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NFATC2 transcription factor regulates cell cycle progression during lymphocyte activation: evidence of its involvement in the control of cyclin gene expression

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ABSTRACT

Upon antigen stimulation, lymphocytes enter in cell cycle and proliferate, and most of the activated T cells die by apoptosis. Many of the proteins that regulate lymphocyte activation are under the control of transcription factors belonging to the NFAT family. As previously demonstrated, NFATC2–/– mice consistently showed a marked increase in lymphocyte proliferation. Here, we evaluate the role of NFATC2 in regulating lymphocyte proliferation and its involvement in the control of cell cycle progression during lymphocyte activation. NFATC2–/– lymphocytes, including $CD4^+$ T cells and B cells, hyperproliferated upon stimulation when compared with NFATC2+/+ cells. Analysis of cell death demonstrated that NFATC2–/– lymphocytes displayed an increased rate of apoptosis after antigen stimulation in addition to the hyperproliferation. Cell cycle analysis after antigen stimulation showed that NFATC2–/– cultures contained more cycling cells when compared with NFATC2+/+ cultures, which is related to a shortening in time of cell division upon activation. Furthermore, hyperproliferation of NFATC2–/– lymphocytes is correlated to an overexpression of cyclins A2, B1, E, and F. Taken together, our results suggest that the NFATC2 transcription factor plays an important role in the control of cell cycle during lymphocyte activation and may act as an inhibitor of cell proliferation in normal cells.

Key words: NFAT • T cell • proliferation • apoptosis

In response to antigen stimulation, lymphocytes proliferate, differentiate, and acquire their effector functions, and most of the activated T cells die by apoptosis. It has been shown that T-cell differentiation and activation-induced cell death are dependent on the cell cycle

progression (1–5). Naive lymphocytes are quiescent and require stimulation to progress in the cell cycle. T cell receptor (TCR) engagement with antigen initiates a series of signal transduction events that lead to a cellular response. Indeed, increase of intracellular free Ca^{2+} is central for T-cell activation by antigen and other stimuli that cross-link the TCR (6). Several observations demonstrate that Ca^{2+} signal stimulates gene transcription associated with the cell cycle progression and also promotes the transition from G1 to S phase (7, 8). Furthermore, accumulating evidence suggests that calcineurin, the Ca^{2+}/calmodulin-activated serine phosphatase, plays a major role in the regulation of cell cycle progression by acting in the early stages of the G1 phase of the cell cycle (9, 10).

Lymphocyte activation is coordinated by an interacting network of transcription factors that determine the expression of different effector proteins that regulate the immune response. Many of the proteins that regulate lymphocyte proliferation and differentiation are under the control of proteins belonging to the NFAT family of transcription factors. The NFAT family encodes five different classes of proteins; NFATC1 (also called NFATc or NFAT2), NFATC2 (NFATp or NFAT1), NFATC3 (NFATx or NFAT4), NFATC4 (NFAT3), and NFAT5 (TonE-BP or NFATL1) (11–13). The NFAT proteins are normally activated by stimulation of receptors coupled to Ca^{2+} mobilization, including the antigen receptors on T and B cells (11). At least four of these proteins are regulated by calcineurin, which is the direct target of the immunosuppressive drugs cyclosporin A and FK506 (11, 14). Binding sites for NFAT proteins are present in the promoter regions of diverse activation-inducible genes, including those encoding cytokines IL-2, IL-4, IL-5, and IFN-γ as well as cell surface proteins CD40L, CTLA-4, and FasL (11, 15, 16). Much evidence suggests that the NFAT family of transcription factors plays an ubiquitous role in cell differentiation (17). However, the specific role of each member of the NFAT family in the control of gene transcription during cell activation and differentiation is not completely clear.

Although different NFAT family members are considered to have a redundant function in gene expression control, data generated by gene disruption experiments suggest that these proteins may have distinct roles in cell physiology (17, 18). Unexpectedly, targeted disruption of NFATC1 resulted in an embryonic lethal phenotype as a result of defective cardiac valve development (19, 20). Complementing RAG-1- or RAG-2-deficient blastocysts with homozygous NFATC1–/– mutant ES cell lines demonstrated that T and B cells from chimeric mice showed reduced proliferation of peripheral lymphocytes when compared with wild-type mice (20, 21). In an *in vitro* model of Th differentiation, the chimeric mice displayed a decreased production of IL-4 but normal production of IFN-γ, demonstrating an impaired Th2 response in mutant T cells (20, 21). These results suggest that NFATC1 is an important positive regulator of Th2 cytokine expression.

NFATC2–/– mice developed normally and did not exhibit any obvious behavioral deficiencies (22, 23). However, NFATC2–/– mice consistently showed a marked increase in their immune response (22, 23). NFATC2–/– mice showed an enhanced Th2 development in both *in vivo* and *in vitro* models of Th differentiation, as evidenced by increased levels of IL-4 production (22, 24, 25). Furthermore, cells from lymph nodes and spleen hyperproliferated in response to different antigen stimulations (23); likewise, $CD4^+$ T cells and B cells hyperproliferated *in vitro* in response to anti-CD3 and anti-IgM antibodies, respectively (22). Although all data on the control

of gene expression by NFATC2 were consistent with the idea that NFATC2 played a positive role in gene transcription (11), the results from NFATC2–/– mice suggested that this transcription factor might play a negative role in the regulation of gene expression in the immune response. Given the importance of lymphocyte activation during the immune response, it is of considerable interest to understand the mechanisms by which NFAT transcription factors exert their profound effects on cell proliferation and differentiation.

In this work, we evaluated the role of NFATC2 in cell cycle progression control during lymphocyte activation, using the NFATC2–/– mice as a model system. We have found that the hyperproliferative phenotype of NFATC2–/– lymphocytes is neither restricted to one specific lymphocyte subset nor dependent on IL-4. Furthermore, NFATC2–/– cells presented an increased rate of apoptosis in addition to the hyperproliferation. Cell cycle analysis showed that NFATC2–/– cells presented a deregulated cell cycle control, which is related to an overexpression of some cyclin genes. Finally, our results suggest that NFATC2 transcription factor play an important role in the control of cell cycle during lymphocyte activation and may act as a negative regulator of cell cycle progression in normal cells.

MATERIALS AND METHODS

Animals and sensitization

Control wild-type (NFATC2+/+) and NFATC2-deficient (NFATC2–/–) mice were generated as previously described (23). Eight- to 12-week-old NFATC2+/+ and NFATC2–/– mice, bred and maintained independently, were used in all experiments. Mice were sensitized in the hind footpad with one s.c. injection of 0.1 ml of ovalbumin (OVA, 200 μg) emulsified in complete Freund's adjuvant as previously described (23, 25). Animals were maintained in the Brazilian National Cancer Institute (INCA) animal facility. Animals are maintained and treated according to the animal care guidelines of the Council for International Organizations of Medical Sciences.

Cell culture

All cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, streptomycin-penicilin, nonessential amino acids, sodium pyruvate, vitamins, HEPES, 2-mercaptoethanol (all from Gibco-BRL, Rockville, MD). Single-cell suspensions were prepared from draining lymph nodes of the sensitized footpad as previously described (23, 25).

Proliferation assay

Cells (2×10^5 cells/well) were plated and left unstimulated or stimulated with different concentrations of OVA (0.1, 0.3, or 0.5 mg/ml); plate-bound anti-CD3 antibody (2C11; 1 μg/ml); or PMA (10 nM) plus ionomycin (1 μM) for different time periods (24, 48, and 72 h) as indicated and pulsed with ^{3}H-thymidine (5 μCi/ml) for 12 h. Cells were then harvested, and ^{3}H-thymidine incorporation was analyzed. For IL-4-neutralizing experiments, cells were *in vitro* stimulated with OVA (0.5 mg/ml) for 72 h in the absence or in the presence of 20, 100, and 200 μg/ml of anti-IL-4 antibody as indicated, and then ^{3}H-thymidine incorporation was analyzed.

Anti-IL-4 antibody was purified from culture hybridoma cells (11B11) by chromatography over protein-G (Amersham-Pharmacia, Piscataway, NJ). Activity of purified anti-IL-4 antibody was tested for binding to IL-4 by ELISA and for neutralization of IL-4 by inhibiting *in vitro* differentiation of $CD4^+$ cells toward Th2, favoring Th1 immune response as previously described (24).

ELISA

Cells (2×10^6 cells/well) were *in vitro* stimulated with OVA (0.5 mg/ml) for 72 h, and the cell-free supernatant was assessed for IL-4 protein level according to the manufacturer's instructions (BD-Pharmingen, San Diego, CA).

Flow cytometry

Cells were stained with specific fluorochrome-labeled antibody as previously described (24), washed, and either fixed with 1% paraformaldehyde or analyzed directly. Labeled monoclonal antibodies anti-CD4, -CD8, and -CD45R (B220) were used (all from BD-Pharmingen). For analysis of apoptosis, cells were stained with annexin-V-FITC (BD-Pharmingen) according to the manufacturer's instructions. Labeled cells were analyzed on a Becton Dickinson FACScan flow cytometer.

Cell cycle analysis

Cell cycle phases were analyzed as previously described (26). In brief, cells (4×10^5 cells/well) were left unstimulated or *in vitro* stimulated with OVA (0.5 mg/ml) and were collected at the indicated time periods (24, 48, and 72 h). Cells were then stained with propidium iodide in the presence of NP-40 and analyzed by flow cytometry. For analysis of cellular replication, lymph node cells (10^7 cells/ml) were labeled with 0.5 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) in phosphate-buffered saline (PBS) for 15 min at 37°C. Staining was stopped by adding an equal volume of DMEM with 10% FCS. The cells were immediately washed, incubated in culture medium for 30 min at 37°C, and then left unstimulated or *in vitro* stimulated with 1 μg/ml of plate-bound anti-CD3 at 2×10^5 cells/well (2C11; BD-Pharmingen). At indicated times (24, 42, and 65 h), cells were analyzed by flow cytometry.

RNA gene expression

Cells (2×10^7 cells) were left unstimulated or *in vitro* stimulated with OVA (0.5 mg/ml) and were collected at the indicated times. Total RNA was immediately extracted with Trizol according to the manufacturer's instructions (Gibco-BRL, Rockville, MD). mRNA expression in total cellular RNA from each sample was analyzed with a multiple probe RNase protection assay kit (Ribo-Quant, BD-Pharmingen). For analysis of expression of genes related to cell cycle, mCYC-1 (cyclin-A2, -B1, -C, -D1, -D2, -D3, -A1, and -B2) and mCYC-2 (cyclin-E, -F, -G1, -G2, -I, and -H) multiprobe sets were used. RNA loading was estimated by measuring the intensities of two housekeeping genes encoding GAPDH and L32. Transcript levels were quantified by densitometry of the autoradiograms, with exposures within the linear range using SigmaGel software (Jandel Scientific, San Rafael, CA).

Statistical analysis

Statistical analysis of values from wild-type and NFATC2–/–, and between control and treated groups, was determined using an unpaired Student's *t* test for single comparison or an ANOVA followed by the Student-Newman-Keuls for multiple comparisons. $P<0.05$ was considered to be statistically significant.

RESULTS

As shown in Figure 1A, lymphocytes from NFATC2–/– mice proliferate more in an antigen dose-dependent manner when compared with lymphocytes from NFATC2+/+ mice. In a time course assay, the maximum difference in proliferation upon antigen stimulation between NFATC2+/+ and NFATC2–/– lymphocytes was observed at 72 h after antigen stimulation (Fig. 1B). Furthermore, NFATC2–/– lymphocytes also hyperproliferate compared with NFATC2+/+ lymphocytes after a polyclonal stimulation that bypasses antigen presentation, such as anti-CD3 antibody or PMA plus ionomycin (data not shown). These results demonstrate that the hyperproliferative phenotype presented by NFATC2–/– cells is dependent on stimulation but might be independent of antigen presentation and costimulation provided by antigen presenting cells (APCs).

To better characterize the hyperproliferative phenotype presented by NFATC2–/– mice, we analyzed the lymphocyte subsets that might be involved with this phenomenon. NFATC2+/+ and NFATC2–/– mice did not show any significant differences in the number of $CD4^+$, $CD8^+$, and B lymphocytes in lymph nodes before or after sensitization with OVA (data not shown). However, after *in vitro* stimulation with OVA, all lymphocyte subsets, including $CD4^+$, $CD8^+$, and B cells, were increased in NFATC2–/– cultures when compared with NFATC2+/+ cultures (Fig. 2). Moreover, in the first 24 h, the most significant difference was observed in $CD4^+$ cells (Fig. 2). These results suggest that the hyperproliferative phenotype presented by NFATC2–/– cells is not restricted to a specific lymphocyte subpopulation; however, $CD4^+$ cells could play a major role in this phenomenon.

It has been shown that $CD4^+$ T cells from NFATC2–/– mice present a preferential differentiation through a Th2 phenotype, characterized by an overexpression of IL-4 (22, 24, 25). Because IL-4 is a potent growth factor for Th2 and B cells, we evaluated the involvement of IL-4 in the hyperproliferation observed in NFATC2–/– lymphocytes. As shown in Figure 3A, lymphocytes from NFATC2–/– mice produce more IL-4 after *in vitro* antigen stimulation when compared with NFATC2+/+ lymphocytes. However, proliferation assays demonstrated that NFATC2–/– cells still proliferate more than NFATC2+/+ cells, even in presence of neutralizing anti-IL-4 antibody (Fig. 3B). This result demonstrates that the hyperproliferation observed in NFATC2–/– lymphocytes is IL-4-independent.

Upon activation, T cells proliferate and die by apoptosis. This active form of apoptosis requires TCR stimulation and involves some death signals, including Fas/FasL engagement (27). In fact, lymphoproliferative disorders have been linked to defects in T-cell apoptosis in mice that possess nonfunctional Fas or FasL genes (28–30). Because NFAT transcription factors regulate

expression of the FasL gene (15, 16), one explanation for the hyperproliferation phenotype of NFATC2–/– mice would be a defect in apoptosis of NFATC2–/– lymphocytes. To address this question, we analyzed cell death. Surprisingly, NFATC2–/– cultures contained an increased number of dead cells after *in vitro* stimulation with antigen when compared with NFATC2+/+ cultures (Fig. 4A). Furthermore, NFATC2–/– cultures also contained more annexin-V-positive cells than NFATC2+/+ cultures upon antigen stimulation (Fig. 4B). Analysis of gene expression showed that NFATC2–/– lymphocytes had no impairment of FasL gene expression (Barboza and Viola, unpublished observations). These results demonstrate that the lymphocyte hyperproliferation of NFATC2–/– mice is not related to diminished apoptosis and, conversely, that NFATC2–/– cells show an enhanced cell death rate in addition to hyperproliferation.

Upon activation, lymphocytes initiate cell cycle progression and proliferate. Accumulating evidence indicates that active apoptosis is related to the cell cycle. Indeed, it has been shown that T cells are more susceptible to apoptosis in late G1 or S phase of the cell cycle (4, 31–33). Based on these results, we asked whether hyperproliferation of NFATC2–/– lymphocytes might be due to cell cycle deregulation. In fact, cell cycle analysis of lymph node cells stimulated *in vitro* with antigen demonstrated that NFATC2–/– cultures contained an increased cell number in S-G2/M phases when compared with NFATC2+/+ cultures at 72 h (Fig. 5A). Furthermore, in a time course analysis, NFATC2–/– cultures contained more cells in S-G2/M phases at 48 and 72 h after OVA stimulation (Fig. 5B).

These data suggest that the NFATC2–/– cell hyperproliferation might be related to an altered cell cycle in NFATC2–/– lymphocytes. One possibility to explain these results would be that NFATC2–/– lymphocytes showed a shortening of time to cell division upon activation. To investigate this hypothesis, we assessed cellular replication by analysis of NFATC2+/+ and NFATC2–/– lymphocytes labeled with CFSE. As shown in Figure 6, only NFATC2–/– cultures contained cells in the first cell division after 42 h of *in vitro* stimulation with anti-CD3. Furthermore, NFATC2–/– cultures also contained more cells at 2, 3, and 4 divisions when compared with NFATC2+/+ cultures after 65 h of stimulation (Fig. 6). These results suggested that hyperproliferation of NFATC2–/– lymphocytes could be related to an altered cell cycle due to a shortening in time of cell division by these cells.

The cell cycle is controlled by a family of protein kinases, which has its activities regulated in response to cellular signals. Complexes of cyclin with cyclin-dependent kinase (CDK) play a central role in the control of cell cycle progression. The expression of specific cyclins dictate formation of distinct cyclin/CDK complexes at different points of the cell cycle. Because cyclins are regulated at the gene expression level, they could represent important candidates for transcriptional control by the NFATC2 transcription factor. To investigate the role of NFATC2 in the expression of cyclin genes, we analyzed cyclin mRNA expression by RNase protection assay. As shown in Figure 7A and 7B, NFATC2–/– lymphocytes present an overexpression of cyclins A2, B1, E, and F when compared with NFATC2+/+ lymphocytes after antigen stimulation. Note that no differences in cyclin expression were observed in unstimulated cells from NFATC2+/+ and NFATC2–/– mice (data not shown). Overexpression of these cyclins support the evidence that the NFATC2–/– lymphocyte hyperproliferation is related to a deregulation in cell cycle control.

DISCUSSION

In this work, we evaluated the role of NFATC2 transcription factor in the regulation of cell cycle progression during lymphocyte activation. Our results demonstrated that NFATC2–/– lymphocytes display an enhanced proliferative response to antigen, suggesting that the NFATC2 transcription factor might exert an inhibitory effect on normal lymphocytes during antigen stimulation. Furthermore, the hyperproliferation observed in NFATC2–/– mice is not restricted to one specific lymphocyte subpopulation. Our data also provide evidence that the hyperproliferation observed in NFATC2–/– lymphocytes is not dependent on APC, because these lymphocytes continue to hyperproliferate upon stimulation that bypasses antigen presentation, such as anti-CD3 or PMA plus ionomycin (data not shown). Together, these results demonstrate that the hyperproliferation phenotype observed in NFATC2–/– cells is mediated by an intrinsic mechanism conserved in all lymphocyte subsets.

It has been demonstrated that NFATC2–/– lymphocytes preferentially differentiate to give a Th2 immune response characterized by overexpression of IL-4 (22, 24, 25). Although IL-4 is a potent growth factor for Th2 and B cells, we demonstrated that the enhanced proliferative response to antigen observed in NFATC2–/– lymphocytes is independent of IL-4 overexpression. Another cytokine that plays a central role in T-cell proliferation is IL-2. The proliferative response of T cells is initiated by engagement of IL-2 with its receptor, and this interaction promotes cell cycle progression of these cells. However, the hyperproliferative response of NFATC2–/– cells does not seems to be related to IL-2 overexpression, because NFATC2–/– lymphocytes present normal levels of IL-2 when compared with NFATC2+/+ lymphocytes upon *in vitro* stimulation with OVA or anti-CD3 (24, 25). Furthermore, no differences in CD25 expression were observed between NFATC2+/+ and NFATC2–/– lymphocytes from naive or OVA-sensitized mice (data not shown). These results strongly support the idea that the hyperproliferative phenotype observed in NFATC2–/– lymphocytes is dependent on a cell-intrinsic mechanism and that the NFATC2 transcription factor is a key regulator of the responsiveness of lymphocytes to antigen.

Cell proliferation is one of the first events that occurs during lymphocyte activation upon antigen stimulation. Naive lymphocytes are quiescent and start the cell cycle progression in response to stimulation. Our results provide strong evidence that the hyperproliferative phenotype observed in NFATC2–/– lymphocytes is related to an altered cell cycle control. Upon antigen stimulation, NFATC2–/– mice present more cells in S-G2/M phases of the cell cycle in comparison with NFATC2+/+ mice. In addition, CFSE analysis demonstrated that NFATC2–/– lymphocytes present an increased rate of cellular replication. These data suggested that the NFATC2–/– cells present a deregulated cell cycle, which could be due to a shortened cell cycle. Recently, it has been shown that the hyperproliferative response of $CD4^+$ cells from a double deficient mice NFATC2–/– x NFATC3–/– is related to a shortening time of cell division (34). In combination, these results suggest that NFAT proteins have an important action in regulating an intrinsic capacity of cellular replication.

Although CFSE analysis demonstrated that NFATC2–/– cultures contained more cells undergoing up to three rounds of divisions when compared with NFATC2+/+ cultures at later time points (65 h), NFATC2+/+ cultures still present a large population of cells that had not suffered any division at this time point. Based on this fact, we may also consider as an additional,

albeit nonexclusive explanation, the possibility that NFATC2–/– lymphocytes, upon activation, reach the first cell division faster than NFATC2+/+ lymphocytes, and thus exit the resting state faster than NFATC2+/+. This result is in accordance to the data from the double deficient mice NFATC2–/– x NFATC3–/– that present a reduced time to reach the first cell division due to an increased TCR responsiveness (34). This finding could explain why NFATC2–/– cultures present more proliferating cells than NFATC2+/+ cultures. In fact, NFATC2–/– cultures contains more $CD4^+$ and $CD8^+$ cells with the $CD44^+CD62L^{low}$ phenotype when compared with NFATC2+/+ cultures (data not shown). Thus, NFATC2 transcription factor may act as a negative regulator of very early inducible genes that controls the commitment of resting cells to become activated upon stimulation, including genes that regulate the cell cycle entrance and T-cell differentiation.

The timing of the cell cycle is regulated by a complex network of proteins, including the cyclins. Interestingly, NFATC2–/– lymphocytes present an overexpression of cyclins A2, B1, E, and F upon stimulation when compared with NFATC2+/+ lymphocytes. Cyclins A2 and E play a key role in the control of cell cycle progression and cell division. Cyclins E and A2 are expressed at early and late stages of G1 phase of the cell cycle, respectively. These two cyclins are implicated in propelling cells to enter into S phase, and cyclin A2 also promotes passage through S into G2 and M phases (35–37). It was reported that overexpression of cyclin E accelerates the G1/S phase transition, which leads cells to present a shortening in time of cell cycle (38–40). Furthermore, cyclin A and cyclin E are overexpressed in some types of lymphoproliferative diseases, such as leukemias (40–42). These data support the evidence that NFATC2–/– lymphocytes may present a shortening time of cell division that might be mediated by the overexpression of cyclins A2 and E.

Thus, our results suggest that the NFATC2 transcription factor may regulate the expression of genes that control cell cycle in lymphocytes. Several data support this evidence. In fact, cyclosporin A inhibits lymphocyte proliferation by arresting the cells at G1 phase of the cell cycle (Silva-Carvalho and Viola, unpublished observations). In addition, cyclosporin A and FK506 inhibited the expression of cyclins A and E but did not have any effect on cyclin D1 expression (43). Calcineurin, which is the major phosphatase that regulates NFAT activation, is the direct target of the immunosuppressive agents cyclosporin A and FK506 (14). Preliminary data demonstrated a putative binding site for NFAT transcription factors on the cyclin A2 proximal promoter (Silva-Carvalho and Viola, unpublished observations). Actually, it has been suggested that NFATC2 transcription factor may have a negative effect on gene transcription, acting as a repressor of the expression of some genes, such as IL-4 (24). This evidence supports the hypothesis that NFATC2 transcription factor could have a direct role in controlling the expression of cell cycle-related genes, and thus may directly play a negative effect in the regulation of some cyclins.

Several observations suggest an involvement of the cell cycle in lymphocyte activation, differentiation, and death by apoptosis. It has been demonstrated that activated induced T-cell death is dependent on the cell cycle, and T cells need to enter the G1 phase of the cell cycle in order to die by apoptosis in response to stimulation (1, 3, 4). Thus, it is expected that lymphocytes that present a hyperproliferative response to antigen would also present an enhanced rate of apoptosis. This evidence is in accordance with our results demonstrating that

NFATC2–/– lymphocytes also present an increased rate of cell death by apoptosis in comparison with NFATC2+/+ lymphocytes. Furthermore, NFATC2–/– lymphocytes present an overexpression of cyclin B1 when compared with NFATC2+/+ lymphocytes. Although there is some evidence that overexpression of this cyclin has no effect on cell cycle dynamics (38), it has been shown that cyclin B1 is related to activation-induced cell death in T cells (1). These data are in agreement with our findings that NFATC2–/– lymphocytes present an enhanced active form of apoptosis.

However, there are conflicting results with regard to the involvement of the cell cycle in Th differentiation. It has been suggested that the IL-4 expression in Th2 cells is dependent of the cell cycle progression (2). However, Grogan and coworkers suggested that the cell cycle is important for the commitment of Th phenotype during the response to stimulation but not for the IFN-γ or IL-4 expression (5). Recently, it has been shown that cell division plays a role in the frequency of IFN-γ- or IL-4-producing cells but that it is not essential for differentiation of Th lymphocytes (44). Although NFATC2–/– mice present an hyperproliferative response in addition to an overexpression of Th2 cytokines, our results are not conclusive about the real involvement of the cell cycle in Th differentiation. However, we can hypothesize that the expression of genes that are involved in cell cycle control and in Th differentiation could be regulated in a coordinated way. The phenotype observed in NFATC2–/– lymphocytes supports this idea. Because NFATC2 is a preexisting transcription factor, this protein has a very early effect on lymphocyte activation (11). Thus, we can speculate that NFAT transcription factors could simultaneously control the expression of early inducible genes that are related to Th differentiation and genes that regulate the cell division "clock." In conclusion, our data provide strong evidence that the NFATC2 transcription factor could play an important role in cell cycle control during lymphocyte activation by regulating cyclin expression. Furthermore, they also suggest that NFAT transcription factor acts as an ubiquitous regulator of gene expression that might be reflected in lymphocyte activation.

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Fig. 1

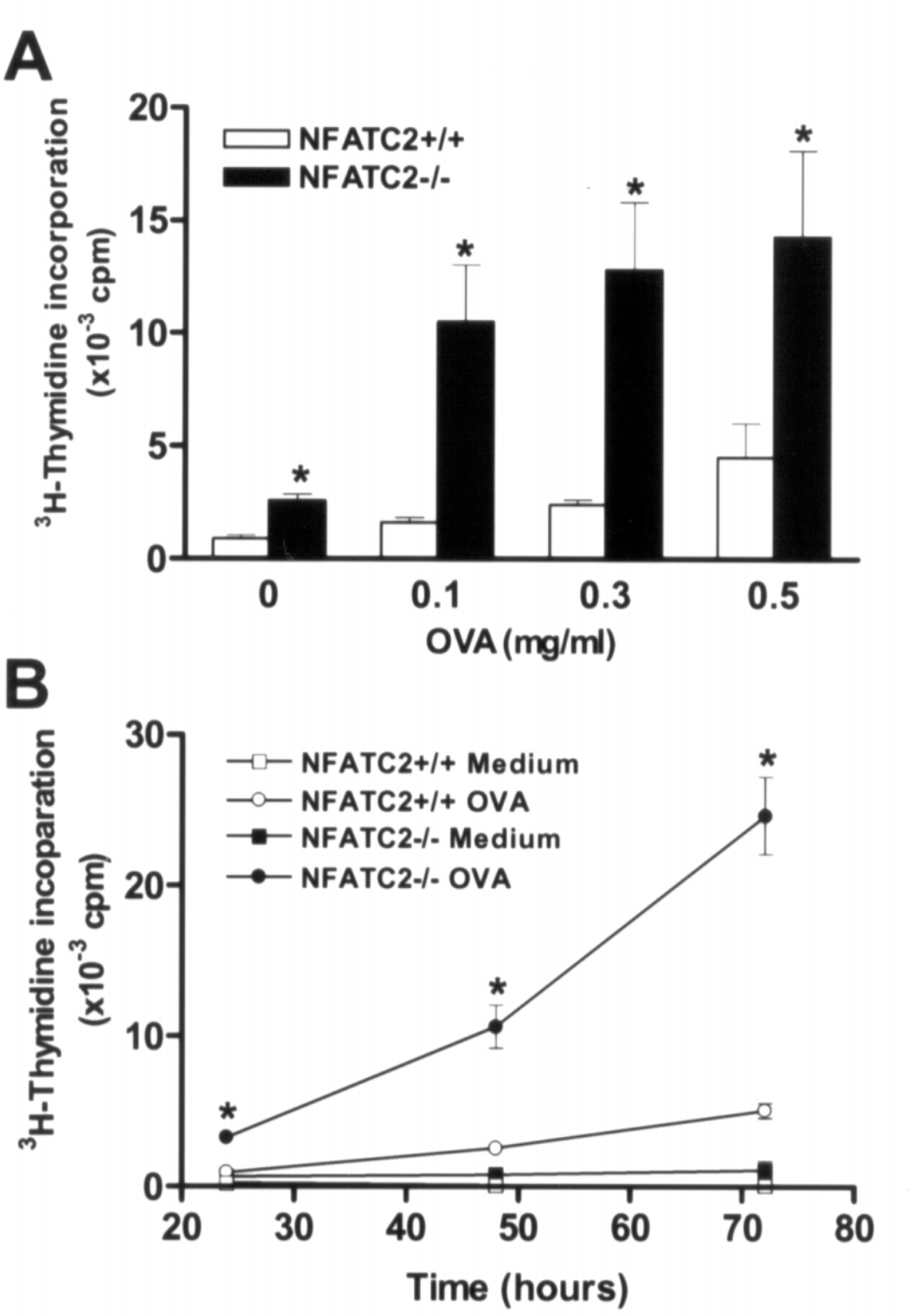

Figure 1. Proliferation assay of lymph node cells from NFATC2+/+ and NFATC2–/– mice. Animals were sensitized and cells (2×10^5 cells/well) were left unstimulated or stimulated *in vitro* with ovalbumin (OVA) as described. **A)** Lymph node cells from NFATC2+/+ and NFATC2–/– were stimulated with different concentrations of OVA (0.1–0.5 mg/ml) or left unstimulated (medium) for 72 h. **B)** Lymph node cells from NFATC2+/+ and NFATC2–/– were stimulated with OVA (0.5 mg/ml) or left unstimulated (medium) for 24, 48, and 72 h. The results are expressed as mean ±SE (n=3) and are representative of four independent experiments. *Data significantly greater than in NFATC2+/+ mice (P<0.05).

Fig. 2

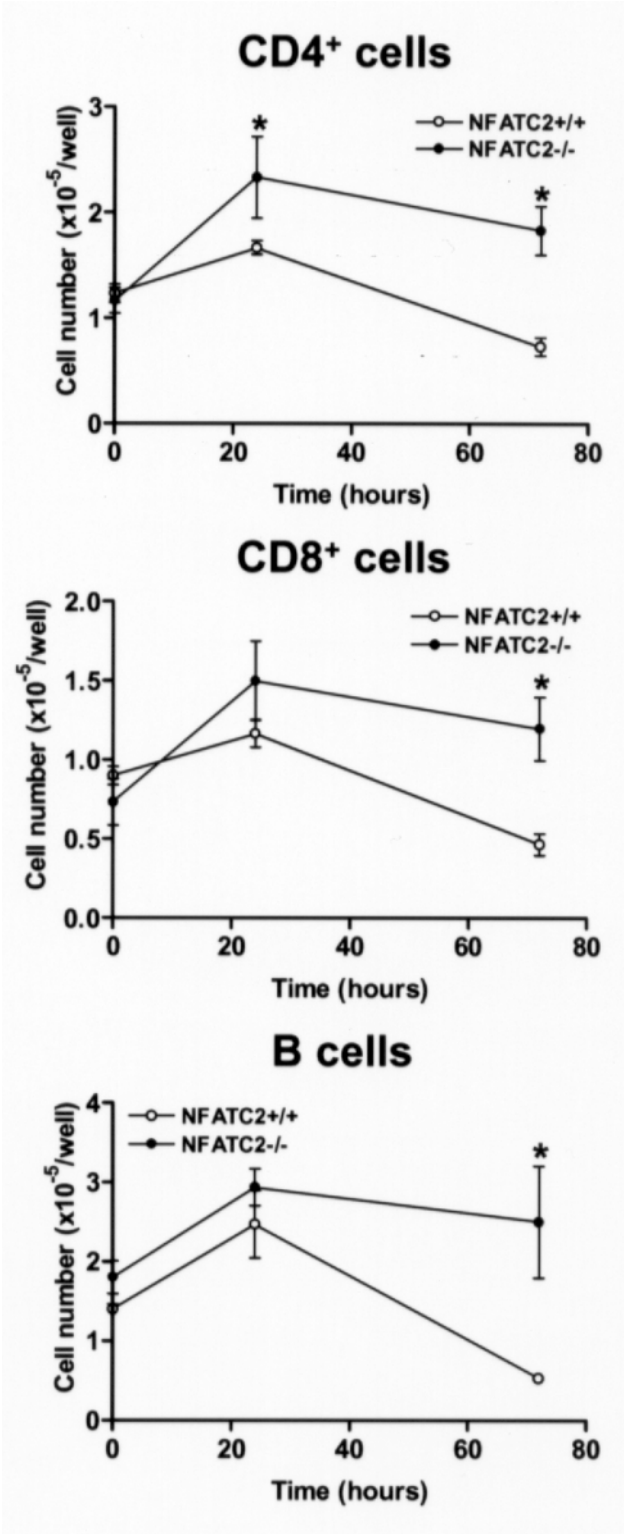

Figure 2. Analysis of subset populations of lymph node cells from NFATC2+/+ and NFATC2–/– mice. Animals were sensitized as described. Fifteen days later, draining lymph nodes were collected and cells (2×10^5 cells/well) were stimulated *in vitro* with ovalbumin (0.5 mg/ml) for 0, 24, and 72 h. At indicated time points, cells were stained with specific fluorochrome-labeled antibodies as described and then analyzed by flow cytometry. The results are expressed as mean ±SE (n=3) and are representative of three independent experiments. *Data significantly greater than in NFATC2+/+ mice (P<0.05).

Fig. 3

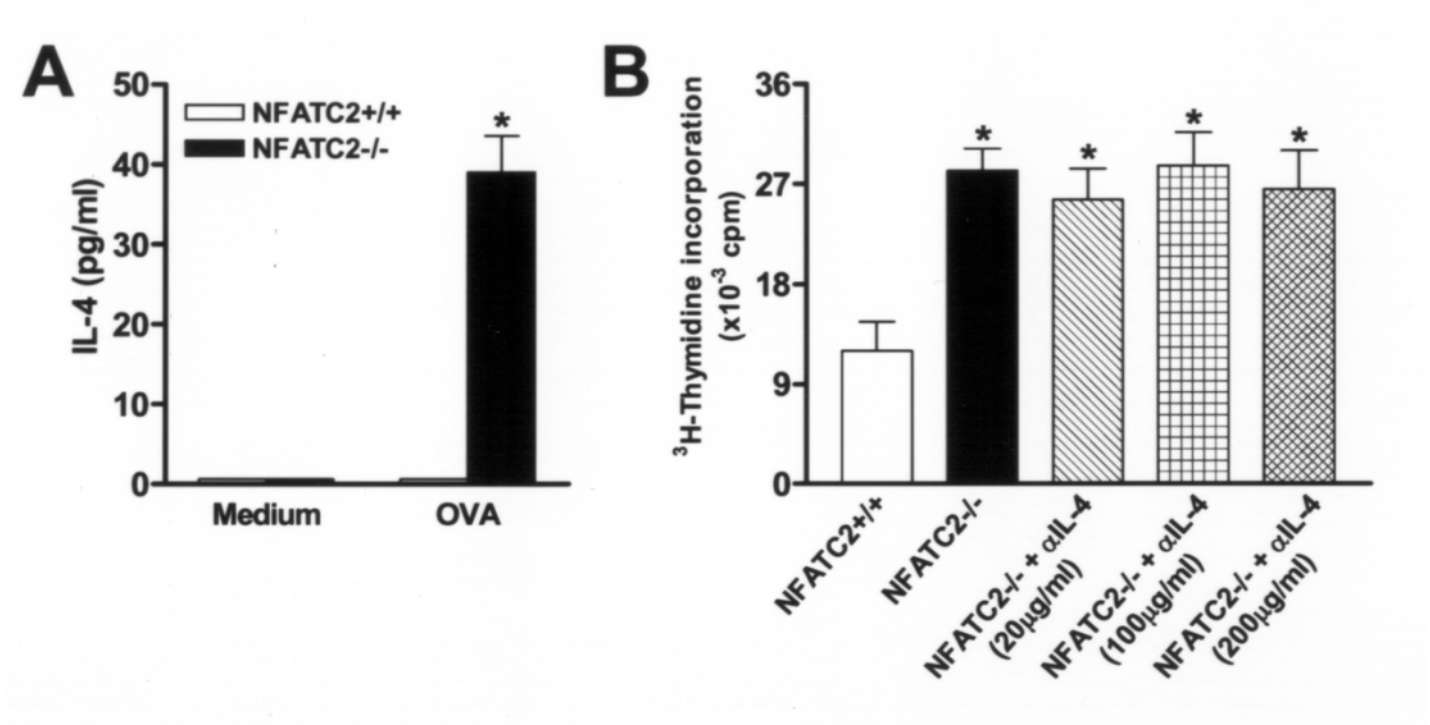

Figure 3. Effect of IL-4 in the hyperproliferation of NFATC2–/– lymphocytes. Animals were sensitized and *in vitro* stimulated with antigen as described. **A)** Analysis of IL-4 production by NFATC2+/+ and NFATC2–/– lymphocytes. Lymph node cells (2×10^6 cells/well) from NFATC2+/+ and NFATC2–/– mice were left unstimulated or stimulated *in vitro* with ovalbumin (OVA) (0.5 mg/ml) for 72 h, and then IL-4 was measured by ELISA as described. **B)** Proliferation assay of lymph node cells treated with neutralizing anti-IL-4 antibody. Lymph node cells (2×10^5 cells/well) from NFATC2+/+ and NFATC2–/– mice were stimulated *in vitro* with OVA (0.5 mg/ml) without or with anti-IL-4 antibody (20, 100, and 200 μg/ml) for 72 h, and then the proliferation was assessed as described. The results are expressed as mean ±SE (n=3) and are representative of two independent experiments. *Data significantly greater than in NFATC2+/+ mice (P<0.05).

Fig. 4

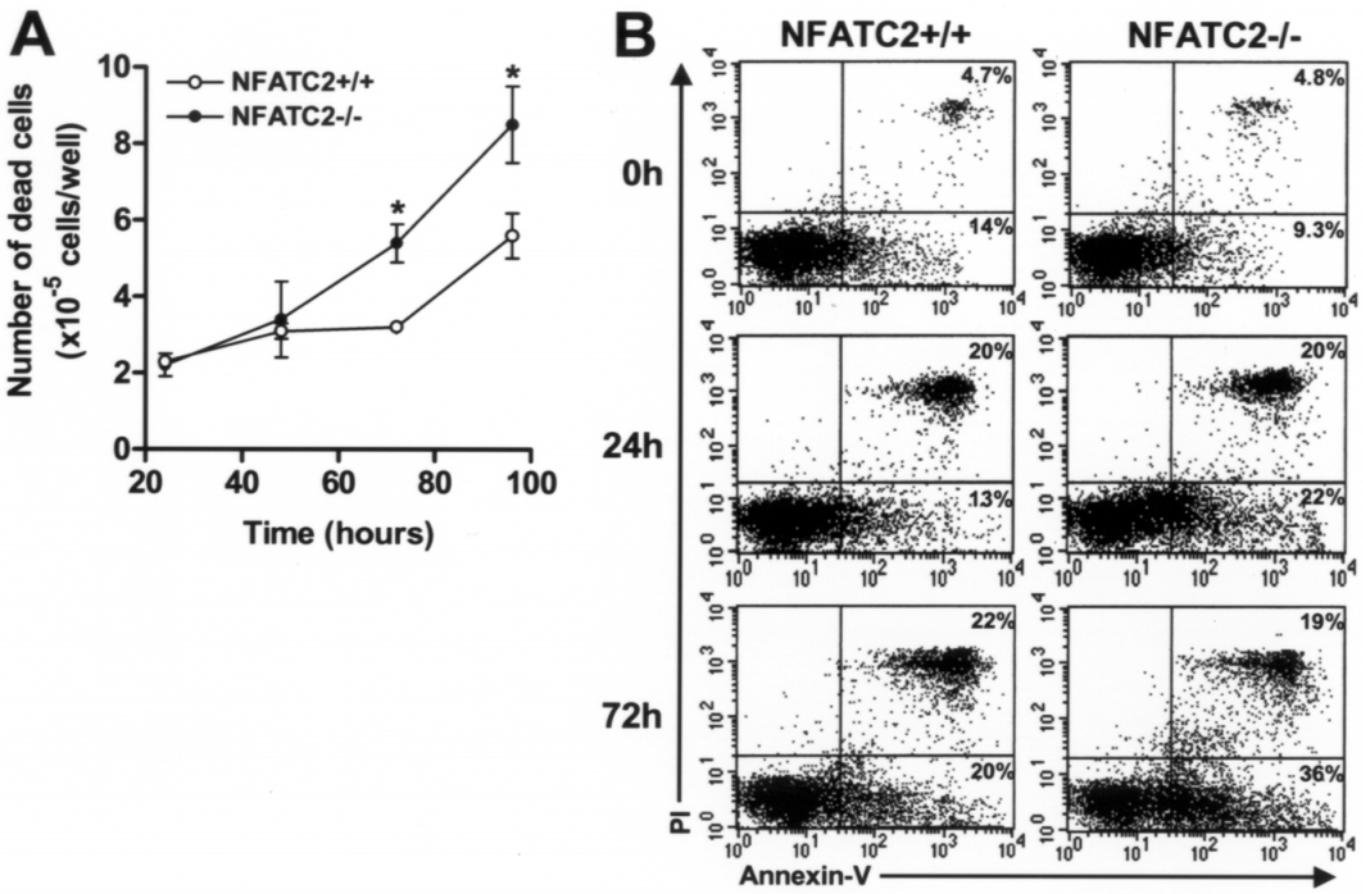

Figure 4. Cell death analysis of lymphocytes from NFATC2+/+ and NFATC2–/– mice. Animals were sensitized and *in vitro* stimulated with ovalbumin (OVA) (0.5 mg/ml) as described. **A)** Kinetics of the number of dead cells after *in vitro* stimulation with OVA. Cells were collected, and their viability was measured by the capacity of Trypan blue exclusion at the indicated time. The results are expressed as mean ±SE (n=3) and are representative of three independent experiments. *Data significantly greater than in NFATC2+/+ mice (P<0.05). **B)** Analysis of cell death by apoptosis after *in vitro* stimulation with OVA. Cells committed to apoptosis were measured by the capacity of annexin V binding. At indicated time points, cells were stained with annexin V/FITC as described and were then analyzed by flow cytometry. The results are from a pool of three mice and are representative of three independent experiments.

Fig. 5

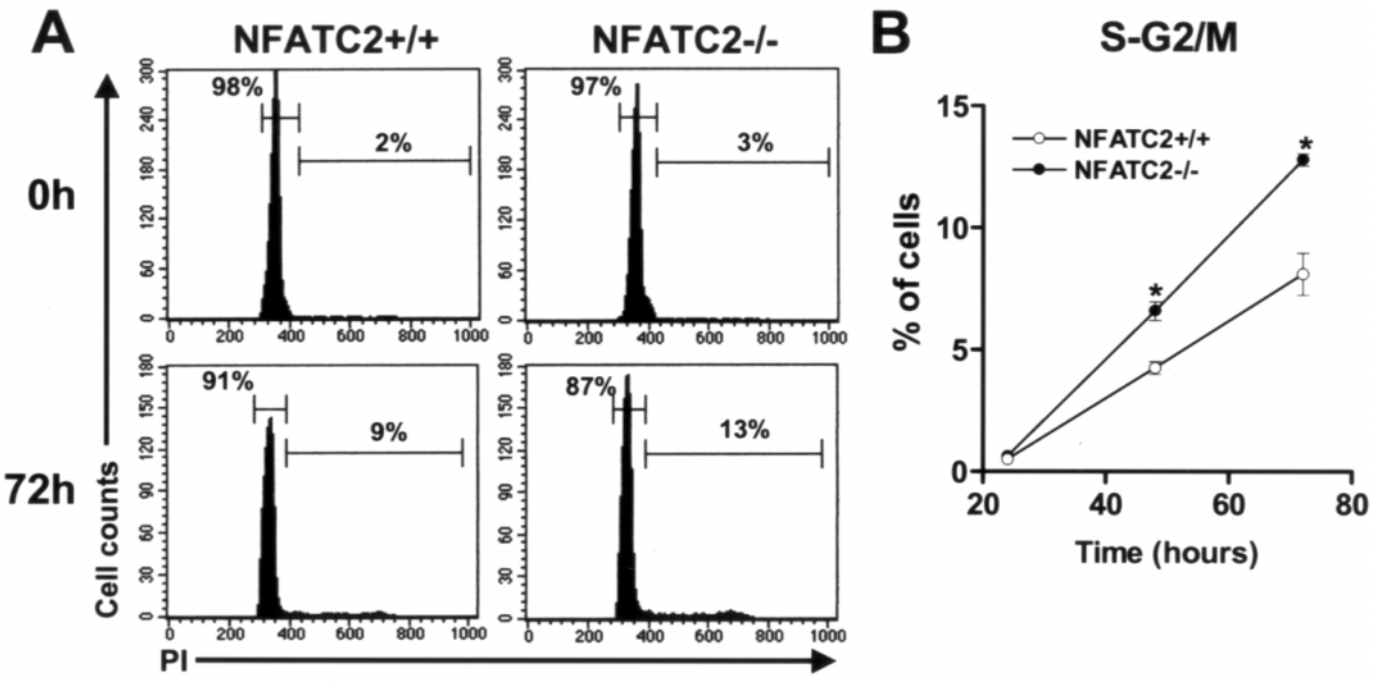

Figure 5. Cell-cycle analysis of NFATC2+/+ and NFATC2–/– lymphocytes. Animals were sensitized as described. Fifteen days later, cells from draining lymph nodes were collected and *in vitro* stimulated with ovalbumin (OVA) (0.5 mg/ml) for 24, 48, and 72 h. At indicated time points, cells (4×10^5 cells/well) were collected and stained with propidium iodide in the presence of NP-40 as described and were then analyzed by flow cytometry. **A)** Representative histogram of 0 and 72 h after OVA stimulation of NFATC2+/+ and NFATC2–/– cells. The results are representative of 12 independent animals. **B)** Distribution of percent number of cells in S-G2/M phases after OVA stimulation at 24, 48, and 72 h. The results are expressed as mean ±SE (n=3) and are representative of four independent experiments. *Data significantly greater than in NFATC2+/+ mice (P<0.05).

Fig. 6

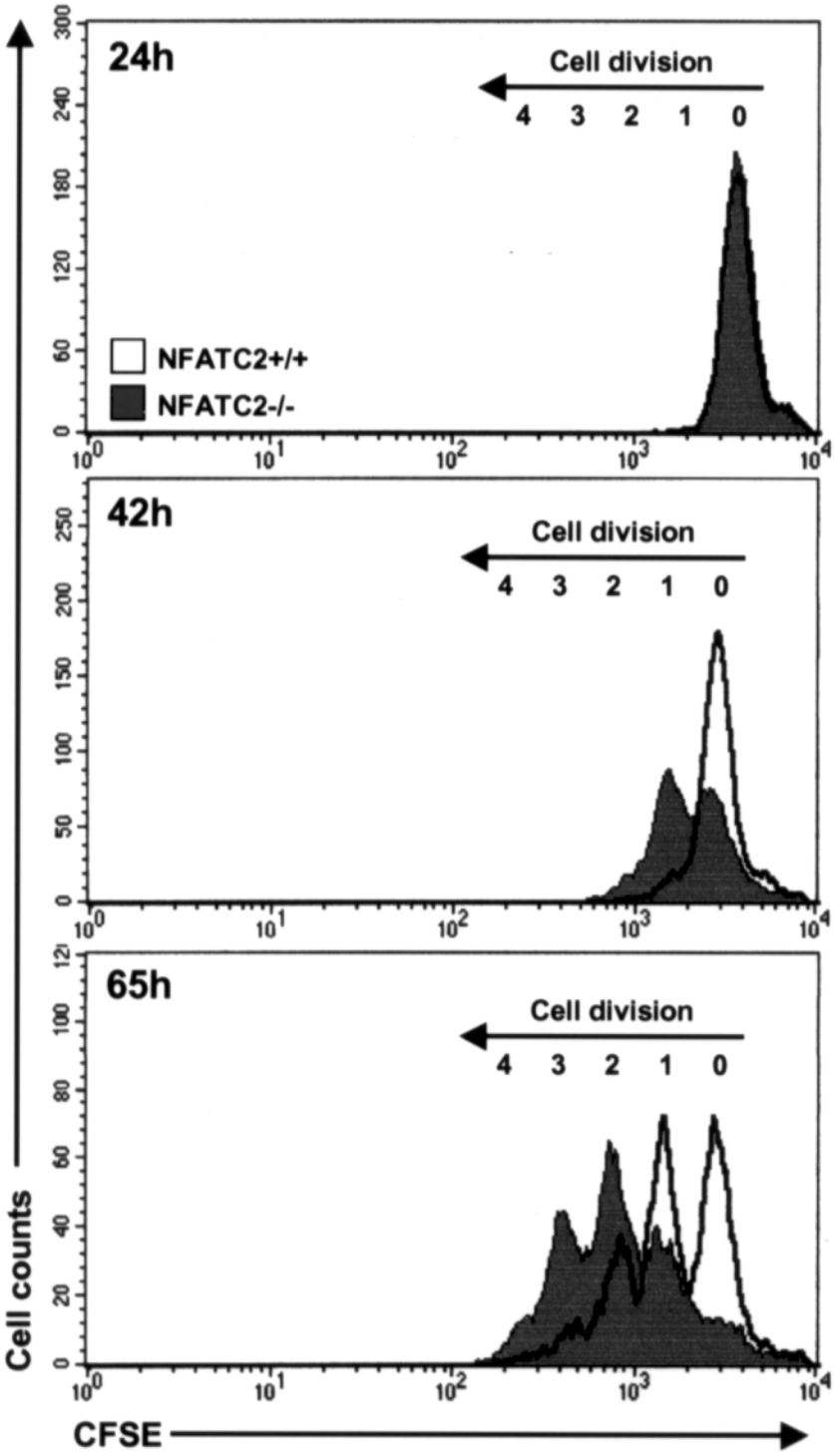

Figure 6. Analysis of cell division upon anti-CD3 stimulation. Animals were sensitized as described. Fifteen days later, cells (10^7 cells/ml) from draining lymph nodes were labeled with carboxyfluorescein diacetate succinimidyl ester as described, and then cells (2×10^5 cells/well) were *in vitro* stimulated with plate-bound anti-CD3 antibody (1 μg/ml) for 24, 42, and 65 h. At indicated time points, cells were analyzed by flow cytometry. The results are representative of six independent animals.

Fig. 7

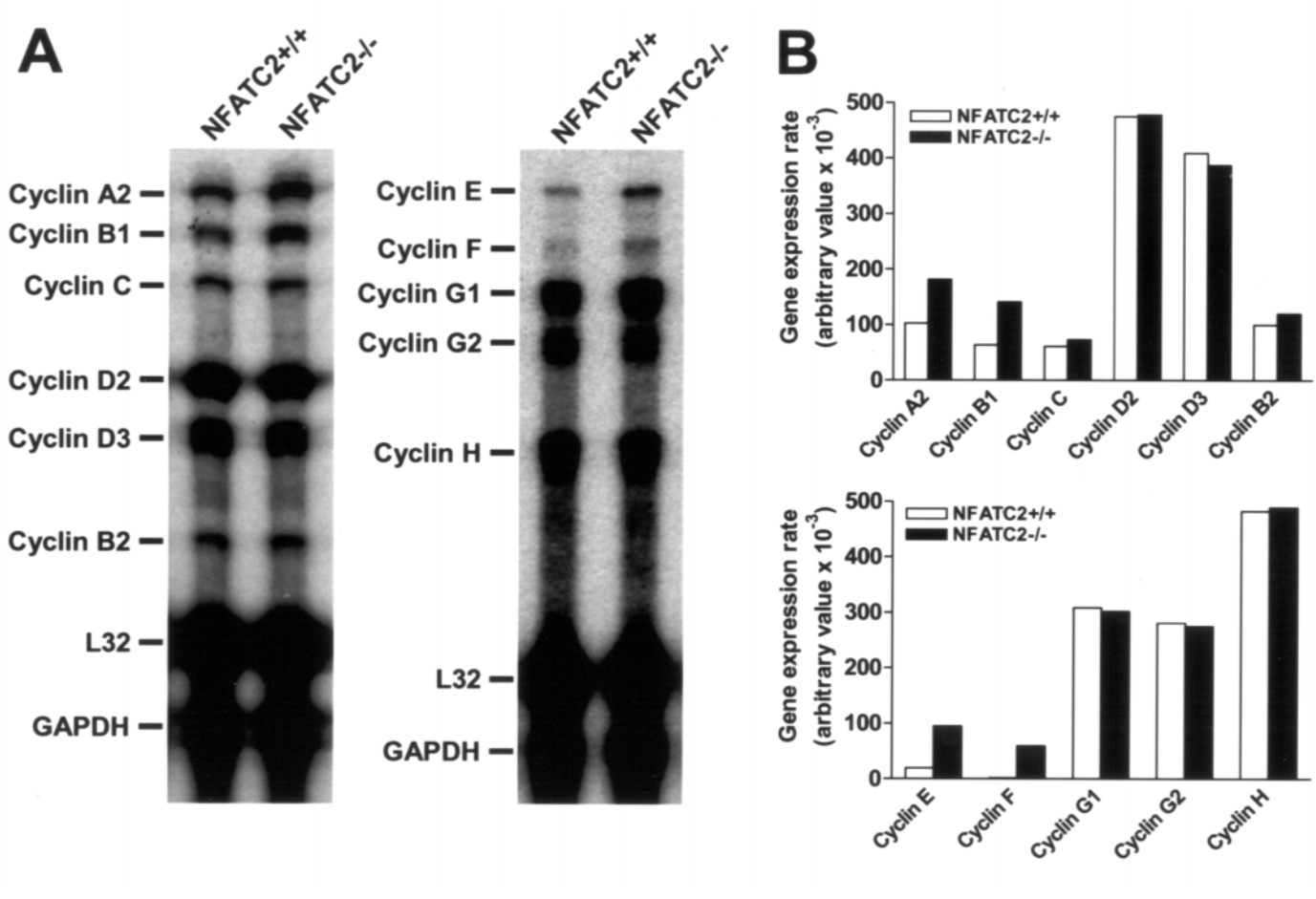

Figure 7. Analysis of cyclins expression of NFATC2+/+ and NFATC2–/– cells. Animals were sensitized and *in vitro* stimulated as described. Cells (2×10^7) were *in vitro* stimulated with ovalbumin (OVA) (0.5 mg/ml) for 24 h and collected, and then total RNA was immediately extracted as described. **A)** Analysis of cyclin mRNA expression by RNase protection assay with a multiprobe assay. RNA loading was estimated by measuring the intensities of two housekeeping genes (L32 and GAPDH). Transcript levels were analyzed by autoradiography. **B)** Quantification of cyclin mRNA levels from **Figure 7A** by densitometry. The values are expressed relative to the housekeeping genes (L32 and GAPDH). The results are from a pool of three mice and are representative of three independent experiments.