 drives the transcription of components of the Skp1-Cullin 1-Fbox ubiquitin ligase complex, which is necessary for degradation of various cell cycle inhibitors (20). Activated FADD-D T cells fail to down-regulate p27 and exhibit very little Retinoblastoma protein (Rb) phosphorylation and CDK2 activities. Furthermore, FADD-D has altered subcellular localization in the context of cell cycle progression. The

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² The abbreviations used are: FADD, Fas-associated death domain; DD, death domain; DN, dominant negative; DP, double positive; BrdUrd, bromodeoxyuridine; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; GST, glutathione S-transferase; Rb, retinoblastoma protein; HSA, heat stable antigen; CKI, casein kinase I; ConA, concanavalin A.

latter is consistent with reports of preferential nuclear accumulation of human FADD protein in several cell lines and that phosphorylation at serine 194 is important for nuclear localization of FADD (19, 21). We concluded that mutation of FADD, which mimics constitutive phosphorylation, adversely affects expression of FoxM1, FADD localization, and subsequently, proper cell cycle entry into the S phase.

EXPERIMENTAL PROCEDURES

Mice—Generation and initial characterization of FADD (S191D) (FADD-D line number 5 in FADD^{-/-} alleles) mice was previously reported (17). The mice have been backcrossed to B6 background for at least five generations. All experiments were performed using littermate controls.

Flow Cytometric Analysis—All organs were isolated, weighed if appropriate, and dissociated through 70- μ m cell strainers. Cells were counted using trypan blue exclusion. Thymocytes, lymph node cells, and splenocytes were then stained with the following antibodies from BD Biosciences: CD4, CD8, CD69, HSA, CD62L, and CD44. Absolute cell numbers were calculated from percentages of DN, double positive (DP), and single positive populations and total thymus cellularity and then normalized to body weight. For intracellular staining of BrdUrd, Ki67, and p130, T cells were column purified by negative selection (R&D Systems), cultured at 2×10^6 cells/ml in RPMI, 10% fetal calf serum and either not stimulated or stimulated with 2.5 mg/ml ConA and 1.25 ng/ml PMA for 2 or 4 days. For Ki67 staining, T cells were fixed in 70% ethanol and processed according to the manufacturer's protocol (BD Biosciences). For p130 (Santa Cruz Biotechnology) staining, cells were processed and stained according to the protocol from Cell Signaling for intracellular staining using unconjugated primary antibodies. For BrdUrd labeling, T cells were labeled with 10 μ M BrdUrd (Sigma) for 1 h at 37 °C, washed twice with PBS, and fixed in 70% ethanol overnight at 4 °C. Processing and anti-BrdUrd fluorescein isothiocyanate (Caltag) staining was carried out following BD Biosciences protocol.

Proliferation and Apoptosis Assay—T cells from lymph nodes and spleens were purified and cultured as stated previously and either not stimulated or stimulated with 2.5 mg/ml ConA and 1.25 ng/ml PMA for the indicated times. At various time points, cells were collected, counted by trypan blue exclusion, and subsequently stained with annexin V (BD Biosciences, according to protocol) and 20 ng/ml propidium iodide for flow cytometric analysis. Apoptotic cells were deemed propidium iodide-negative, annexin V-positive.

Western Blotting—T cells were purified, cultured, and stimulated for various times as already described. Cells were pelleted and washed with PBS, and protein lysates were prepared in 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.5, 1 mM Na₃VO₄, 1% Nonidet P-40 plus protease inhibitors.

Protein was quantified using the Bio-Rad BCA reagents, and 5–10 μ g of total protein was loaded onto either 8 or 12% SDS-PAGE gels. Protein was transferred onto nitrocellulose, blocked in 5% bovine serum albumin in Tris saline buffer (0.1% Tween), and blotted for the various cell cycle proteins. The following antibodies were from Santa Cruz Biotechnology: p21, cyclin D2, CDK6, CDK2, cyclin E, cyclin A, cdc2, p27, c-Myc, and

p-Rb(Ser795). Anti-actin was from AbCam, and anti-FoxM1 was from Abnova. Generation of anti-FADD rabbit polyclonal antisera has been published previously (4).

Immunoprecipitation Kinase Assay—T cells were not stimulated or stimulated with 1:1000 each of plate-bound anti-CD3 (500A2) and anti-CD28 (37N). Protein lysates were prepared in the following lysis buffer: 50 mM Hepes, pH 7.5, 10 mM NaCl, 1% Triton, 10% glycerol, 5 mM MgCl₂, 1 mM EGTA, 2 mM Na₃VO₄, plus protease inhibitors. CDK2 complexes were immunoprecipitated overnight at 4 °C using mouse monoclonal anti-CDK2 (Santa Cruz Biotechnology) and captured with protein G beads (Pierce). CDK2 immunoprecipitations were resuspended in kinase buffer (50 mM Hepes, pH 7.4, 10 mM MgCl₂, 5 mM MnCl₂, 1 mM dithiothreitol) with the addition of 10 μ Ci of [γ -³²P]ATP or 20 μ g/ml GST-Rb substrate, where appropriate. Kinase assays were performed for 30 min at 30 °C, stopped with an equal volume of 2 \times reducing sample buffer, boiled, and run on 8% SDS-PAGE gel. Gels were dried, exposed, and read on a PhosphorImager. The GST-Rb substrate was purified on glutathione beads as per a standard GST fusion protein preparation.

Immunofluorescence—T cells were treated as indicated, harvested, and spun onto slides using the Cytospin system. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed three times with PBS, and then permeabilized/blocked in blocking solution (1 \times PBS, 0.5% fetal bovine serum, 0.2% Triton, 3% bovine serum albumin) for 20 min at room temperature. T cells were then stained with anti-FADD (1:1000, rabbit polyclonal) in blocking solution for 30 min at room temperature, washed three times with 1 \times PBS, 0.1% Triton, stained with 1:100 anti-rabbit Alexa Fluor 488 secondary antibody (Molecular Probes) for 30 min at room temperature, and washed three times as described above. 4',6-Diamidino-2-phenylindole (Sigma) was diluted into secondary antibody solution for nuclear stain. Slides were mounted with anti-fading solution (90% glycerol, 20 mM Tris-HCl, pH 8, 2.33% 1,4-diazabicyclo (2,2,2)-octane), sealed with nail polish, and analyzed on a confocal microscope at $\times 60$ magnification.

RESULTS

Normal Positive Selection in 2–4-week-old FADD-D Mice—Using the previously described FADD-D mice, we sought to understand the mechanism of the proliferative defects in FADD-D T cells. To determine whether improper thymocyte development in FADD-D mice might contribute to the mature T cell phenotype, we analyzed various aspects of the thymic compartments of FADD-D animals. As seen in Fig. 1A (left), FADD-D animals are runted, and this phenotype is exacerbated with age. The body weight of 2–4-week-old FADD-D mice is significantly less than that of littermate controls, and this difference increases with the 6–8-week age group. FADD-D thymi exhibit an apparent reduced cellularity, but since FADD-D animals are runted, we calculated the ratio of thymus size to body size (Fig. 1A). Although FADD-D thymi and body sizes were smaller than those of control mice in both age groups, the ratio of thymus weight to body weight was not significantly different between control and FADD-D mice, especially for the 2–4-week-old mice. These data suggest that the reduced thymic cellularity of 2–4-week-old FADD-D mice is in

FADD(S191D) and Cell Cycle Machinery

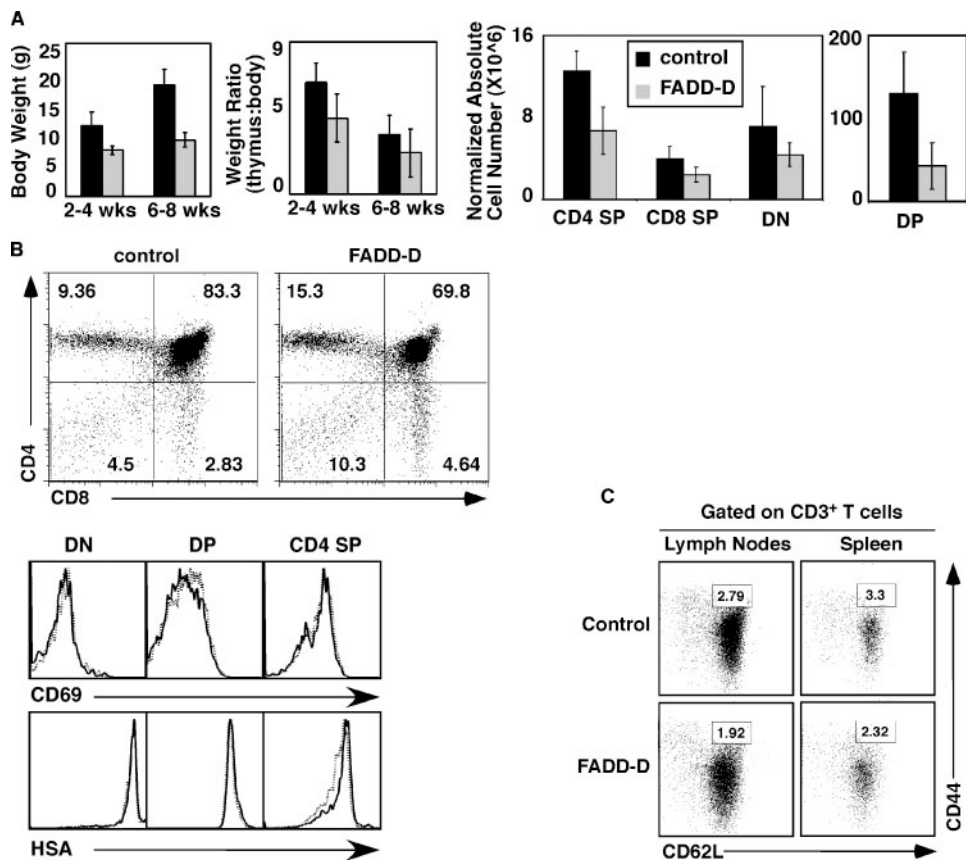


FIGURE 1. Thymocyte-positive selection in 2-4-week-old FADD-D mice appears normal. *A*, the averages of body weights and the ratio of thymus weight:body weight were calculated and plotted for control (black bars) and FADD-D (gray bars) mice representing two age groups: 2-4 weeks ($n = 5$) and 6-8 weeks ($n = 3$). The absolute cell numbers for the CD4/CD8 thymic subsets of control and FADD-D 2-4-week-old mice were calculated and then normalized to body weight. *B*, flow cytometric analysis of thymocytes from 2-4-week-old mice. *Upper panel*, a flow profile of thymocytes stained with CD4 versus CD8. *Lower panel*, the double negative (DN), DP, and CD4 single positive (SP) thymocyte compartments were analyzed for expression of the positive selection markers CD69 and HSA. (Control = solid line; FADD-D = dotted line). *C*, CD3⁺ T cells from lymphocytes and splenocytes of 3-week-old mice were analyzed for expression of CD44 and CD62L. Boxes denote where an increased cell population in FADD-D mice would have been found if homeostatic proliferation were to occur.

proportion to the overall decrease in mouse size. This is further supported by a relatively normal CD4/CD8 profile of FADD-D thymocytes (Fig. 1*B*). Taking into account the normalized absolute cell numbers for the four FADD-D thymic subsets (Fig. 1*A*), these young FADD-D mice have relatively normal numbers of CD4 and CD8 single positive cells, a small decrease of DN thymocytes, and a 2-fold decrease of DP thymocytes. The drop in the number of DP thymocytes is exacerbated for older mice (data not shown), possibly reflecting the increasing requirement for FADD in DN to DP transition in older but not young animals (8).

To determine whether FADD-D T cells were being properly selected, we assayed for the expression of cell surface markers associated with thymocyte-positive selection. CD69 is expressed at very low levels on DN thymocytes but is up-regulated during positive selection. After positive selection, CD69 expression gradually decreases. In contrast, HSA is expressed at high levels on DN and DP thymocytes but is down-regulated gradually following positive selection (22). These typical expression patterns of CD69 and HSA are seen in the FADD-D thymocytes (Fig. 1*B*), demonstrating that positive selection is

proceeding normally in FADD-D thymi. It was previously observed that FADD-D mice are slightly lymphopenic (17). Since a lymphopenic environment could cause naive T cells to proliferate, we analyzed the peripheral T cells of 3-week-old mice for evidence of homeostatic expansion. Homeostatic proliferating T cells express high levels of CD44 and, in contrast to antigen-activated T cells, maintain CD62L expression (23). There was no increase in the representation of the CD44^{high} CD62L^{high} population of T cells observed in the lymph nodes or spleen of either control or FADD-D animals (Fig. 1*C*), suggesting that homeostatic proliferation of FADD-D T cells is not occurring.

FADD-D T Cells Exhibit Selective Cell Cycle Defects—As evidenced by the cell numbers following T cell activation over a 4-day period, FADD-D T cells do not proliferate (Fig. 2*A*) (17). To more specifically assess this proliferative defect of FADD-D T cells, we analyzed for cell cycle progression using Ki67 staining and BrdUrd incorporation. Ki67 is a nuclear-associated antigen that is expressed solely in proliferating cells (24). Naive control T cells do not express Ki67, but after 4 days of activation, the majority of T cells express Ki67, indicating that they are proliferating (Fig. 2*B*, top). However, Ki67 expression remains extremely low in FADD-D T cells after activation. BrdUrd incorporation at 2 days after activation exhibits similar results as described for Ki67 (Fig. 2*B*, middle). As both Ki67 expression and BrdUrd incorporation measure entry into G₁ and/or S phase, we concluded that FADD-D T cells do not proliferate because of defects in cell cycle regulation associated with the G₁ to S transition.

Interestingly, in contrast to wild-type T cells, a small portion of non-stimulated FADD-D T cells expresses Ki67 and incorporates BrdUrd. This basal level of proliferation is not increased upon activation (Fig. 2*B*). The spontaneously cycling population of FADD-D T cells is further confirmed by intracellular staining for p130, a marker of quiescence (25-28). Naive T cells from control mice express high levels of p130, which becomes down-regulated upon activation and entry into cell cycle (Fig. 2*B*, bottom). However, a small population of naive FADD-D T cells has already down-regulated p130 expression. It is interesting to consider that wild-type FADD is phosphorylated within hours of T cell stimulation, thus correlating with exit from quiescence and entry into cell cycle (Fig. 3*A*, top) (18). Taken together, these data suggest that the phosphorylation of FADD

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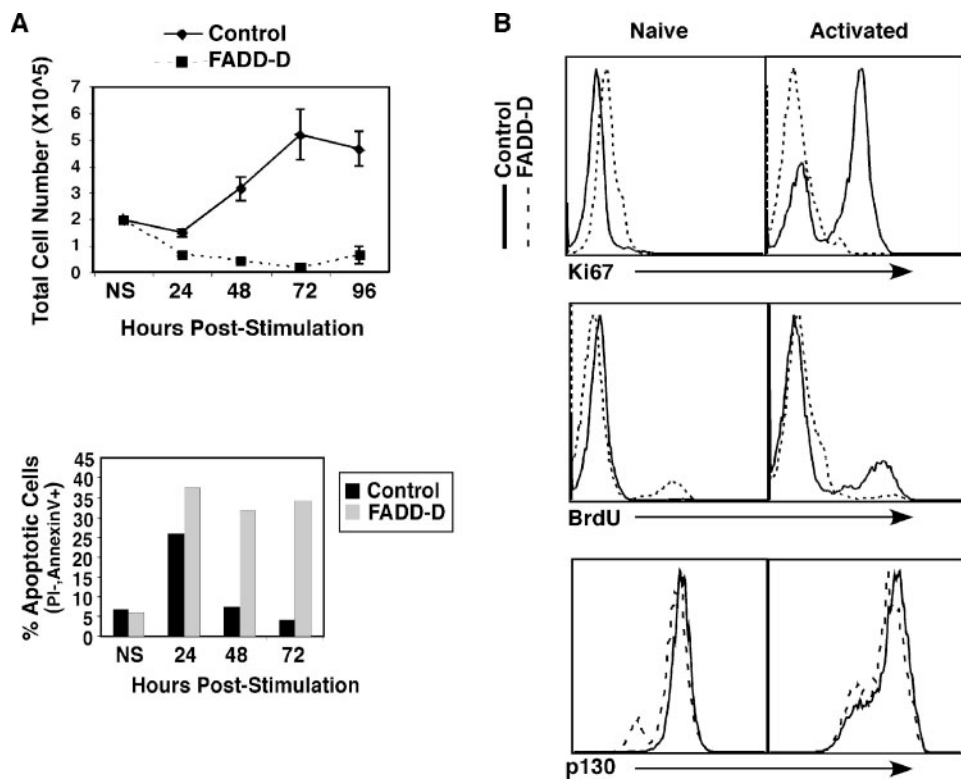


FIGURE 2. Analysis of peripheral T cells in 2–4-week-old mice. *A*, upper panel, T cells were column-purified from control and FADD-D lymph nodes and spleens and cultured at 2×10^6 cells/ml for 4 days after stimulation with ConA/PMA. Live cells were counted by trypan blue exclusion every 24 h after stimulation. Each point represents the average value of triplicate samples, and error bars indicate the corresponding standard deviations. Lower panel, purified T cells from control and FADD-D mice were either not activated or activated with ConA/PMA treatment. At the indicated times after stimulation, apoptosis was assessed by annexin V/propidium iodide staining. The apoptotic cells were identified as annexin V-positive, propidium iodide-negative. *B*, purified naive T cells from control or FADD-D animals were either not stimulated or stimulated with ConA/PMA. At 2 days after activation, T cells were analyzed for BrdUrd incorporation and p130 expression, whereas at 4 days after activation, T cells were intracellularly stained for Ki67.

may be an important event associated not only with the G_1/S but also with the G_0/G_1 transition.

The failure of FADD-D T cells to go through cell cycle is accompanied by subsequent apoptosis. By annexin/propidium iodide staining, higher percentages of FADD-D T cells undergo apoptosis beginning at 24 h after stimulation. Thus, it appears that FADD-D T cells die in response to failed cell cycle progression (Fig. 2A, lower panel).

FADD-D Interferes with FoxM1 Expression and Subsequent Downstream Events Necessary for S Phase Progression—To define how FADD-D interferes with cell cycle regulation, and thereby characterize the role of FADD in cell cycle, we compared expression patterns of a panel of cell cycle proteins by Western blot in naive and activated T cells from control and FADD-D mice. Although most cell cycle proteins exhibit normal expression patterns in FADD-D T cells (Fig. 3A), p27 and Rb are specifically affected. p27, a member of the Cip/Kip family of CDK inhibitors, binds to and blocks CDK activity (29). Rb is a repressor of E2F transcription factors, which are important for regulating transcription of various cell cycle proteins (29–34). Degradation of p27 and Rb phosphorylation cause release of CDK inhibition and E2F repression, respectively, events crucial for progression from G_1 to S phase (30). Although p27 degradation and phosphorylation of Rb are evident in activated control T cells, these events are largely absent in activated

FADD-D T cells, indicating the block in FADD-D T cell proliferation occurs at the G_1/S transition (Fig. 3, A and B). These data correlate with the previously described Ki67 and BrdUrd defects. CDK2 kinase is a key controller of both p27 and Rb regulation. Since CDK2 protein expression was fairly normal in FADD-D T cells, we assessed the activity of CDK2 in an *in vitro* immunoprecipitation/kinase assay (Fig. 3C). CDK2 kinase complexes were immunoprecipitated from cell lysates of naive and activated control and FADD-D T cells. Using GST·Rb as the substrate, CDK2 kinase activity was measured by radiolabeled ATP incorporation. Although CDK2 complexes from activated control T cells are able to phosphorylate the GST·Rb substrate, CDK2 complexes from FADD-D activated T cells exhibit very little kinase activity. As a control, co-immunoprecipitated cyclin E was visualized by Western blot analysis. Thus, reduced CDK2 kinase activity likely contributes to the observed p27 and pRb defects of FADD-D T cells.

FoxM1, a member of the Forkhead box family, is a major transcriptional regulator of the cell cycle (20, 35–37). Its targets include cell cycle proteins associated with the G_1/S transition, as well as the mitosis. FoxM1 is highly expressed in proliferating cells and is induced in T cells upon activation and entry into cell cycle (38). Because p27 degradation, Rb phosphorylation, and CDK2 activation are events downstream of the transcriptional network of FoxM1, we examined FoxM1 expression in FADD-D T cells. As seen in Fig. 4, naive T cells from control animals express a low level of FoxM1 protein, which increases after activation. In contrast, FADD-D T cells fail to express any detectable FoxM1 protein at any point before or after activation. To further substantiate the proliferation defect being specific to peripheral T cells of FADD-D mice, we found that FoxM1 is highly expressed in both FADD-D and control thymocytes. This suggests that FADD-D specifically impairs mature T cells in their ability to express FoxM1. To see whether this effect is specific for FoxM1, we examined c-Myc, another transcription factor associated with proliferation (39, 40). In contrast to FoxM1, both naive and activated FADD-D T cells exhibit a high level of c-Myc expression (Fig. 4). Thus, FADD-D T cells appear to be specifically defective in FoxM1 expression, which most likely explains their inability to proliferate in response to T cell stimulation.

Constitutive Phosphorylation Causes Mislocalization of FADD during Cell Cycle Progression—Subcellular localization of FADD has been a controversial issue. For its function at the death receptors, FADD is cytoplasmic. However, FADD has

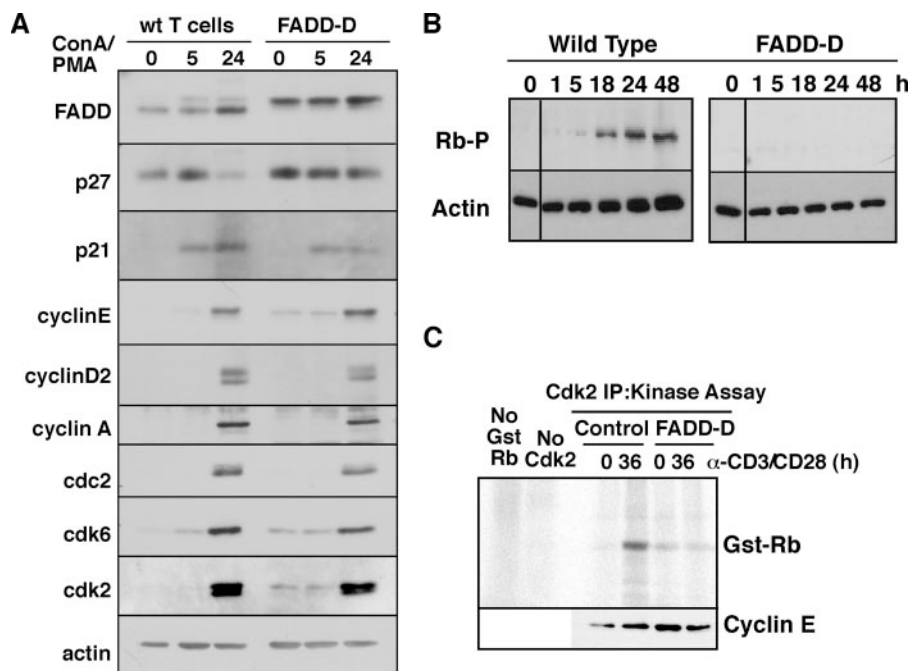


FIGURE 3. FADD-D T cells manifest specific cell cycle-associated defects in p27 degradation, Rb phosphorylation, and CDK2 kinase activation. A and B, protein lysates from naive T cells (0) or those that had been stimulated with ConA/PMA for the indicated times (in h) were used in Western blot analysis of various cell cycle proteins. Actin was included as a loading control. wt denotes wild-type. Rb-P, phosphorylated Rb. C, T cells from control and FADD-D mice were not stimulated or stimulated for 36 h with plate-bound anti-CD3/anti-CD28 antibodies. Anti-CDK2 immunoprecipitations were performed on the total cell extracts and subsequently used for an *in vitro* kinase assay that utilized GST-Rb as the substrate and [γ - 32 P]ATP as the label. Kinase assays lacking either the substrate (No *Gst Rb*) or the immunoprecipitation (No *CDK2*) were used as controls. The cyclin E Western blot shows equal loading of CDK2 protein complexes. These experiments have been repeated at least two times with similar results.

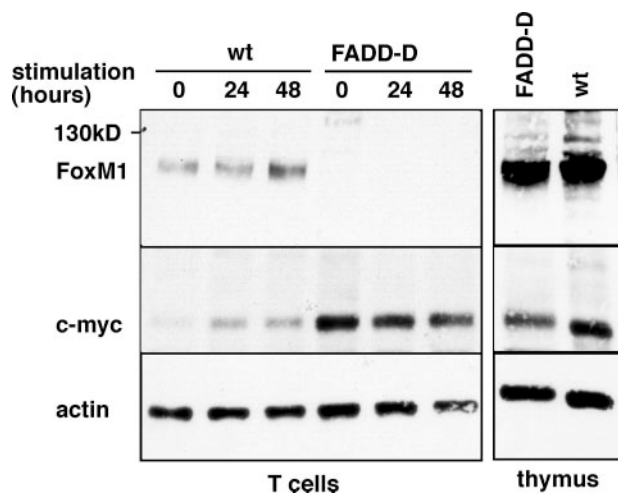


FIGURE 4. FADD-D T cells exhibit defects in FoxM1 and c-Myc regulation. Protein lysates were prepared from control (wt) and FADD-D T cells before and following ConA/PMA stimulation for the indicated times and from total thymocytes. Western blotting was carried out using FoxM1- or c-Myc-specific antibodies. Actin was used as a loading control.

also been shown to be nuclear in many cell types (19, 21, 41–43). Thus, nuclear localization of FADD may contribute to its role in cell cycle regulation. Using immunofluorescence microscopy on primary mouse T cells, we examined the localization of FADD in the context of cell cycle progression and phosphorylation status (Fig. 5). In naive wild-type T cells, FADD is primarily localized within the nucleus, although

some must reside in the cytoplasm as naive T cells are sensitive to FasL-induced death (44). After activation and subsequent phosphorylation, FADD translocates significantly to the cytoplasm. Like FADD, FADD-D protein is also primarily localized in the nucleus of naive T cells. However, in contrast to wild-type FADD, FADD-D protein fails to accumulate in the cytoplasm during cell cycle progression. Constitutive phosphorylation of FADD at serine 191 thus disrupts the proper localization of FADD during cell cycle.

DISCUSSION

FADD is one of many molecules in a growing list that possesses roles in both apoptosis and proliferation (1, 45). To balance such intricately linked, yet opposing, processes requires exquisite control. Recent work in the field indicates that phosphorylation and subcellular localization may be important in the ability of FADD to compartmentalize its roles (19, 21, 42, 43). Using the previously characterized FADD-D

mice (17), we now provide the first molecular evidence for how FADD specifically regulates the early phases of cell cycle. Constitutive phosphorylation of FADD results in problems at both the G_0/G_1 and the G_1/S transitions of the T cell cycle. Unstimulated FADD-D T cells spontaneously enter cell cycle, but upon activation, fail to progress through cell cycle due to molecular defects and mislocalization associated with G_1/S transition. In human cell lines, the level of FADD phosphorylation is highest at G_2/M and lowest at G_1/S . This phosphorylation at G_2/M is mediated by the CKI α kinase (19). We show here that FADD is also important in the early phases of the cell cycle. Our data for FADD-D support the notion that regulation of FADD phosphorylation is important for cell cycle progression. It is interesting to note that unlike primary T cells, the proliferative function of FADD has not been observed in transformed cell lines (9, 46). Because of this difference, we hypothesize that the proliferative role of FADD may be associated with the initial cell cycle entry, which is overcome in immortalized cell lines. Considering both the timing of FADD phosphorylation after T cell activation and the premature spontaneous entry of FADD-D T cells into cell cycle, our data appear to corroborate a role for FADD in the exit from quiescence. It is possible that improper exit from quiescence contributes to the subsequent G_1 defects. Alternatively, FADD serine phosphorylation might be biphasic in primary T cells, occurring during G_0 to G_1 followed by a dephosphorylation event during the G_1 to S transition and subsequent rephosphorylation at G_2/M (as observed in cell lines). However, this phenomenon may be masked in a Western blot analysis due to

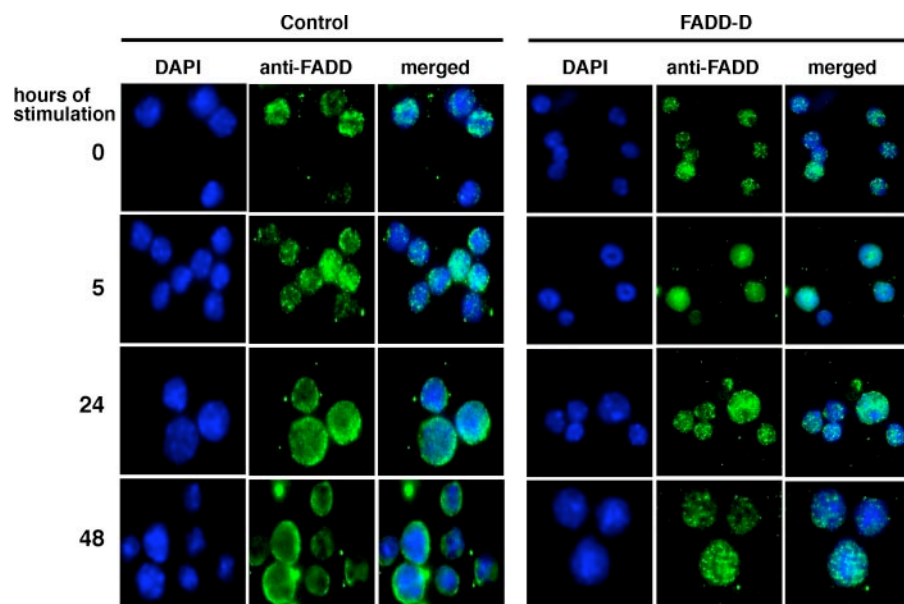


FIGURE 5. Immunofluorescence analysis of FADD localization in peripheral T cells. Purified T cells from control and FADD-D mice were either not stimulated (0) or stimulated for 5, 24, or 48 h with ConA/PMA, spun onto slides, and stained for FADD (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue, nuclear). Pictures were taken on a confocal microscope at $\times 60$ magnification.

an asynchronous nature of T cell activation. Consistent with this hypothesis, we observed that the CKI α pharmacological inhibitor, CKI-7, fails to inhibit the early FADD phosphorylation (within 5 h after stimulation), although it does block the late FADD phosphorylation at 24 h after stimulation (19).³ These data suggest that another kinase distinct from CKI α phosphorylates FADD in the early phase of cell cycle entry.

We have identified the molecular defects of FADD-D T cells at the G₁/S transition. The abnormal regulation of p27, pRb, and CDK2 can ultimately be attributed to the lack of FoxM1 expression, although c-Myc and other unknown cell cycle defects may also contribute to the FADD-D phenotype. The exact molecular link between FADD and FoxM1 is not clear. Interestingly, although FADD-D T cells constitutively express c-Myc, T cells from FADD-DN mice have been shown to have reduced levels of c-Myc after activation (47). This difference might be partly due to the presence of constitutively cycling FADD-D T cells. Like FADD-D T cells, FADD-DN T cells also exhibit proliferative defects in response to activation, but FADD-DN T cells do not spontaneously enter the cell cycle. Alternatively, the accumulation of c-Myc protein in FADD-D T cells could be due to the failure of stimulated FADD-D T cells to reach the late G₁ stage of the cell cycle where derepression of GSK-3 kinase normally occurs (48, 49). In late G₁ of normal cells, active GSK-3 phosphorylates c-Myc at threonine 58, leading to c-Myc protein ubiquitination and subsequent protein degradation. Finally, it is equally plausible that FADD exerts effects on other, as of yet unidentified, aspects of c-Myc and FoxM1 regulation.

The failure of FADD-D T cells to pass through S phase is also accompanied by subcellular mislocalization. Consistent with the observation that nuclear localization of human FADD in

some cells is dependent upon phosphorylation of serine 194 (the equivalent position of mouse serine 191) (21, 42, 43), FADD-D protein is localized primarily to the nucleus. However, unlike wild-type FADD, FADD-D protein fails to significantly accumulate in the cytoplasm following activation. Consistent with this datum, human phosphorylation-deficient FADD (S194A) protein, in contrast to wild-type human protein, resides mostly in the cytoplasm (21). Due to the presence of unmapped threonine phosphorylation sites in mouse FADD (4), phosphorylation-deficient FADD mutant mice have not yet been generated, and FADD(S191A) T cells are phenotypically normal (17). The failure of FADD-D protein to distribute appropriately between nuclear and cytoplasm might be related to the proliferation-associated molecular

defects. FADD-D T cells still maintain the capacity to induce apoptosis through the death receptors, and thus, a small pool of FADD and FADD-D must normally reside within the cytoplasm. Presumably, this small cytoplasmic pool is undetectable by immunofluorescence. Although our understanding of the mechanism of the role of FADD in cell cycle is far from complete, we conclude that serine phosphorylation status, and consequently, appropriate localization, is a major mechanism by which FADD functions to control cell cycle entry. As FADD has no known enzymatic activities, one possible scenario is for serine phosphorylated FADD to act as an adapter protein for cell cycle related proteins. Regulation of the FADD serine phosphorylation, its subcellular localization, and the nucleation of the associated cell cycle proteins might then regulate different phases of the cell cycle in activated T cells. Identification and characterization of these FADD-interacting proteins will be necessary to fully understand how FADD functions as a proliferation apoptosis coupler.

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