A Dominant Negative Fas-associated Death Domain Protein Mutant Inhibits Proliferation and Leads to Impaired Calcium Mobilization in Both T-cells and Fibroblasts*

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Death domain-containing members of the tumor necrosis factor (TNF) receptor family ("death receptors") can induce apoptosis upon stimulation by their natural ligands or by agonistic antibodies. Activated death receptors recruit death domain adapter proteins like Fas-associated death domain protein (FADD), and this ultimately leads to proteolytic activation of the caspase cascade and cell death. Recently, FADD has also been implicated in the regulation of proliferation; functional inhibition of FADD results in p53-dependent impairment of proliferation in activated T-cells. In this study we have further analyzed T-cells derived from transgenic mice expressing a dominant negative FADD mutant (FADD DN) under control of the lck promoter in vitro so as to identify the signaling pathways that become engaged upon T-cell receptor stimulation and that are regulated by death receptors. FADD DN expression inhibits T-cell proliferation, both at the G0 → S transition and in the G1 phase of continuously proliferating cells. We observe a decrease in the release of calcium from intracellular stores after T-cell receptor stimulation, whereas influx of extracellular calcium seems to be unaffected. FADD DN-expressing fibroblasts show a similarly inhibited cell growth and impaired calcium mobilization indicating that the modulation of proliferation and calcium response by death receptors is not cell type-specific.

The TNF1/nerve growth factor receptor family consists of members that all share a cysteine-rich sequence motif in their extracellular ligand-binding domains (for review see Refs. 1 and 2). The ligands of these receptors comprise a corresponding family of structurally homologous proteins that exist both as homotrimeric soluble molecules and in the form of cell-bound type II transmembrane proteins and that induce activation by imposing receptor aggregation. Ligand-receptor interactions within the TNF-TNF receptor family induce pleiotropic cellular responses including activation, proliferation, differentiation, and apoptosis (1, 3).

A subgroup of the TNF receptor family, the death receptors, shares a homologous intracellular protein-protein interaction motif of about 80 amino acids length, the death domain (DD), that ensures downstream cell death signaling (4–6). Originally identified in the CD95 (FAS/APO-1) and TNF-R1 (P55/CD120a) receptors, the death domain is also present in a growing number of family members that includes DR-3 (TRAMP/WSI/APO-3/LARD), TRAIL-R1 (DR-4), TRAIL-R2 (DR-5/TRICK-2/KILLER), p75 nerve growth factor receptor, and CAR-1 (7). The death domain transduces an apoptotic signal by recruiting death domain-containing adapter molecules such as the FAS-associated protein FADD/MORT-1 or the TNF-receptor-associated protein TRADD (8–10). Whereas FADD binds directly to CD95, it is recruited to other death receptors like TNF-R1 and DR-3 via the intermediary adaptor TRADD (11, 12). FADD then links these receptors to the apoptotic caspase effector machinery.

At its N terminus the FADD protein contains another homophilic interaction domain, the death effector domain, which binds to an analogous domain repeated in tandem within the zymogen form of two cellular proteases, caspase 8 (FLICE/MACH (13, 14)) and caspase 10b (FLICE-2 (15)). FADD thereby recruits caspase 8 to the death receptor C-terminal cytoplasmic tail to form a death-inducing signaling complex (16); this in turn leads to oligomerization and activation of caspase 8 by self-cleavage (17). Caspase 8 is then the apical caspase that proteolytically activates downstream effector caspsases such as caspase 9 and caspase 3 (18), thus initiating the so-called caspase cascade which in certain cell types needs the involvement of mitochondrial changes including cytochrome c release (type II cells (19)). Caspase activation is generally considered to be the "point of no return" during apoptosis as it results in the proteolytic cleavage of many cellular target proteins (20).

In certain instances, death receptors mediate alternative functions distinct from apoptosis (1). For example, engagement of TNF-R1 by TNF rarely triggers apoptosis but rather activates the transcription factors NF-κB and AP-1, leading to induction of proinflammatory and immunomodulatory genes in

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¶ The abbreviations used are: TNF, tumor necrosis factor; DN, dominant negative; TCR, T-cell receptor; PBS, phosphate-buffered saline; CFSE, carboxyfluorescein succinimidyl ester; FACS, fluorescence-activated cell sorter; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PMA, phorbol myristate acetate; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; FADD, Fas-associated death domain protein; TRADD, TNF receptor-associated death domain protein; RB, retinoblastoma; JNK, Jun N-terminal kinase; NFAT, nuclear factor of activated T-cells; CRAC, calcium release-activated channel.
response to infections (21). Several recent studies have also indicated a role for death receptors in proliferation. Thus, the receptor activator of NF-κB (RANK) stimulates naive T-cell proliferation following interaction with its ligand (22); TNF and CD95 antibody can both induce proliferation in human fibroblasts (23), and cross-linking of CD95 by specific monoclonal antibodies has been implicated in co-stimulation of CD3 ligand induced T-cell proliferation (24). In addition, CD95 activation stimulates lupus peripheral T-lymphocytes (25).

Recently, we established transgenic mouse lines that over-express a dominant interfering deletion mutant of FADD (FADD DN) in both thymocytes and mature T-cells under control of the Ick promoter (Ick FADD DN mice) (26). FADD DN lacks the N-terminal death effector domain and thereby blocks those TNF receptor family members that use FADD as an adapter (13). By using this approach we hoped to circumvent the potential problem of functional redundancy among the death receptor family that might limit any distinctive phenotype in single receptor knockout models (see e.g. Refs. 27 and 28). As expected, CD95-induced apoptosis was inhibited in FADD DN-expressing cells. However, to our surprise we also observed a p53-dependent inhibition of thymocyte proliferation in vivo, and analogous inhibition of activation induced proliferation of thymocytes and mature T-cells in vitro (26). The inhibition of proliferation was not caused by any increase in cell death. This unexpected result has been confirmed by other groups using similar transgenic mouse models (29, 30). In addition, the FADD gene knockout causes embryonic lethality (31, 32), and thymus reconstitution in RAG-1-deficient chimeric mice has confirmed a defect in activation-induced T-cell proliferation in the absence of FADD. These data confirm that the proliferation defect in Ick FADD DN transgenic cells is due to functional inactivation of FADD, and they strongly support a growth-promoting function for one or more death domain-containing members of the TNF receptor family that associate with FADD.

The aim of this study was to characterize in more detail the molecular mechanism responsible for the proliferation defect in FADD DN-expressing cells. To do this we have analyzed various signal transduction pathways known to be stimulated by TCR activation that lead to proliferation. We find no differences in activation of any tested TCR-activated pathways in the presence of FADD DN with the exception of calcium mobilization, which was severely depressed. FADD DN blocks both quiescent cells from entering the cell cycle (the G0 → S transition) and the proliferation of continuously cycling T-cells in G1. Furthermore, we observe that FADD DN exerts a similar growth-inhibitory effect on fibroblasts, indicating that this effect is not limited to T-cells and so is likely to act on more general, cell type-independent proliferation pathways. FADD DN expression also in fibroblasts results in impaired calcium mobilization upon growth factor stimulation. These data should help to identify the cellular components that constitute the molecular link between death receptors and the proliferative machinery.

**EXPERIMENTAL PROCEDURES**

*Transgenic Mice—*Generation of Ick FADD DN transgenic mouse lines and their initial characterization have been reported (26). The mice have been maintained on a CBA × C57BL/6 background.

*Plasmids and Fibroblast Transfection—*S3T3 fibroblasts constitutively expressing a dominant interfering mutant of FADD (S3T3/FADD DN) were obtained by infection of S3T3 cells expressing the plasmid pB Hygro NF4D containing a cDNA encoding FADD residues 80–208, followed by hygromycin selection and cloning as described in Hueber et al. (33). In these S3T3/FADD DN cells, FADD was not activated by addition of tamoxifen.

*Purification and Activation of Mouse T-cells from Spleen—* Mouse T-lymphocytes were purified from dispersed spleenocytes using Mouse T-cell Enrichment Columns from R & D Systems (high affinity negative selection) according to the manufacturer's instructions. The cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 0.1% (v/v) 2-mercaptoethanol, 1 mM sodium pyruvate, penicillin (50 units/ml), and streptomycin (50 μg/ml). For activation of purified T-cells (5 × 10⁶ cells/ml), 2 ml of RPMI medium without serum. After an incubation of 30 min at 37 °C, 1 ml of RPMI, complemented with 5% fetal calf serum, was added to the cells and mixed. The cells were re-plated for further 24 h on anti-CD3/anti-CD28-coated plates in the presence of IL-2-containing supernatant (recovered from the cells before sorting) and then analyzed again for their CFSE profiles by flow cytometry.

**Flow Cytometric Analysis of Intracellular Calcium Concentrations—**To assay intracellular Ca²⁺ concentration, 5 × 10⁶ freshly isolated thymocytes or peripheral T-cells from lymph nodes were loaded with 3 μM Indo-1 acetoxyethyl ester (INO1; Molecular Probes) in 1 ml of RPMI medium without serum. After an incubation of 30 min at 37 °C, 1 ml of RPMI, complemented with 5% fetal calf serum, was added to the cells and mixed. The cells were re-plated for further 24 h on anti-CD3/anti-CD28-coated plates in the presence of IL-2-containing supernatant (recovered from the cells before sorting) and then analyzed again for their CFSE profiles by flow cytometry.

**Western Blot Analysis—**Proteins for Western blotting were separated using a Mini-Protean III system (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. After blocking, the membranes were then incubated with primary antibodies (anti-CD3, anti-CD28, anti-CD8, anti-CD4, and anti-CD2) at 4 °C overnight. The membranes were then washed three times with PBS and incubated with biotinylated secondary antibody (1:200 dilution) for 1 h at 37 °C. After washing, membranes were incubated with peroxidase-conjugated streptavidin (1:1000 dilution) for 1 h at 37 °C. Membranes were washed and developed using SuperSignal West Pico (Pierce Chemical Co., Rockford, IL).

**Cytokine Assays—**Cytokine levels were measured in supernatants using a rat cytokine multiplex kit (Millipore, Bedford, MA).

**Flow Cytometric Analysis of Intracellular Calcium Concentration—**Calcium mobilization was analyzed using Fura-2 AM (Molecular Probes) as described previously. Cells were incubated with Fura-2 AM for 1 h at 37 °C, washed, and resuspended in fresh medium at 1 × 10⁶ cells/ml. Flow cytometric analysis and stimulation of the cells with bombesin (Sigma; dissolved in Me₂SO at 10 μg/ml) was performed as described by Novak and Rabinovich (35).

**CFSE and Hoechst 33342 Staining of T-cells—**The CFSE staining was performed as described previously (Ref. 36 and references herein). Briefly, T-cells were washed once with PBS, 0.1% bovine serum albumin (BSA) and resuspended in 1 ml of the same buffer containing 10 μg/ml CFSE (obtained from Molecular Probes) for 10 min at 37 °C. The cells were then washed twice with fresh medium at 1 × 10⁶ cells/ml. The cells were analyzed by flow cytometry after indicated times (usually 72 h). A control of CFSE-stained T-cells incubated for the same time without activation helped to identify the population of cells in the CFSE profile which had not yet divided.

**Purification and Activation of Mouse T-cells from Spleen—**Mouse T-lymphocytes were purified from dispersed spleenocytes using Mouse T-cell Enrichment Columns from R & D Systems (high affinity negative selection) according to the manufacturer's instructions. The cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 0.1% (v/v) 2-mercaptoethanol, 1 mM sodium pyruvate, penicillin (50 units/ml), and streptomycin (50 μg/ml). For activation of purified T-cells (5 × 10⁶ cells/ml), 2 ml of RPMI medium without serum. After an incubation of 30 min at 37 °C, 1 ml of RPMI, complemented with 5% fetal calf serum, was added to the cells and mixed. The cells were re-plated for further 24 h on anti-CD3/anti-CD28-coated plates in the presence of IL-2-containing supernatant (recovered from the cells before sorting) and then analyzed again for their CFSE profiles by flow cytometry.

**Western Blot Analysis—**Western blots were performed according to standard protocols as specified by the manufacturers of each commercially available antibody. For standard protein analysis, cells were lysed in 125 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2.5% SDS, and 10% β-mercaptoethanol. To analyze the phosphorylation status of cellular proteins, T-cells were collected by brief centrifugation and resuspended in a small volume of 2× phosphatase inhibitor solution (60 mM sodium...
wild type cells. This shows that the G0 mice than in cells from their wild type counterparts. Specifi-
cular, 10 mM iodoacetamide, 500 mM leupeptin, 20 μg/ml aprotinin. An equal volume of 10% SDS was added, and the lysed cell pellet was immediately boiled for 5 min. After electrophoresis, proteins were electroblotted onto a polyvinylidene fluoride membrane (Millipore), incubated sequentially with primary and horseradish peroxidase-coupled secondary antibodies, and developed with a chemiluminescence reagent (ECL from Amersham Pharmacia Biotech). Antibodies were obtained from New England Biolabs (phospho-RR-(Ser-780) and phospho-RR-(Ser-807/Ser-811), phospho-p38 MAPK, phospho-AKT-(Ser-473)), Santa Cruz Biotechnology (p21, p27, phospho-JNK, Alexi (c-FLIP), and Pro-
mega (phospho-ERK1/2 (Anti-Active™ MAPK polyclonal antibody)). The anti-NFAT1 antibody (anti-67.1 antiserum) was a generous gift from Dr. Patrick Hogan, and the anti-Pan-MYC and anti-EGR-1 anti-
bodies have been described earlier (32, 37). Murine BCL-2 expression was assayed using a rabbit antisera raised against the murine Bcl-2 peptide GAAPPTGIFSFQPE.

IL-2 ELISA—To quantify the amount of IL-2 secreted into the supernatant, an ELISA kit for quantification of mouse IL-2 was used according to the manufacturer's protocol (Genzyme).

RNase Protection Assay—To analyze mRNA levels of mRNAs encod-
ing various cytokines and apoptosis regulator molecules, the RiboQuant multi-probe RNase Protection Assay system from Pharmingen was used following the manufacturer's protocol (template set was mCK-1). Total RNA was prepared from unstimulated and CD3/CD28 co-
activated purified T-cells using TRIzol reagent from Life Technologies, Inc.

RESULTS

FADD DN Inhibits G0 → S Entry into the Cell Cycle and Also Blocks Cell Cycle Progression in G1—Overexpression of FADD DN suppresses TCR-stimulated proliferation of mature T-cells, and this suppression is not due to increased cell death (26, 30). To investigate this proliferation block in greater detail, we used the fluorescent dye CFSE. In its diacetate form this non-toxic, non-fluorescent molecule passively diffuses into cells, whereupon cellular esterases cleave the acetyl groups to generate the active CFSE fluorophore (Ref. 36 and references therein). The highly amine-reactive succinimidyl ester then forms dye-protein adducts that are retained within or at the surface of the cell. As a consequence, CFSE segregates equally between daughter cells upon cell division, resulting in a sequential halving of cellular fluorescence intensity with each successive generation. When analyzed by flow cytometry, this sequential halving of fluorescence appears as distinct peaks or populations of cells, permitting the tracking of cell division in populations of proliferating cells.

We isolated and labeled mature T-cells from the spleens of both wild type and lck-FADD DN transgenic mice with CFSE and analyzed their fluorescence after 72 h of CD3/CD28 co-
stimulation (see Fig. 1, A and B). As predicted the CFSE profiles, obtained by gating on the living cells in the forward/ sideward scatter, showed that far fewer cell divisions had oc-
curred in activated T-cells derived from FADD DN transgenic mice than in cells from their wild type counterparts. Specif-
ically, 54% of FADD DN expressing T-cells had not yet divided at all 72 h after co-stimulation as compared with only 19% of wild type cells. This shows that the G0 → G1 entry into the cell cycle upon activation is inhibited in FADD DN T-cells.

To determine whether cell proliferation is similarly inhibited in continuously proliferating T-cells that have already passed the G0 → G1 transition, we sorted CFSE-labeled purified T-
cells 72 h after CD3/CD28 co-stimulation to obtain homogene-
ous cell populations that had divided either once (P1) or not at all (P0) (as shown for wild type cells in Fig. 1C). Cell sorting was performed in the presence of recombinant IL-2 and soluble anti-CD3 and anti-CD28 antibodies to maintain the mitogenic stimulus. Sorted cells were then cultured on anti-
CD3/anti-CD28-coated plates in the presence of IL-2 for a fur-
ther 24 h, and their CFSE fluorescence profiles were compared again. As expected, many more cells from the wild type P0 population (which had not divided at all after the first 72 h) started proliferating during the following 24 h compared to FADD DN T-cells (data not shown). However, we also observed a similar inhibition of proliferation in FADD DN P1 T-cell populations that had already entered the cell cycle before sorting and replating (see Fig. 1D). Only 1.5% of wild type cells had not divided again since replating compared with 41% of FADD DN cells.

This result was confirmed by cell cycle analysis of cycling cells (see Fig. 1E): 72-h CD3/CD28 co-stimulated and CFSE-
stained T-cells from either FADD DN transgenic mice or their wild type littermates were incubated with the DNA intercalat-
dye Hoechst 33342, and both their CFSE and cell cycle profiles were obtained by flow cytometric analysis. CFSE staining demonstrated the characteristic inhibition of FADD DN T-cell proliferation compared with wild type cells. Cell cycle distribution analysis of cycling cells (gated on the CFSE profile) indicated that 51% of the wild type cells were in G1 phase (25% in S and 24% in G2/M) compared with 61% of FADD DN T-cells (31% in S and 7% in G2/M). This result was confirmed in several independent experiments. Together, these data demon-
strate that FADD DN not only inhibits the entry of activated T-cells into the cell cycle but also blocks the cell cycle progres-
sion of already proliferating cells in G1, phase of the cell cycle.

FADD DN Expression Has No Detectable Influence on Several Molecules Implicated in T-cell Proliferation and Cell Cycle Progression—To search for molecules and signaling pathways that might be responsible for the proliferative defect in FADD DN overexpressing T-cells, we analyzed expression levels and activities of several key molecules implicated in activation-
induced T-cell proliferation. The earliest characterized bio-
chemical response elicited by the TCR is the activation of TCR-associated protein kinases (for review of TCR signal transduction pathways see Refs. 38 and 39). However, the overall crude pattern of tyrosine-phosphorylated proteins pres-
ent after 5 min of co-stimulation by soluble anti-CD3 and anti-CD28 antibodies appeared the same in activated T-cells purified from both FADD DN transgenic and wild type mice (data not shown).

p21RK couples the TCR to the MAPK pathway (38, 39), a kinase cascade involving c-RAF activating MEK which acti-
uates the MAP kinases ERK1 and ERK2, both of which then phosphorylate additional target kinases (40). The cascade classically culminates in the phosphorylation of various transcrip-
tional factors such as ELK-1, which participates in the transcrip-
tional control of c-fos and other cell cycle regulators. We observed no inhibition of phosphorylation/activation of either ERK1 and ERK2 by FADD DN expression during CD3/CD28-
mediated co-activation of T-cells (see Fig. 2A).

We also investigated any effect FADD DN expression might exert on induction of the early growth response gene egr-1, a target of the MAPK pathway (41, 42) which regulates IL-2 transcription by synergistic interaction with the nuclear factor of activated T-cells, NFAT (43). We observed a severalfold induction of EGR-1 expression upon CD3/CD28 co-stimulation in purified T-cells that appeared the same in both wild type and FADD DN transgenic T-cells (see Fig. 2B).

Apart from the ERKs, the MAPK family includes two further subgroups of kinases: the Jun N-terminal kinase, JNK (also known as stress-activated protein kinase), and the p38 MAPK (Ref. 44 and references therein). In T-cells, TCR ligation is sufficient to induce maximally ERK and p38 activity, whereas JNK requires a co-stimulatory signal (such as CD28 ligand binding) for efficient JUN phosphorylation (45). Both the p38 and the JNK pathway contribute to IL-2 gene expression in T-lymphocytes (46). Fig. 2C reveals that p38 activation by phosphorylation upon CD3/CD28 co-stimulation is unaffected.
by FADD DN expression. Likewise, FADD DN expression does not affect JNK phosphorylation (data not shown).

Cell cycle progression initiated by IL-2 in an IL-2-dependent T-cell line has been shown to be mediated by phosphatidylinositol 3-kinase and its effector kinase PKB/Akt, ultimately leading to phosphorylation of E2F (47). We therefore assayed activation of PKB upon CD3/CD28 co-stimulation using a phospho-specific anti-PKB antibody in Western blots (see Fig. 2D). Phosphorylation of PKB was indeed apparent during activation of primary T-cells, but it was unaffected by expression of FADD DN (the difference in the amount of phosphorylated PKB between activated wild type and FADD DN T-cells seen in Fig. 2B is due to different loading of protein).

In T-cells, the Ser-807/Ser-811 site in the cell cycle regulator RB becomes phosphorylated upon CD3/CD28 co-stimulation. This phosphorylation was unaffected by expression of FADD DN (data not shown). The Ser-780 site on RB is already substantially phosphorylated in resting T-cells. However, CD3/CD28 co-activation increases the level of Ser-780 phosphorylation even further, and this is also unaffected by FADD DN expression (data not shown).

We could not detect any differences in the expression levels of c-MYC and BCL-2 between activated wild type and FADD DN transgenic cells (data not shown). In addition, the cell cycle inhibitor p27 is equally down-regulated, whereas another inhibitory protein, p21, is equally up-regulated in co-stimulated wild type and FADD DN-expressing cells (data not shown).

In summary, our analysis of a broad range of molecules implicated in T-cell proliferation indicated no differences between wild type T-cells and those whose proliferation was blocked by expression of FADD DN.

Expression of FADD DN in T-cells Impairs Calcium Flux upon TCR Activation, Although This Does Not Interfere with Normal NFAT1 Dephosphorylation and IL-2 Up-regulation—Two major pathways emanating from the TCR synergize to initiate T-cell activation and the synthesis of IL-2 as follows: activation of p21RAS leading to MAPK stimulation and contributing to NFAT activation, and the mobilization of intracellular

![Image of FADD DN inhibits cell cycle entry and progression in mature T-cells.](http://www.jbc.org/)

**Fig. 1.** FADD DN inhibits cell cycle entry and progression in mature T-cells. A, CFSE FACs profile of non-stimulated T-cells 72 h after purification. The single peak represents all cells that have not divided. B, CFSE profiles of wild type (thick line) and FADD DN transgenic (thin line) purified T-cells after 72 h of CD3/CD28 co-stimulation. The relative size of the different peaks is shown in percent for the wild type (act) and FADD DN transgenic (tg) T-cells. Each peak represents a cell population that has divided a certain number of times; cells in the rightmost peak with the highest fluorescence intensity (1) have not divided at all. Similar results were obtained in five different experiments. C, cell sorting of CFSE-labeled purified T-cells from wild type mice after 72 h of CD3/CD28 co-stimulation. Two cell populations, P0 and P1, were isolated, representing cells that had divided either not at all (P0; thin black line) or just once (P1; broken line). The thick line shows the CFSE profile of all cells; the P0 and P1 peaks of the sorted cells are superimposed. Similar cell populations P0 and P1 were obtained from FADD DN transgenic T-cells (data not shown). D, overlay of CFSE profiles of sorted cell populations P1 from wild type (thick line) and FADD DN transgenic (thin line) T-cells (see C) 24 h after cell sorting and further co-stimulation by CD3/CD28. The P1 cells had already entered the cell cycle before sorting. Relative peak areas for the different cell populations are given below for wild type (WT) and FADD DN (transgenic, TG) T-cells. E, overlay of CFSE profiles of sorted cell populations P1 from wild type (thick line) and FADD DN transgenic (thin line) 24 h after cell sorting and further co-stimulation by CD3/CD28. The P1 cells had already entered the cell cycle before sorting. Relative peak areas for the different cell populations are given below for wild type (WT) and FADD DN (transgenic, TG) T-cells.
calcium $[\text{Ca}^{2+}]$, (see Ref. 48 and references therein). Sustained elevation of $[\text{Ca}^{2+}]$, is achieved by $\text{Ca}^{2+}$ entry through the plasma membrane mediated by the initial depletion of luminal $\text{Ca}^{2+}$ stores and ultimately leads to the activation of calcineurin, a $\text{Ca}^{2+}$/calmodulin-dependent serine/threonine phosphatase. Activation of calcineurin, in turn, results in dephosphorylation and translocation of preformed NFAT from the cytoplasm to the nucleus, where it either binds directly to the proximal NFAT-binding site of the IL-2 promoter or combines with FOS and JUN proteins at the distal NFAT-binding site in the IL-2 promoter.

To examine TCR-induced calcium flux in the context of FADD DN overexpression, cells were preloaded with the calcium dye Indo-1, and flow cytometry was used to monitor intracellular calcium concentrations after cross-linking of the TCR-CD3 complex with anti-CD3 antibody. In wild type cells TCR cross-linking elicits an expected initial peak of increased calcium in the cytoplasm followed by a sustained intracellular calcium elevation (Fig. 3, A and B). In contrast, FADD DN cells show a severely reduced initial peak of elevated cytoplasmic calcium, whereas the following sustained elevation looks similar to wild type cells (Fig. 3, A and B). When using a lower concentration of the stimulating anti-CD3 antibody (4 $\mu$g/ml), wild type T-cells display a slower and more flattened elevation in intracellular calcium, whereas FADD DN cells fail to respond altogether (Fig. 3, C and D).

These differences are not due to defective dye loading in the transgenic cells as ionomycin used at high doses (2 $\mu$g/ml) induces equivalent large increases in both cell types (data not shown). A similar reduction was observed in thymocytes (Fig. 3, A and C) and peripheral T-cells (Fig. 3, B and D).

The overall pattern of calcium elevation in T-cells results from two components of the calcium response as follows: the initial transient peak is due to the release of calcium from intracellular stores (50), and the resulting depletion of these stores triggers the opening of the plasma membrane calcium channels, permitting calcium influx and sustained elevation in intracellular calcium (51). The intracellular calcium profile obtained with the FADD DN transgenic cells showed the main difference in the first transient step compared with wild type cells that suggested at least a defect at the level of calcium release from intracellular stores. To check whether such a defect is due to TCR proximal events, we used thapsigargin, a compound specifically inhibiting the calcium-ATPase in the endoplasmic reticulum membrane, which leads to a complete depletion of intracellular calcium stores and by that triggers extracellular calcium entry (52, 53). Surprisingly, as in the case with TCR cross-linking, we observed that the calcium response upon thapsigargin treatment was impaired in FADD DN transgenic cells (Fig. 3E) arguing against the possibility that FADD DN only affects TCR signaling close to the membrane. This result is in accordance with our finding that the calcium defect in FADD DN transgenic cells is not T-cell-restricted (see below).

The difference in calcium mobilization between wild type and transgenic T-cells was also seen when using a low concentration of the calcium ionophore ionomycin (0.05 $\mu$g/ml) to empty intracellular calcium stores (data not shown) (54), excluding the possibility that the defect is simply linked to a lower efficiency of thapsigargin to block the ER-$\text{Ca}^{2+}$-ATPase in presence of FADD DN.

In order to pinpoint further the defect in calcium mobilization, calcium flux was studied in FADD DN transgenic cells under different conditions. If the thapsigargin stimulation was performed in the presence of EGTA in order to chelate the extracellular calcium present in the medium, a different level of calcium elevation in wild type and transgenic thymocytes was observed (Fig. 3F). Notably, this difference (shown as a zoom in Fig. 3H), although appearing quite small in Fig. 3F due to the weak amplitude of calcium release from internal stores, was highly reproducible in the six independent experiments performed. In addition, by looking at the percentage of cells responding over time, this difference was even more obvious: 50% of the wild type cells respond after thapsigargin stimulation in the presence of EGTA compared with only 10% of FADD DN transgenic cells (Fig. 3G). This result confirms the defect in the initial peak observed following TCR cross-linking and emphasizes that FADD DN negatively influences intracellular calcium release. Interestingly, when extracellular calcium was added in the middle of the experiment, calcium influx occurred in a similar manner in the FADD DN and wild type cells (Fig. 3F).

We were surprised to see that the inhibition of calcium release from intracellular stores did not lead to any obvious difference in the consecutive calcium entry step. This apparent contradictory result could be explained in the light of a paper recently published by Lepple-Wienhues et al. (55), showing...
FADD DN Inhibits Calcium Response in T-cells and Fibroblasts

Thymus

A

anti CD3
15µg/ml

B

anti CD3
15µg/ml

C

anti CD3
4µg/ml

D

anti CD3
4µg/ml

Time (min)

Lymph Nodes

E

tg

F

CaCl2

TG/EGTA

G

CaCl2

TG/EGTA

H

Time (min)
that CD95/FAS signaling blocks calcium influx (see "Discussion").

To determine whether FADD DN cells were capable of activating calcineurin and dephosphorylating NFAT, T-cells were purified and activated for 5 min with phorbol myristate acetate (PMA)/ionomycin. Cell lysates were then assayed for NFAT phosphorylation status using an anti-NFAT1 antibody (for review on NFAT family members see Ref. 56). However, despite the marked inhibition of proliferation in FADD DN expressing T-cells upon PMA/ionomycin treatment, NFAT1 dephosphorylation in these stimulated cells was the same in both wild type and FADD DN transgenic T-cells (Fig. 4A). We were also unable to detect any impairment in IL-2 production and excretion by FADD DN expression in T-cells (see Fig. 4B) treated with either PMA/ionomycin or CD3/CD28, as determined by IL-2 ELISA. This last observation is consistent with the observation that addition of exogenous IL-2 to purified T-cells upon activation fails to prevent FADD DN-mediated inhibition of proliferation (26). Finally, we used RNase protection assay to analyze IL-2 mRNA induction in wild type and FADD DN transgenic T-cells following CD3/CD28 treatment. As shown in Fig. 4C, FADD DN-expressing T-cells exhibited normal induction of IL-2 (and IL-6) mRNAs.

Taken together, these data indicate that the reduction in the increase in intracellular \([\text{Ca}^{2+}]\) caused by FADD DN expression, while presumably significant, is not sufficient to suppress NFAT1 activation or IL-2 induction upon TCR stimulation.

Overexpression of FADD DN in Fibroblasts Inhibits Proliferation and Calcium Mobilization—We were interested to see whether the anti-proliferative effect of FADD DN is restricted to T-lymphocytes. Therefore we expressed FADD DN in mouse Swiss 3T3 fibroblasts by retroviral infection (33). Infected cells expressing FADD DN were observed to grow very slowly, already indicative of an impaired proliferative capacity. This was confirmed by labeling an asynchronously growing culture of Swiss 3T3/FADD DN cells with CFSE followed by flow cytometric analysis 24 and 48 h later (see Fig. 5A). The CFSE profile showed that FADD DN-expressing cells proliferated far less than cells infected with an empty control vector, a difference that could not be accounted for by increased cell death (data not shown). These results show that the anti-proliferative effect of FADD DN is not restricted to lymphoid cells.

Fibroblasts also display a well defined increase in intracellular calcium levels after ionomycin treatment or mitogenic stimulation by bombesin (57). To investigate whether the impairment of calcium mobilization by FADD DN is a non-cell type-specific, general phenomenon, we treated FADD DN-expressing Swiss 3T3 fibroblasts with ionomycin or bombesin and compared intracellular calcium peaks with stimulated control cells infected with the empty retroviral expression vector. Fig. 5B shows that FADD DN inhibits the calcium response in fibroblasts after ionomycin or bombesin treatment suggesting that not only the inhibition of proliferation but also the impairment of calcium mobilization by FADD DN is not cell type-specific.

Fig. 3. Intracellular calcium flux is reduced in FADD DN transgenic T-cells. Thymocytes (A and C) or peripheral T-cells from lymph nodes (B and D) isolated freshly from either wild type (solid lines) or FADD DN transgenic (dashed lines) mice were preloaded with Indo-1. The increase in intracellular calcium concentrations after stimulation by either anti-CD3 antibody (15 μg/ml A and B) or by thapsigargin (TG; 50 nM) (E, F, G, and H) was analyzed. A–F and H, the relative amount of cytosolic calcium in individual cells (determined by the ratio of Indo-1 violet/blue fluorescence) is shown; G, the percentage of responding cells as a function of time is displayed. F and G, EGTA (0.7 mM) was added simultaneously with thapsigargin to bind calcium in the medium; extracellular calcium in the form of CaCl₂ (0.7 mM) was added back midway through the experiment. H represents a magnification of the left half F. Arrows indicate the time point of stimulation. The experiment was repeated at least four times with similar results.
DISCUSSION

In this paper we have assessed the effect of FADD DN expression on several signal transduction and effector pathways implicated in activation-induced T-cell proliferation. Since we did not observe any difference between wild type and FADD DN T-cells in the overall pattern of tyrosine-phosphorylated proteins after TCR stimulation (data not shown), we assume that the TCR-regulated protein tyrosine kinase cascade, which is the starting point for different signaling pathways like NFAT or MAPK activation (38, 39), is not directly affected by expression of the transgene. More specifically, FADD DN expression caused no detectable change in activation of ERK1/2, JNK, or p38, arguing that the MAPK pathways are not affected. This notion is corroborated by the fact that induction of the early growth response gene egr-1, a target of the MAPK pathway (41, 42), also appears normal in FADD DN cells.

One particularly provocative observation is that the calcium response, as defined by the rise of intracellular calcium concentrations after TCR activation or thapsigargin treatment, is significantly reduced in FADD DN-expressing T-cells. Although we show that this impairment in Ca\textsuperscript{2+} flux is insufficient to prevent dephosphorylation of NFAT1 (a prerequisite for its nuclear translocation), it nevertheless could affect other Ca\textsuperscript{2+}-dependent processes underlying inhibition of cell proliferation by FADD DN.

It has previously been shown that Ca\textsuperscript{2+} from intracellular stores may be mobilized upon CD95 ligation (58), formally establishing a link between calcium signaling and death receptors. Intracellular calcium stimulates a number of transcription factors implicated in T-cell mitogenesis, such as c-FOS, c-JUN, the cyclic AMP-response element, and the serum response element (59), and it is also involved in inactivation of the transcriptional repressor DREAM (60).

Elevation of intracellular inositol trisphosphate controls both the release of stored calcium and the influx of external calcium (59) through the calcium release-activated channel (CRAC (61)) when the internal Ca\textsuperscript{2+} stores are drained. Our results show that the mobilization of calcium from intracellular stores was defective in FADD DN-expressed T-cells, whereas the entry of extracellular calcium was preserved. This last observation is intriguing since a defect in the release of intracellular calcium is expected to result in a decreased influx of extracellular calcium. We believe that the secondary effect of FADD DN on calcium influx is masked by another phenomenon: the recent paper by Leeple-Wienhues et al. (55) shows that CD95 stimulation blocks CRAC and calcium influx in lymphocytes through the activation of acid sphingomyelinase and ceramide release in a cell population refractory to death induction (55). One attractive hypothesis based on this observation is that in vivo in a specific T-cell subpopulation continuous interaction between Fas and its ligand at the cell surface membrane (either in cis or in trans) does not lead to cell death but rather to CRAC inhibition and consequently to a failure in activation-induced proliferation.

Therefore, the incapacity to see a defect in the calcium influx...
in our stimulated transgenic cells could be due to the fact that expression of FADD DN prevents CRAC inhibition by FAS engagement in vivo. Indeed, FAS-induced ceramide release (leading to CRAC blockage) has been shown to be a FADD-dependent signal (62).

A link between depolarization-activated ion channels and death domain-containing proteins has already been reported. A conserved N-terminal sequence of the pro-apoptotic Drosophila melanogaster protein REAPER for which a homology with the FADD death domain has been suggested (63) can block K⁺ flux through pores of voltage-gated K⁺ channels (64).

Two human cases of primary T-cell immunodeficiency have been linked to a failure of T-cell proliferation due to defective transmembrane calcium influx, although Ca²⁺ release from internal stores was apparently normal (65, 66). In one of these cases, the same defect was also present in other hematopoietic cell lineages as well as in fibroblasts (66). The precise defect leading to the absence of Ca²⁺ influx is unclear but is most probably located in the pathway linking calcium release from intracellular stores to the opening of the calcium channels present in the plasma membrane and may possibly involve dysfunction of the Ca²⁺ channels themselves. This phenotype of inhibited proliferation together with an impaired Ca²⁺ influx is remarkably similar to the one observed in activated FADD DN-expressing T-cells. It raises the interesting speculation whether the molecular defect leading to impaired Ca²⁺ influx in these cases of primary immunodeficiencies might lie in the regulation of calcium signaling by TNF receptor family members.

In our search for molecules that are implicated in cell cycle progression and that might be regulated by death receptors, we checked the activity of several obvious candidates. But neither up- and down-regulation of the CDK-inhibitory proteins p21 and p27 upon activation nor the expression level of c-MYC or the level of RB phosphorylation was different between wild type and FADD DN transgenic activated T-cells. We also could not detect any differences in the up-regulation of CD25 (IL-2 receptor), CD95, and CD95 ligand nor in the TCR down-regulation as determined by immunoblotting (data not shown). Most remarkably, calcium analysis with ionomycin-treated FADD DN-expressing cells and FADD DN overexpression Swiss 3T3 fibroblasts. Despite long periods of propagation in culture, these cell clones never lost FADD DN expression as determined by immunoblotting (data not shown). Most remarkably, calcium analysis with ionomycin-treated FADD DN-expressing fibroblasts has shown that the inhibition of proliferation in this cell type also correlates with a decreased calcium mobilization. These data collectively suggest that neither the impairment of proliferation nor the decrease in intracellular calcium mobilization upon overexpression of FADD DN is T-cell-specific.

Inhibition of proliferation by FADD DN argues for the existence of a proliferation pathway emerging from the FADD/CD95 complex. Another interesting possibility is suggested by the recent finding that the CD95 ligand by itself can transduce a growth-inhibiting signal through its intracellular domain (71). Perhaps the regulation of cell proliferation occurs at the level of TNF ligand family members, and in the case of FADD DN-expressing cells with impaired proliferative capacity, the ligand-induced anti-proliferative signal transduction pathway becomes "unmasked" because FADD DN is blocking the predominant death receptor-induced apoptosis pathway. Future experiments with already existing transgenic mouse models will help to understand the relative contribution of TNF receptor and ligand family members to the regulation not only of cell death but also of proliferation.

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