

Mammalian nitrilase 1 homologue Nit1 is a negative regulator in T cells

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Abstract

The mammalian Nit1 protein is homologous to plant and bacterial nitrilases. In flies and worms, Nit1 is fused to the 5' end of Fhit, suggesting that Nit1 may functionally interact with the Fhit pathway. Fhit has been shown to play a role of a tumor suppressor. Somatic loss of Fhit in human tissues is associated with a wide variety of cancers. Deletion of Fhit results in a predisposition to induced and spontaneous tumors in mice. It has been suggested that Nit1 collaborates with Fhit in tumor suppression. Similar to mice lacking Fhit, Nit1-deficient mice are more sensitive to carcinogen-induced tumors. It was previously shown that ectopic expression of Nit1 or Fhit led to caspase activation and apoptosis, and that both proteins may play a role in DNA damage-induced apoptosis. In this study, we analyzed the physiological function of Nit1 in T cells using Nit1-knockout mice. Nit1-deficient T cells can undergo apoptosis induced by DNA damage due to irradiation and chemical treatment. However, apoptosis induced by Fas or Ca⁺⁺ signals appeared to be compromised. Additionally, Nit1 deficiency resulted in T cell hyperproliferative responses induced by TCR stimulation. The expressions of T cell activation markers were elevated in Nit1^{-/-} T cells. There was a spontaneous cell cycle entry and enhanced cell cycle progression in Nit1^{-/-} T cells. These data indicate that Nit1 is a novel negative regulator in primary T cells.

Introduction

Nit1 was initially identified as a gene fused to Fhit in *Drosophila melanogaster* and *Caenorhabditis elegans* (1). However, in mammals the Nit1 and Fhit proteins are encoded by separate genes on different chromosomes. The human Fhit gene is localized to chromosome 3p14.2, which contains the fragile site FRA3B and is frequently rearranged in many human cancer cells (2). Somatic inactivation of Fhit has been observed in a majority of lung, esophageal, bladder, cervical, as well as head and neck carcinomas (3,4,5,6,7,8). Deletion of one or both alleles of Fhit rendered mice highly susceptible to tumor development induced by *N*-nitrosomethylbenzylamine (NMBA) (9,10). In addition, there is a high incidence of spontaneous tumors in Fhit^{-/-} mice (11). These data suggest that Fhit has the properties of a tumor suppressor.

The human Fhit gene encodes a protein of 147 amino acids which is homologous to the histidine triad (Hit) nucleotide-binding protein family including diadenosine 5', 5''' P¹, P⁴-tetraphosphate asymmetrical hydrolase of *Schizosaccharomyces pombe* (2,12). The NH₂ terminus of Fhit in *Drosophila* and *C. elegans* was found to contain an extra sequence of ~300 amino acids, which is 48% identical to the mammalian Nit1 protein and 22% identical to the nitrilase in bacteria and plants (1). Prokaryotic nitrilases cleave various nitriles into the corresponding acids and ammonia and are mainly involved in the utilization of various nitrogen sources (13,14,15). Plant nitrilases participate in the production of the hormone indole-3-acetic acid (16). Whether the mammalian Nit1 protein has similar biochemical functions remains unclear. Structural analysis of the *C. elegans* Nit1Fhit protein

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revealed that a Nit1 tetramer binds two Fhit dimers (17). Since it exists as a fusion to the mammalian Fhit homologue in flies and worms, Nit1 was postulated to be functionally related to Fhit, particularly as a tumor suppressor (1). Recent studies showed that Nit1 deficiency rendered mice more susceptible to forestomach tumor induction by NMBA (18).

Overlapping expression of Nit1 and Fhit has been observed in many tissues, including the lymphoid organs (18). While no abnormality has been reported in young *Fhit*^{-/-} mice, spontaneous lymphoid malignancies including B cell lymphoma and lymphocytic infiltrations develop in aged *Fhit*^{-/-} mice (11). It remains to be determined whether Nit1 deficiency leads to spontaneous malignancy in the immune system. Previous studies have tried to understand the biological pathways that are regulated by Nit1 and Fhit. One of the likely mechanisms by which Fhit and Nit1 act as tumor suppressors is through pro-apoptotic pathways. Transfection of Nit1 or Fhit has been shown to induce apoptosis in cell lines (18,19,20,21,22,23). Epithelial cells lacking Nit1 or Fhit were resistant to apoptosis induced by DNA damages (18). One of the major apoptotic pathways is mediated by the mitochondria, involving cytochrome C and members of the Bcl-2 family proteins (24). Aberrantly elevated expression of Bcl-2 results in lymphoma due to a lack of apoptosis in lymphocytes (25,26). However, previous data indicated that Fhit-induced apoptosis was independent of Bcl-2 and Bcl-x(L) and instead required FADD-mediated pathway (27). FADD is a mediator of extrinsic apoptotic signaling pathways induced by death receptors including Fas which is essential for homeostasis in the immune system (28,29,30,31). It has not been determined whether Nit1 plays a role in primary lymphocyte apoptosis or other responses such as proliferation induced by antigen receptors.

In this study, we analyzed the function of Nit1 in T cells using conditional Nit1-knockout mice in which Nit1 deletion was induced in either T cells or germ cells using the *lckcre* or *cmvcre* transgenes, respectively. The data show that a lack of Nit1 has no obvious effect on T cell numbers and subset ratios in the thymus or periphery. No defects in DNA damage-induced apoptosis are detected in *Nit1*^{-/-} T cells. However, apoptosis induced by Fas or Ca⁺⁺ flux is slightly reduced in *Nit1*^{-/-} T cells. Interestingly, *Nit1*^{-/-} T cells display enhanced proliferation, elevated activation marker expression, accelerated cell cycle progression and aberrant expression of some cell cycle proteins. Therefore, this study reveals a function of Nit1 in the negative regulation of T cell proliferation.

Methods

Deletion of the Nit1 gene in T cells and germ line cells

The conditional Nit1-knockout mice (*Nit1*^{fl/fl}) have been described (18). To generate T cell-specific Nit1-deficient mice, *Nit1*^{fl/fl} mice were crossed with *lckcre* mice purchased from Taconic (32). The resulting heterozygous *Nit1*^{fl/+} *lckcre* mice were intercrossed to obtain *Nit1*^{fl/fl} *lckcre* mice. To induce deletion of *Nit1* in germ cells, *Nit1*^{fl/fl} mice were crossed with *cmvcre* mice purchased from The Jackson Laboratory to produce *Nit1*^{-/-} mice. PCR-based genotyping was performed to detect the three *Nit1* alleles and the *lckcre* trans-

genes using mouse ear tissue lysates and the following primers: Nit-CKO (5'-GTACCGGATACCGATTACTTCGA-3'), Nit-R (5'-GTGCTGGGATTAAAGGTGTGCA-3') and Nit-WT/Floxed (5'-GTTGGTCTAGCAATCTGTTATGA-3'). The *lckcre* transgene was typed by PCR using two primers (F12Cre, 5'-CCAGCTAAACATGCTTCATCGTC-3' and B21Cre, 5'-CCTGATCCTGGCAATTCGG-3'), as previously described (33). All animal studies were approved by appropriate institutional committees.

Flow cytometry

Cells were isolated from the thymus, spleen and lymph nodes of 6- to 8-week old mice and single-cell suspensions depleted of RBC by hypotonic lysis and were enumerated using a hemocytometer. Cell surface protein expression was determined by staining cells with the appropriate fluorochrome-conjugated antibodies in PBS containing 3% BSA, 1 mM EDTA and 0.05% NaN₃ and analyzed using a Coulter Epics XL flow cytometer (Beckman Coulter).

T cell apoptosis assay

Cells were isolated from the thymus, seeded into 96-well flat bottom plates (1 × 10⁵ per well) in complete RPMI 1640 and incubated at 37°C without additional stimulation for the indicated times or with various concentrations of Fas ligand (FasL) (Alexis) plus anti-FLAG M2 antibodies (1 µg ml⁻¹; Sigma-Aldrich) and cycloheximide (1 µg ml⁻¹) for 16 h. Thymocytes were treated with the indicated concentrations of dexamethasone (Sigma-Aldrich) for 16 h at 37°C as described (34,35). Irradiation-induced apoptotic responses were determined as previously described (35). Thymocytes (1 × 10⁵) were seeded in triplicate into 96-well round bottom plates in 100 µl complete RPMI 1640, irradiated (200 rad) and incubated at 37°C for the indicated times. Thymocytes were treated with ionomycin or staurosporine at the indicated concentrations as described (36). After incubating for the indicated times at 37°C, propidium iodide (PI) (1 µg ml⁻¹) was added to the cultures and cell death determined by flow cytometry as described previously (33).

Cell proliferation assays

Total lymph node cells and splenocytes (1 × 10⁵) were seeded into 96-well round bottom plates in 100 µl of complete RPMI 1640. After addition of anti-CD3 ascites at various dilutions and anti-CD28 ascites (1:1000 dilution), cells were incubated for 36 h at 37°C, followed by an 8-h pulse with 0.5 µCi of [³H] thymidine. Cells were collected using a 96-well harvester, and thymidine incorporation was determined by using a liquid scintillation counter. T cells were purified from the spleen and lymph nodes using MACSTM beads (Miltenyi Biotec) and stimulated with anti-CD3 plus anti-CD28 antibodies. T cell accumulations were determined by tritiated thymidine incorporation or enumeration using a hemocytometer and proliferation shown as bar graphs and growth curves, respectively.

IL-2 expression assays

Peripheral T cells were seeded (3 × 10⁶ cells per well) in 4-well plates coated with 20 µg ml⁻¹ anti-CD3 antibodies

(BD Biosciences), and biotinylated anti-CD28 antibodies (BD Biosciences) were added to a final concentration of $2 \mu\text{g ml}^{-1}$. After 6 h stimulation at 37°C in 5% CO_2 incubator, cells were collected for intracellular staining with IL-2. Intracellular staining procedure was performed using the BD Cytfix/Cytoperm Plus kit with Golgistop™ (BD Biosciences) according to the manufacturer's instruction. Cells (1.5×10^6 per staining) were washed once with staining buffer (PBS with 1% FBS and 0.05% sodium azide), then stained with FITC-conjugated anti-CD4 and Tri-color-conjugated anti-CD8 on ice for 20 min. These cells were washed once with PBS and fixed with $100 \mu\text{l}$ BD cytofix/cytoperm at 4°C for 20 min. After two washes with $250 \mu\text{l}$ BD perm/Wash solution, cells were stained with PE-conjugated anti-IL-2 (BD Biosciences) on ice for 30 min. Cells were washed once with BD Perm/Wash solution and analyzed by flow cytometry.

Cell division and cell cycle profile analyses

To analyze cell division kinetics, T cells (4×10^6) were purified from lymph nodes by panning with anti-IgM antibodies, re-suspended in 0.9 ml PBS/5% FBS and mixed with 0.1 ml PBS/5% FBS containing $50 \mu\text{M}$ of carboxyfluorescein succinimidyl ester (CFSE; $5 \mu\text{M}$ final concentration). After 5 min of incubation at 37°C , cells were washed twice with PBS/5% FBS, once with complete RPMI 1640 medium and stimulated with anti-CD3 ascites (1:5000 dilution) plus anti-CD28 ascites (1:1000 dilution) in round-bottom 96-well plates. For control, mimosine ($62.5 \mu\text{g ml}^{-1}$; Sigma) was added to the cells and analyzed by flow cytometry (33). For cell cycle profile analysis, T cells were purified from the spleen and lymph nodes by panning with anti-mouse IgM antibodies and seeded into 96-well round-bottom plates (10^5 per well) in complete RPMI 1640 medium containing anti-CD3 antibodies (1:10000 dilution) plus anti-CD28 ascites (1:1000 dilution) in $100 \mu\text{l}$ complete RPMI 1640. At the indicated times, cell cycle profiles were analyzed by determining DNA contents as previously described (37). Briefly, cells were washed once with PBS containing 1% BSA and 0.01% sodium azide, dispersed in $100 \mu\text{l}$ of a staining solution (0.1% sodium citrate, 0.1% Triton X-100, $50 \mu\text{g ml}^{-1}$ PI, $100 \mu\text{g ml}^{-1}$ RNaseA) and subsequently analyzed on a flow cytometer.

Western blot analyses

Cells ($1\text{--}2 \times 10^7$) were lysed at 4°C for ~ 15 min in $50\text{--}100 \mu\text{l}$ ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonylfluoride and a protease inhibitor cocktail (Roche). Cell lysates were centrifuged at maximum speed for 10 min, the supernatant mixed with sample buffer and proteins resolved in a 10% SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane, blocked with 5% milk in PBS/T and incubated overnight at 4°C with primary antibodies. Blots were washed three times with PBS/T and incubated with HRP-conjugated goat secondary antibodies (Pierce) as per the manufacturer's recommendations. The Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) was used to develop and detect protein signals on X-ray films (Kodak).

Quantitative reverse transcription-PCR and protein turnover assays

T cells were purified from the spleen and lymph nodes using MACS™ beads (Miltenyi Biotec), and total RNA was prepared using RNeasy Mini Kit (Qiagen) from untreated T cells (0 h) and T cells stimulated with anti-CD3 plus anti-CD28 antibodies for 24 h. First strand cDNAs were synthesized using SuperScript™ III reverse transcriptase kit (Invitrogen). Primers used to evaluate relative expression were as follows: cyclin (Cyc) E (forward 5'-tccagaaaaggaaggcaaa-3' and reverse 5'-agacttcgcacacctccatt-3'), CDC2 (forward 5'-ctggcagttcatggattct-3' and reverse 5'-ggccacacttcgtttagg-3') and β -actin (forward 5'-ccctgaagtacccattgaa-3' and reverse 5'-acgctcggtcaggatcttc-3'). Quantitative reverse transcription (RT)-PCR was performed and the levels of Cyc E and CDC2 RT-PCR products were normalized against the levels of β -actin RT-PCR products. To determine protein turnover, T cells were stimulated with anti-CD3 plus anti-CD28 antibodies for 24 h. Cycloheximide ($50 \mu\text{g ml}^{-1}$) was then added for 30 and 60 min. Total cell proteins were extracted and standard western blotting was performed using anti-Cyc E and anti-CDC2 antibodies.

Results

The effect of Nit1 deficiency on lymphocyte development

To determine whether the Nit1 protein is expressed in the murine lymphoid system, we performed western blot analyses of the thymus, spleen and lymph nodes of wild-type *Nit1^{+/+}* mice. Polyclonal anti-Nit1 antisera detected a protein of ~ 30 kDa in thymocytes, splenocytes and lymph node cells (Fig. 1A). To investigate the physiological function of Nit1 in lymphocytes, we used conditional Nit1-knockout (*Nit1^{fl/fl}*) mice in which exons 4, 5, 6 and 7 of the *Nit1* gene were flanked with two *loxP* sites (18). To induce Nit1 deletion in T cells, mice bearing the T lineage-specific *Ickcre* transgene were crossed with *Nit1^{fl/fl}* mice. The thymus was isolated from the resulting *Nit1^{fl/fl} Ickcre* mice and used in western blotting. As shown in Fig. 1(A) (left), thymocytes from *Nit1^{fl/fl} Ickcre* mice appear to express minimal levels of the Nit1 protein, when compared with control *Nit1^{+/+} Ickcre* and *Nit1^{fl/fl} Ickcre* mice. This result indicates that *Nit1* was efficiently deleted in T cells in the thymus through *Ickcre*. Nit1 deletion was also apparent in peripheral T cells, as the levels of the Nit1 protein were dramatically reduced in splenocytes and lymph node cells in *Nit1^{fl/fl} Ickcre* mice (left; Fig. 1A). The Nit1 protein is also expressed in B cells and non-lymphoid cells (bottom; Fig. 1A and data not shown).

To determine the effect of Nit1 deficiency on T cell development, flow cytometric analyses were performed with T cell-specific *Nit1^{-/-}* mice. Thymocytes, splenocytes and lymph node cells were isolated and stained with fluorochrome-conjugated antibodies to the T cell co-receptors CD4 and CD8. In the thymus, mature T cells express either CD4 or CD8 (CD4^+ and CD8^+), immature T cells express both CD4 and CD8 ($\text{CD4}^+\text{CD8}^+$) and pro/pre-T cells lack these co-receptors ($\text{CD4}^-\text{CD8}^-$). As shown Fig. 1B, these T cell populations are present in *Nit1^{fl/fl} Ickcre* mice at levels similar to those in control *Nit1^{+/+} Ickcre* mice. The majority of the T cells present in the peripheral lymphoid organs such

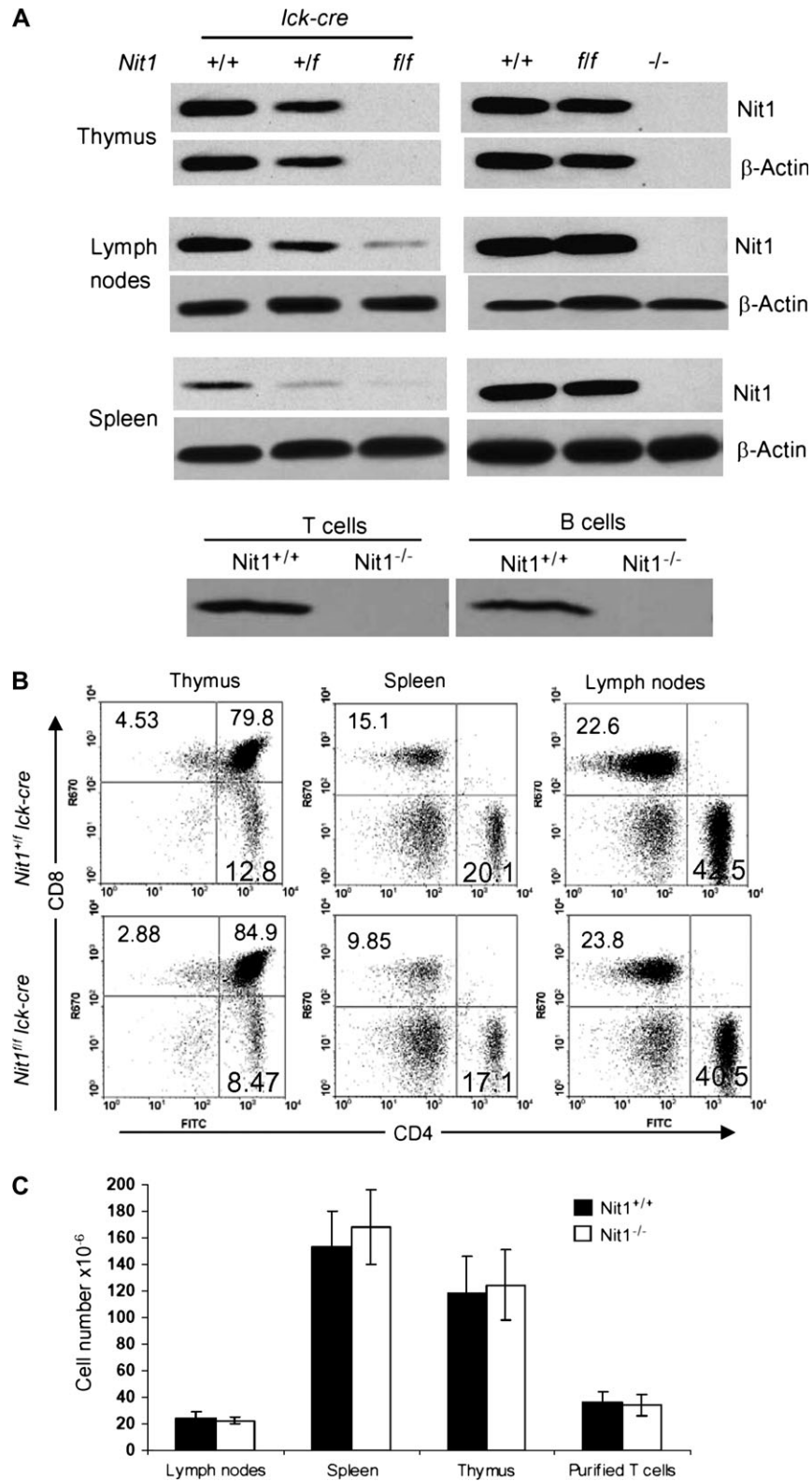


Fig. 1. Western blot and flow cytometric analyses of *Nit1*^{-/-} mice. (A) Thymocytes, splenocytes and lymph node cells were isolated from T cell-specific *Nit1*-deficient (*Nit1*^{fl/fl} *Lck-cre*) and *Nit1*^{-/-} mice in which *Nit1* was deleted in germ cells using *cmvcre*. *Nit1*^{+/+} and *Nit1*^{+/f} mice expressing *Lck-cre* and *Nit1*^{fl/fl} mice were used as controls. T cells and B cells were purified by using MACS beads. Total cellular proteins were fractionated by SDS-PAGE and western blotting performed using polyclonal anti-*Nit1* antibodies. (B) Cells were isolated from the indicated organs of T cell-specific *Nit1*-deficient and control mice and stained for the CD4 and CD8 co-receptors. Flow cytometric dot plots are shown with the percentages of T cell subsets indicated. (C) Average cell numbers of the lymph nodes, spleen, thymus and purified peripheral T cell population in *Nit1*^{-/-} mutant and *Nit1*^{+/+} mice control ($n = 5$).

as the spleen and lymph nodes are the mature CD4⁺ or CD8⁺ subsets. The percentage of each of these T cell lineages in *Nit1^{fl/fl} lckcre* mice is comparable to those in control *Nit1^{+/+} lckcre* control littermates (Fig. 1B). The total number of thymocytes, splenocytes, lymph node cells and purified T cells was not significantly affected by a lack of Nit1 in T cells (Fig. 1C). There was no phenotypic difference between *Nit1^{+/+}*, *Nit1^{+/+} lckcre* and *Nit1^{fl/fl} lckcre* control mice.

The *lckcre* expression is initiated at the CD4⁺CD8⁻ double negative stage (DN2) during thymic development. Although *lckcre*-induced deletion of *Nit1* does not appear to have an impact on T cell development, it is unclear whether Nit1 is required at earlier stages during hematopoiesis. Therefore, we crossed *Nit1^{fl/fl}* mice with mice carrying the *cmvcre* transgene, which is expressed in germ cells. In the resulting *Nit1^{-/-}* mice, the Nit1 protein was not detectable in thymocytes, splenocytes, lymph node cells or purified T or B cells as shown by western blot analyses (Fig. 1A). Similar to the T cell-specific knockout *Nit1^{fl/fl} lckcre* mice, wild-type levels of CD4⁺ and CD8⁺ T cells were present in *Nit1^{-/-}* mice (data not shown). These results indicate that deletion of Nit1 in stem cells or after lineage commitment does not affect the development of T cells. The data shown hereafter were obtained from *Nit1^{-/-}* mice.

Apoptotic responses in *Nit1^{-/-}* T cells

It has been shown that ectopic over-expression of Nit1 could lead to caspase activation and apoptosis (18). Therefore, we determined the effect of Nit1 deficiency on primary T cell apoptosis induced by various stimuli. T cells were isolated from the thymus of *Nit1^{-/-}* mutant and *Nit1^{+/+}* control mice. Initially, T cells were cultured in complete medium and cell death was determined at various times for up to 48 h. It appeared that the kinetics of cell death in *Nit1^{-/-}* T cells induced by a lack of stimulatory signals were comparable to that of control *Nit1^{+/+}* T cells (Fig. 2A). This indicates that a lack of Nit1 does not alter *in vitro* survival or apoptosis of T cells. Wild-type T cells undergo apoptosis induced by triggering the death receptor Fas with FasL, and *Nit1^{-/-}* mutant T cells were somewhat less sensitive to FasL than *Nit1^{+/+}* control T cells (Fig. 2B). Steroids such as dexamethasone produced in the thymus can induce apoptosis in T cells and may play a role in thymic selection (38). To determine whether Nit1 deficiency has an effect on this response, *Nit1^{-/-}* T cells were incubated with increasing concentrations of dexamethasone. This treatment initiated similar dose-dependent apoptosis in *Nit1^{+/+}* control and *Nit1^{-/-}* mutant T cells (Fig. 2C). Previous studies indicated that Fhit and Nit1 are required in apoptosis induced by DNA damages (18, 39). Therefore, we irradiated T cells with γ -rays and found that this treatment induced apoptosis in *Nit1^{+/+}* control and *Nit1^{-/-}* mutant T cells with similar kinetics (Fig. 2D). Chemically induced Ca²⁺ flux or treatment with the protein kinase C inhibitor, staurosporine, can also lead to apoptosis in T cells (36). The data show that *Nit1^{-/-}* T cells had a slightly lower rate of apoptosis than *Nit1^{+/+}* control T cells when treated with the calcium ionophore, ionomycin (Fig. 2E). A similar rate of apoptosis occurred in *Nit1^{+/+}* control and *Nit1^{-/-}* T cells stimulated with staurosporine (Fig. 2F).

Apoptotic responses in splenic and lymph node T cells were also determined. Similar to thymic T cells, peripheral T cells were slightly resistant to FasL (Fig. 2G) and ionomycin (Fig. 2H). There was no significant defect in apoptosis in peripheral T cell treated with staurosporine, γ -radiation or dexamethasone (Supplementary Figure S1, available at *International Immunology* Online). Taken together, these results indicate that Nit1 does not play a major role in apoptosis in T cells.

Proliferative responses and expression of activation markers and IL-2 in *Nit1^{-/-}* T cells

Freshly isolated mouse lymphocytes are in a resting state and can undergo massive proliferation when antigen receptors are stimulated. To analyze the effect of Nit1 deficiency on T cell proliferative responses, spleen and lymph node, cells were isolated from *Nit1^{-/-}* and control *Nit1^{+/+}* littermates and treated with agonistic anti-CD3 plus anti-CD28 antibodies to cross-link the TCR. After stimulation, relative cell accumulation was measured by an 8-h radio-pulse with [³H] thymidine. This analysis revealed that proliferation of *Nit1^{-/-}* mouse splenocytes and lymph node cells induced by TCR stimulation was significantly increased when compared with control *Nit1^{+/+}* mice (Fig. 3A). To confirm this result, T cells were purified from spleens and lymph nodes and stimulated with various concentrations of anti-CD3 plus anti-CD28 antibodies. At 48 h after TCR stimulation, *Nit1^{-/-}* T cells proliferated to a greater extent than control *Nit1^{+/+}* T cells (left; Fig. 3B). In addition, cell numbers were determined at various times post-stimulation and growth curves generated. As shown in Fig. 3B (right), more *Nit1^{-/-}* T cells than control *Nit1^{+/+}* T cells were present at 48 h. At later time points, the difference becomes more apparent. Therefore, it appears that a lack of Nit1 resulted in enhanced proliferative responses in T cells.

TCR stimulation induces the expression of activation markers and co-stimulatory proteins on T cells. The hyperproliferative responses to antigen receptor engagement on Nit1-deficient T cells suggested that these cells might either be in a hyperactive state or exhibit a lower threshold of activation. To examine whether Nit1 deficiency altered the expression of T cell activation markers, we performed flow cytometric analyses. IL-2R α (CD25), CD28, CD44 and CD69 are all rapidly induced within 18 h after T cell activation in wild-type T cells (Fig. 4). Interestingly, a greater percentage of Nit1-deficient T cells displayed higher levels of CD25, CD28, CD44 and CD69 without prior stimulation as compared with wild-type littermate control mice (0 h). Importantly, Nit1 deficiency resulted in a significant increase in the percentage of cells expressing these T cell activation markers as compared with *Nit1^{+/+}* T cells (Fig. 4). Therefore, it appears that freshly isolated *Nit1^{-/-}* T cells are somewhat activated and display enhanced induction of activation markers upon TCR engagement. To determine whether a lack of Nit1 affects TCR signaling-induced IL-2 production in T cells, we performed intracellular staining and flow cytometric analyses. As shown in Fig. 4 (bottom), both resting and activated *Nit1^{-/-}* T cells appeared to contain higher levels of IL-2 than *Nit1^{+/+}* control T cells.

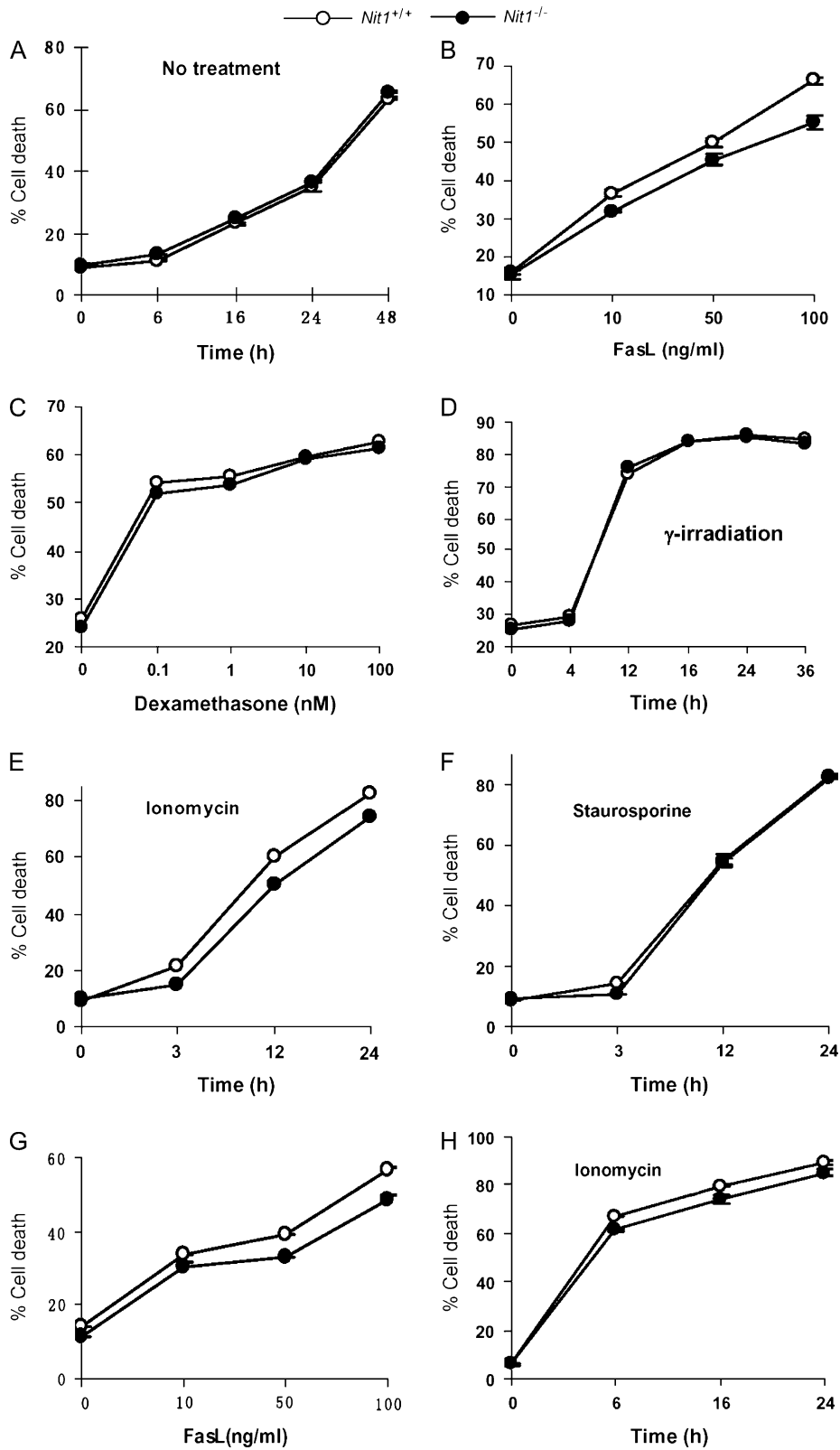


Fig. 2. Analysis of apoptotic responses in T cells. T cells were isolated from the thymus and cultured in complete medium without additional treatment for the time indicated (A), treated with FasL at the indicated concentrations for 16 h (B), with dexamethasone at the indicated concentrations for 16 h (C), 200 rad γ irradiation (D), ionomycin ($1 \mu\text{g ml}^{-1}$; E) or staurosporine ($1 \mu\text{g ml}^{-1}$; F) followed by incubation for the indicated times. Peripheral T cells were isolated from the spleen and lymph nodes and treated for 16 h with FasL at the indicated concentrations (G) or with ionomycin ($1 \mu\text{g ml}^{-1}$), followed by incubation for the indicated time (H). Line graphs are shown to indicate percentages of cell death.

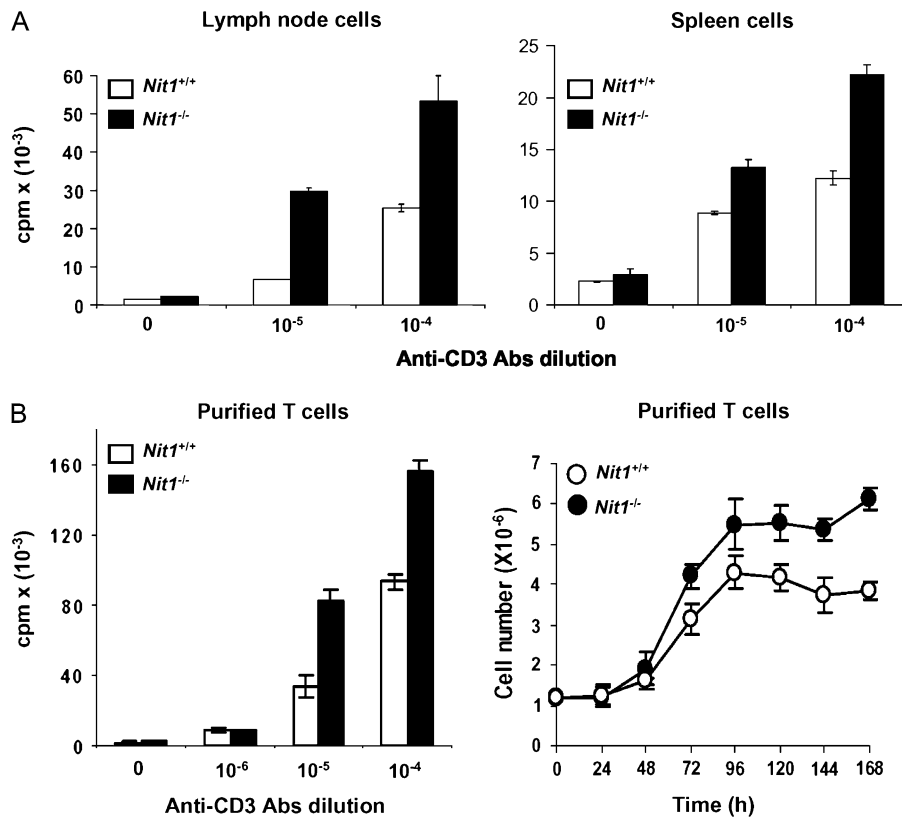


Fig. 3. Analysis of lymphocyte proliferation responses. (A) Total splenocytes and lymph node cells were stimulated with anti-CD3 plus anti-CD28 antibodies and proliferation determined by [³H] thymidine incorporation assays. (B) Purified T cells were stimulated with anti-CD3 plus anti-CD28 antibodies and proliferation determined by [³H] thymidine incorporation assays (left). Growth curves were generated by counting cell numbers at times indicated (right).

Analysis of T cell division kinetics and cell cycle progression

To further analyze the T cell hyperproliferative phenotype in *Nit1*^{-/-} mice, we determined cell division kinetics. Purified T cells were labeled with CFSE and stimulated with the agonistic anti-CD3 plus anti-CD28 antibodies. As T cells divide, the intensity of CFSE fluorescence is reduced by half with each cell division. Therefore, the numbers of cells which have undergone cell divisions could be determined by flow cytometry. At 24 h after TCR stimulation, few T cells (2–8%) had undergone one or more divisions (Fig. 5A), and at 48 h, more *Nit1*^{-/-} CD4 T cells (71%) than *Nit1*^{+/+} control CD4 T cells (40%) had divided one or more times. Concomitantly, there were more undivided control *Nit1*^{+/+} CD4 T cells (60%) than undivided mutant *Nit1*^{-/-} CD4 T cells (29%). A similar phenotype was present in CD8 T cells. At 48 h post-stimulation, there were more mutant *Nit1*^{-/-} CD8 T cells that had divided two or more times as compared with *Nit1*^{+/+} CD8 T cells (Fig. 5A). These results indicate that Nit1 deficiency leads to a more rapid cell division in activated T cells.

Because of the enhanced cell division phenotype, we determined whether Nit1 deficiency affected cell cycle progression. Purified T cells were stimulated with anti-CD3 plus anti-CD28 ascites antibodies and cell cycle profiles generated by staining nuclear DNA with PI. The percentage of cells at various cell cycle stages was determined by measuring the relative DNA content in the cells, as indicated by PI

fluorescence using a flow cytometer. Unstimulated wild-type T cells (0 h) were mostly in the G_{0/1} phase (>90%; Fig. 5B). However, there was a consistently higher percentage of cells in the G₂/M phase in unstimulated mutant *Nit1*^{-/-} T cells (2.6%) than in control *Nit1*^{+/+} T cells (1.1%). At 36 h after stimulation, the percentage of *Nit1*^{-/-} T cells in the S and G₂/M phases was nearly twice that of control *Nit1*^{+/+} T cells. This difference also occurred at 48 h after stimulation (Fig. 5B). Furthermore, there was a lower percentage of *Nit1*^{-/-} T cells in the sub-G_{0/1} and G_{0/1} phase than in control *Nit1*^{+/+} T cells. The sub-G_{0/1} phase cells usually represent those that are undergoing apoptosis. Taken together, the data show that a lack of Nit1 results in spontaneous T cell cycle entry, enhanced cell cycle progression and reduced cell apoptosis in activated T cells.

Cell cycle protein expression in *Nit1*^{-/-} T cells

It has been shown that *Nit1*^{-/-} kidney cells express elevated levels of Cyc D1 (18). Wild-type T and B cells do not express Cyc D1, as indicated in previous studies (40,41,42). To determine the effect of Nit1 deficiency on cell cycle protein expression, western blotting was performed with total splenocytes and purified T cells. Wild-type control mouse embryonic fibroblasts expressed much higher levels of Cyc D1 than wild-type splenocytes (Fig. 6A). Wild-type T cells did not express detectable Cyc D1, and a lack of Nit1 did

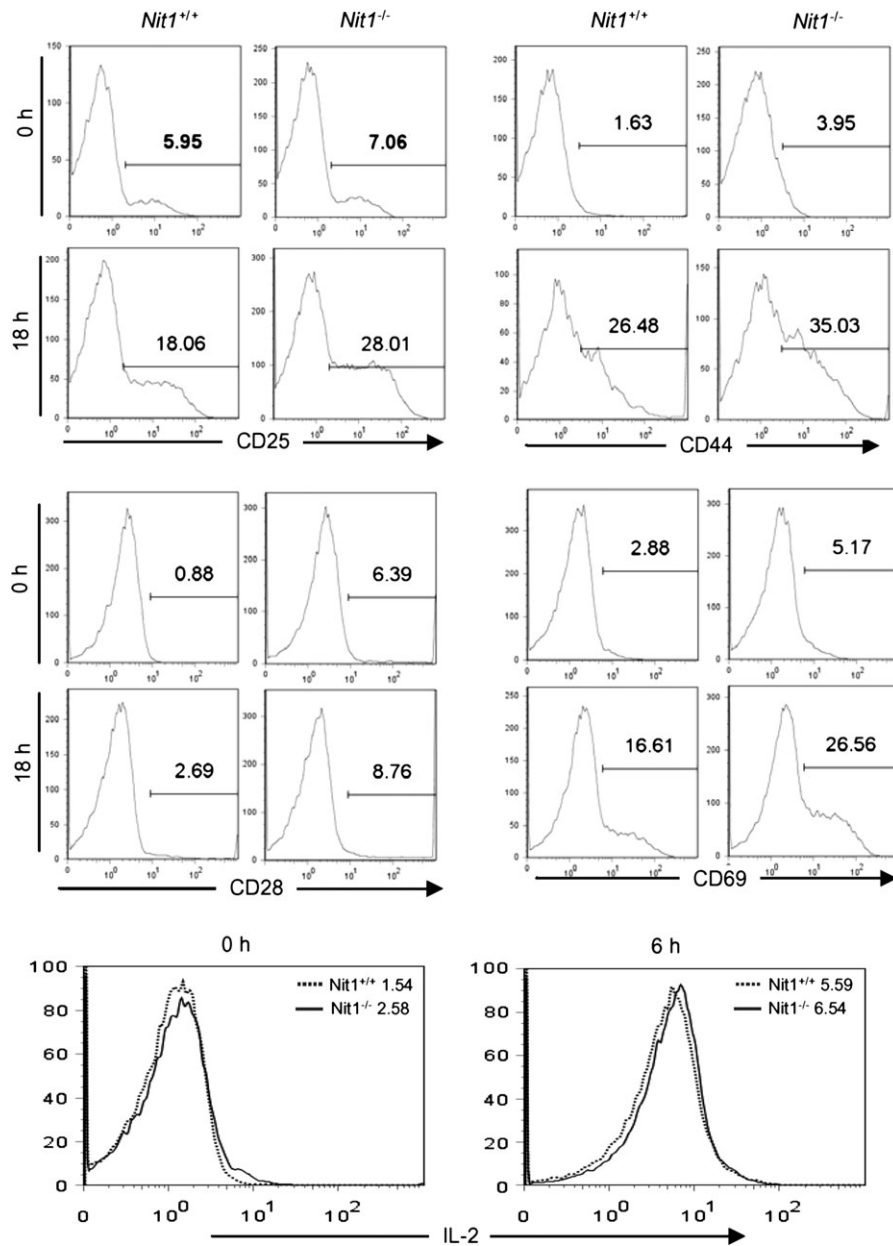


Fig. 4. Analysis of T cell activation markers. Freshly isolated, resting T cells (0 h) and purified T cells stimulated with anti-CD3 plus anti-CD28 antibodies (18 h) were stained for CD25, CD28, CD44 and CD69 and analyzed by flow cytometry. The expression levels are depicted as flow cytometric histograms. The numbers indicate percentages of T cells that express each activation marker. The IL-2 levels were determined by intracellular staining of unstimulated (0 h) or T cells that have been stimulated with anti-CD3 plus anti-CD28 antibodies for (6 h), followed by flow cytometry. The numbers indicate mean fluorescent intensities.

not lead to the expression of Cyc D1 in either unstimulated or activated T cells at 28 h after anti-CD3 plus anti-CD28 antibodies stimulation (Fig. 6A).

To determine whether *Nit1* deficiency affects other cell cycle proteins, western blotting was performed at both early (10 and 20 h; left) and late time points (24, 48 and 72 h; right) following TCR stimulation with anti-CD3 plus anti-CD28 antibodies (Fig. 6B). In both experiments, unstimulated T cells (0 h) were used to determine basal level expression. Wild-type unstimulated T cells express minimal Cyc A, Cyc B1, Cyc D3, Cyc E, CDC2, CDK2 and CDK6 (Fig. 6B). There

was a slight but reproducible increase of Cyc E and CDC2 in unstimulated *Nit1*^{-/-} T cells, as compared with unstimulated control *Nit1*^{+/+} T cells. Elevated levels of Cyc E were also detected in activated *Nit1*^{-/-} T cells when compared with *Nit1*^{+/+} T cells particularly at 20 h or later time points after TCR stimulation (Fig. 6B). CDC2 expression in activated *Nit1*^{-/-} T cells was not overtly different from that in activated *Nit1*^{+/+} T cells. The basal expression and induction of Cyc A, Cyc B1, Cyc D3, CDK2 and CDK6 were not consistently altered in *Nit1*^{-/-} mutant T cells, as compared with *Nit1*^{+/+} T cells. Resting T cells contain high levels of the cell cycle

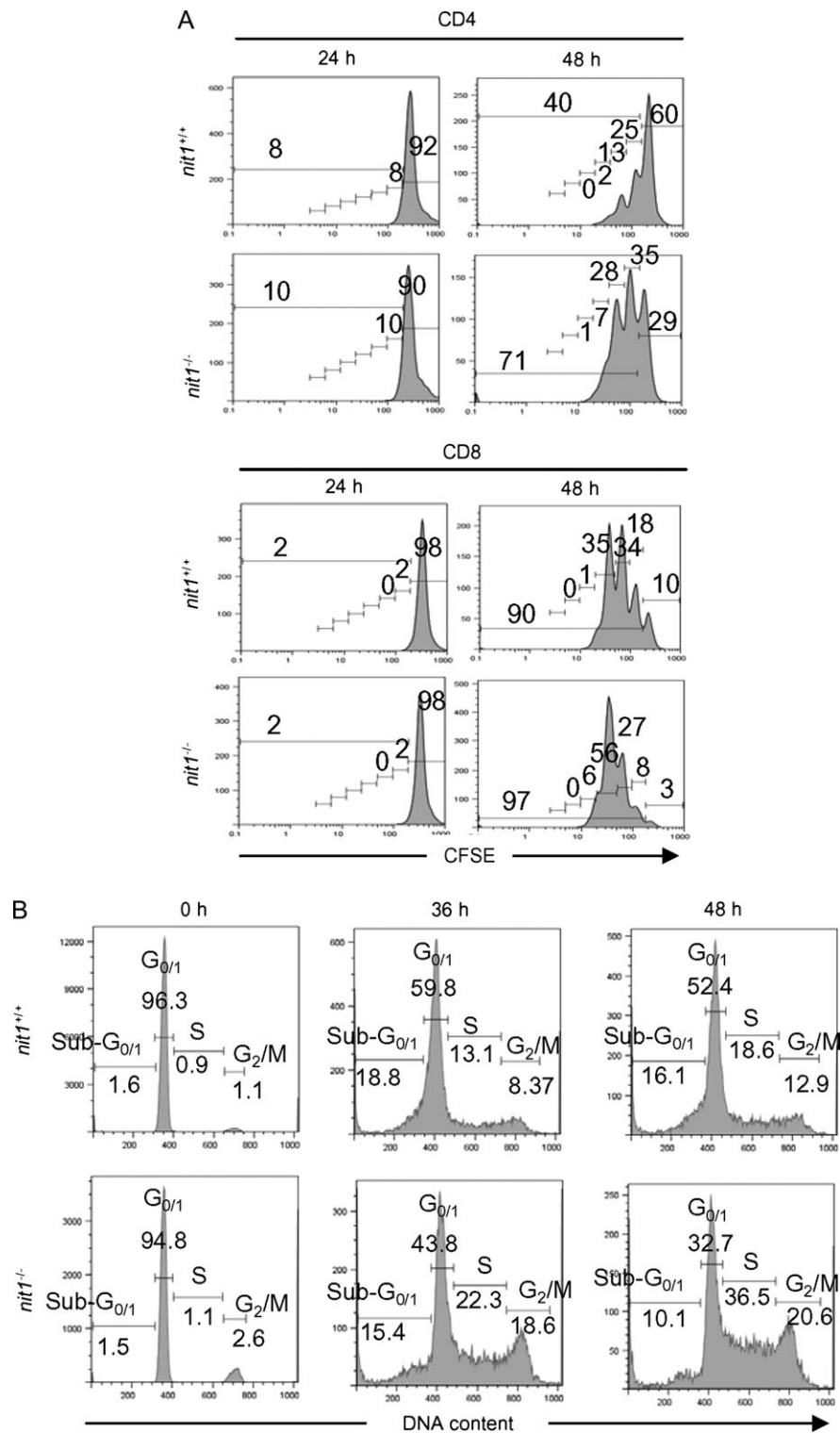


Fig. 5. Analysis of T cell division kinetics and cell cycle progression profiles. (A) Purified T cells were labeled with CFSE, stimulated with anti-CD3 plus anti-CD28 antibodies, analyzed by flow cytometry at the indicated times and cell division visualized using flow cytometric histograms. The numbers present on each histogram indicate the percentage of cells that had undergone specific cell divisions. (B) Purified T cells were stimulated with anti-CD3 plus anti-CD28 antibodies and at the indicated times cells stained with PI and analyzed by flow cytometry. Relative DNA content (PI) at each time point is presented in histograms, and the percentages of T cells in each cell cycle stage are indicated.

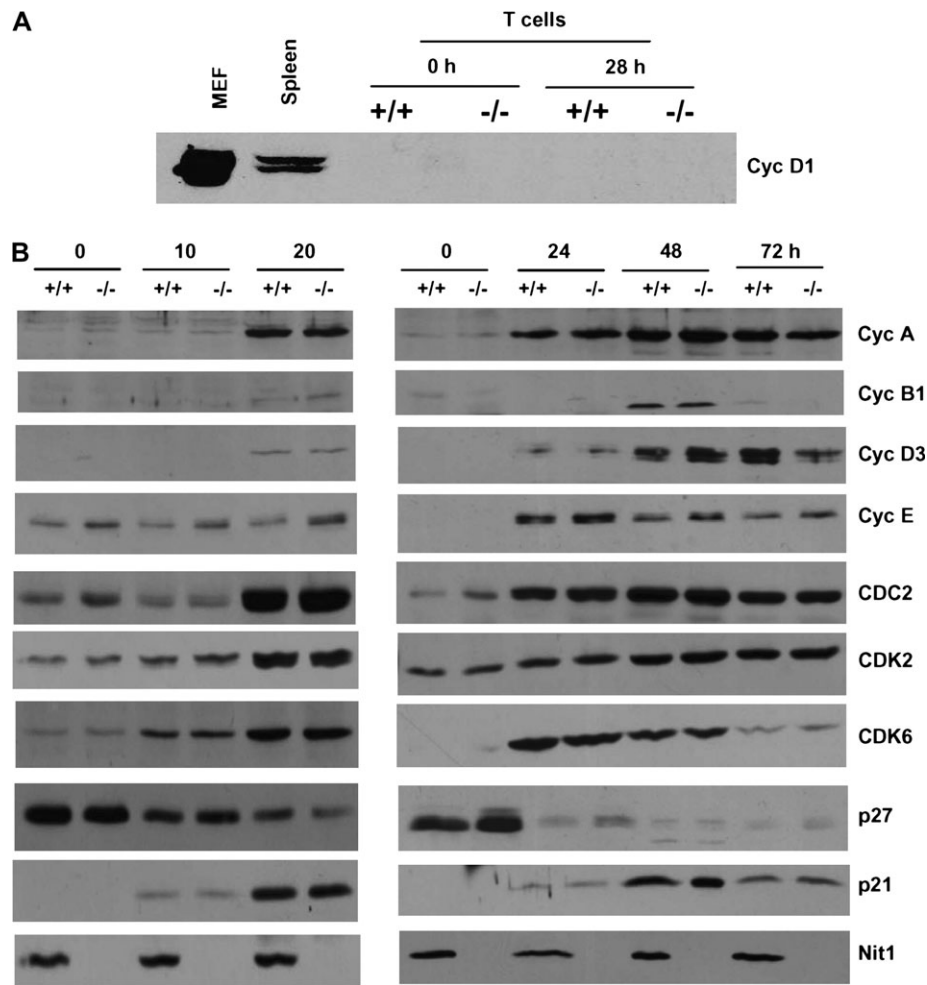


Fig. 6. Western blot analyses of cell cycle proteins in *Nit1*^{-/-} T cells. (A) Expression of Cyc D1 in total splenocytes and freshly isolated T cells (0 h) and purified T cells stimulated with anti-CD3 plus anti-CD28 antibodies for 28 h. Wild-type mouse embryonic fibroblasts (MEFs) were used as positive controls. (B) Purified T cells were stimulated with anti-CD3 plus anti-CD28 antibodies, and at the indicated times cells were harvested, proteins extracted and western blotting performed with the indicated antibodies.

inhibitor p27, which is quickly degraded in proliferating T cells following TCR stimulation. This process was not affected by *Nit1* deficiency (Fig. 6B). p21 (Cip1/Waf1) can be an activator of CDKs, dependent on the level of expression (43). Western blotting revealed that p21 was expressed at minimal levels in wild-type resting T cells and induced in activated T cells (Fig. 6B). There was no obvious defect in the expression of p21 in *Nit1*^{-/-} T cells, compared with control *Nit1*^{+/+} T cells (Fig. 6B). A lack of *Nit1* in *Nit1*^{-/-} T cells was confirmed by western blotting with anti-*Nit1* antibodies (bottom, Fig. 6B). The mRNA levels of Cyc E and CDC2 were also analyzed, and the data showed that activated *Nit1*^{-/-} mutant T cells (24 h) contained significantly higher levels of Cyc E and CDC2 mRNA than in *Nit1*^{+/+} control T cells (Fig. 7A and supplementary Figure S2, available at *International Immunology* Online). To determine protein turnover kinetics, T cells were activated to induce Cyc E and CDC2, and cycloheximide was then added to inhibit protein synthesis. As shown in Fig. 7B (and supplementary Figure S2, available at *International Immunology* Online), both Cyc E and CDC proteins

can undergo degradation with similar kinetics in both *Nit1*^{+/+} and *Nit1*^{-/-} T cells. These data indicate that *Nit1* negatively regulates Cyc E and CDC2 mRNA expression in T cells.

Discussion

A tight regulation of proliferation and apoptosis is critical for homeostasis in the immune system. Insufficient death and/or excessive proliferation in lymphocytes may lead to the development of cancer and autoimmune diseases. In this study, we investigated the physiological function of the putative tumor suppressor protein, *Nit1*, in T cells by using conditional *Nit1*-deficient mice. T cell-specific deletion of *Nit1* using *lckcre* did not appear to affect either thymic development or establishment of the peripheral T cells pool (Fig. 1). The *lckcre* transgene is expressed as early as the pre-T stage (32). The lack of a phenotype displayed by T cell-specific *Nit1*^{-/-} mice is not due to incomplete deletion of *Nit1*, as the *Nit1* protein is undetectable in thymic T cells (Fig. 1A). Furthermore, deletion of *Nit1* in germ cells leading to complete loss of *Nit1* in all cells had no major impact on

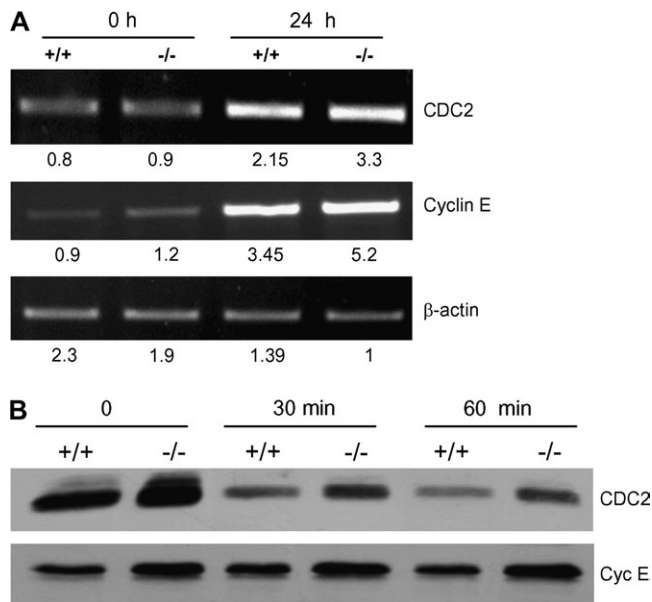


Fig. 7. mRNA and protein turnover of Cyc E and CDC2. (A) RNA was extracted from resting (0 h) and activated (24 h) T cells, and semi-quantitative RT-PCR performed to determine the relative levels of mRNA of Cyc E and CDC2. Density was determined using ImageJ (National Institutes of Health) and normalized against β -actin. Numbers indicated fold over the level of β -actin in activated (24 h) $Nit1^{-/-}$ T cells, which is set as 1. (B) T cells were stimulated with anti-CD3 plus anti-CD28 antibodies, and at 24 h after stimulation, cycloheximide was added, cells were harvested at the indicated times and the levels of the Cyc E and CDC2 proteins analyzed by western blotting.

the generation of various subsets of T cells. Therefore, Nit1 appears to be dispensable during T cell development. Although Nit1 was suggested to function as a putative tumor suppressor (18), no lymphoid malignancy has been observed among those analyzed $Nit1^{-/-}$ mice (1–6 month olds). It is likely that establishment of additional genetic lesions is necessary for the development of lymphoid malignant phenotypes in $Nit1^{-/-}$ mice.

It has been postulated that there are genetic and functional interactions between Nit1 and Fhit (1, 18). Previous studies indicate that Nit1 and Fhit can be pro-apoptotic especially when they are over-expressed in transformed cells, which requires caspase activation (18, 27). Over-expression of a dominant negative mutant of FADD or caspase 8 impaired apoptosis induced by Fhit, indicating that the death receptor-mediated extrinsic pathways are involved. It has not been determined whether FADD and caspase 8 dominant negative mutants affect apoptosis induced by Nit1. FADD and caspase 8 are essential for lymphocyte apoptosis induced by Fas (28,29,30). As shown in Fig. 2(B), Nit1 deficiency resulted in a mild reduction in Fas-induced apoptosis. Fas expression is normal in $Nit1^{-/-}$ T cells (data not shown). $Nit1^{-/-}$ T cells also display a slightly decreased apoptotic response to Ca^{++} flux (Fig. 2E), which signals through the pro-apoptotic Bcl-2 family member Bim (44). T cells lacking Bim are also impaired in apoptosis induced by a lack of stimulation or by dexamethasone treatment (44). However, $Nit1^{-/-}$ T cells undergo normal apoptosis

when left untreated or in the presence of dexamethasone (Fig. 2A and C). Previous studies suggest that Bim, Fhit and Nit1 play a role in apoptosis induced by DNA damage due to chemical or irradiation treatments (18, 39, 44). We show that a lack of Nit1 did not have effects on T cell apoptosis induced by γ irradiation (Fig. 2D) or by treatment with the DNA damage-inducing drug etoposide (data not shown). Staurosporine-induced apoptosis was intact in $Nit1^{-/-}$ T cell (Fig. 2F). $Nit1^{-/-}$ mice (1–8 month olds) did not develop obvious autoimmune lymphoproliferative diseases as seen in mice lacking Fas or Bim. However, Nit1 deficiency may have a predisposing effect on cancer and/or lymphoproliferation disease development, due to the compromised apoptosis in response to FasL and Ca^{++} signals (Fig. 2).

Stimulation of the TCR leads to proliferation of T cells. This proliferative response is significantly enhanced in $Nit1^{-/-}$ T cells when compared with control $Nit1^{+/+}$ T cells (Fig. 3A and B). In addition, the expression of T cell activation markers including CD25, CD28, CD69, CD44 and IL-2, which are largely dependent on TCR signaling, is elevated by Nit1 deficiency (Fig. 4). The enhanced proliferation of $Nit1^{-/-}$ T cells is further confirmed by CFSE dilution assays that show more rapid cell division of $Nit1^{-/-}$ T cells stimulated by TCR cross-linking (Fig. 5A). Furthermore, Nit1-deficient T cells display significantly accelerated cell cycle progression (Fig. 5B). Strikingly, Nit1 deficiency appears to lead to spontaneous cell cycle entry in unstimulated T cells (0 h; Fig. 5B). Collectively, these results implicate Nit1 as a novel negative regulator of T cell proliferation and cell cycle progression.

Cyc D1 has been shown to be elevated in $Nit1^{-/-}$ kidney cells (18). However, our analyses of primary T cells show that they do not express Cyc D1 and that Nit1 deficiency do not result in aberrant Cyc D1 induction in $Nit1^{-/-}$ T cells (Fig. 6A). This indicates that the mechanism of Nit1 regulation of the T cell cycle is distinct from that in kidney cells. T cells from healthy mice are in a resting stage with over 95% cells in the $G_{0/1}$ phase of cell cycle (Fig. 5B). After TCR stimulation, T cells enter the cell cycle due to the rapid induction of cyclins and CDKs. Concomitantly, there is an induction of degradation of the major cell cycle inhibitor p27. In contrast to the p27 expression pattern, the cell cycle inhibitor p21 was minimally expressed in resting T cell and was induced by TCR signaling (Fig. 6B). It has been shown that p21 can function as an inhibitor, as well as an activator, for CDKs (43, 45). Our data show that the expression pattern of Cyc A, B1, D3, CDK2 and 6, p21 and p27 was not altered in $Nit1^{-/-}$ T cells (Fig. 6B). However, slightly elevated expression of Cyc E and CDC2 was consistently detected in $Nit1^{-/-}$ T cells with or without TCR stimulation (Fig. 6B). These results suggest that the enhanced cell cycle entry and progression in $Nit1^{-/-}$ T cells are partly due to increased levels of Cyc E and CDC2. Possibly, additional defects exist in $Nit1^{-/-}$ T cells. Future analyses will help understand the molecular mechanisms involved in the regulation of proliferation by Nit1-mediated signaling in T cells.

Supplementary data

Supplementary Figures S1 and S2 are available at *International Immunology Online*.

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Abbreviations

CFSE	carboxyfluorescein succinimidyl ester
Cyc	cyclin
FasL	Fas ligand
NMBA	<i>N</i> -nitrosomethylbenzylamine
PI	propidium iodide
RT	reverse transcription

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