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The Duration of Nuclear Residence of NFAT Determines the Pattern of Cytokine Expression in Human SCID T Cells

Stefan Feske,* Ruth Draeger,† Hans-Hartmut Peter,† Klaus Eichmann,‡ and Anjana Rao2*

The expression of cytokine genes and other inducible genes is crucially dependent on the pattern and duration of signal transduction events that activate transcription factor binding to DNA. Two infant patients with SCID and a severe defect in T cell activation displayed an aberrant regulation of the transcription factor NFAT. Whereas the expression levels of the NFAT family members NFAT1, -2, and -4 were normal in the patients’ T cells, dephosphorylation and nuclear translocation of these NFAT proteins occurred very transiently and incompletely upon stimulation. Only after inhibition of nuclear export with leptomycin B were we able to demonstrate a modest degree of nuclear translocation in the patients’ T cells. This transient activation of NFAT was not sufficient to induce the expression of several cytokines, including IL-2, IL-3, IL-4, and IFN-γ, whereas mRNA levels for macrophage inflammatory protein-1α, GM-CSF, and IL-13 were only moderately reduced. By limiting the time of NFAT activation in normal control cells using the calcineurin inhibitor cyclosporin A, we were able to mimic the cytokine expression pattern in SCID T cells, suggesting that the expression of different cytokine genes is differentially regulated by the duration of NFAT residence in the nucleus. The Journal of Immunology, 2000, 165: 297–305.

The NFAT family of transcription factors consists of five members, NFAT1, -2, -3, -4, and -5 (1, 2). NFAT1, -2, and -4 are expressed in immune system cells, including T, B, and NK cells; mast cells; macrophages; and basophils, where they play a central role in inducible gene transcription during an immune response. NFAT family members can also be expressed outside the immune system, e.g., NFAT3 in testis, muscle cells, and the heart (for reviews, see Refs. 3–5). Binding sites for NFAT have been found in the regulatory domains of many inducible genes as high affinity binding sites for NFAT alone, as binding sites for conventional Rel family proteins, or as so-called composite binding sites to which NFAT binds as a complex with AP-1 (Fos-Jun) proteins (reviewed in Ref. 3). Among the genes described to be regulated by NFAT in T cells are IL-2 (6, 7), IL-3 (8), IL-4 (9–11), IL-5 (12), GM-CSF (13, 14), IFN-γ (15, 16), TNF-α (17, 18), IL-13 (19), Fas ligand (FasL) (20), and CD40 ligand (21).

Gene transcription by the NFAT proteins NFAT1, -2, -3, and -4 is continuously controlled by the calcium-regulated phosphatase calcineurin. The immunosuppressants cyclosporin A (CsA) and FK506 are potent and specific inhibitors of calcineurin phosphatase activity and thus of NFAT-mediated gene induction (22, 23).

Calcineurin-mediated dephosphorylation of these NFAT proteins is not only responsible for their translocation from the cytoplasm to the nucleus, but also controls their affinity for DNA (24). Thus, both the length of NFAT residence in the nucleus as well as the transcriptional activity of nuclear NFAT depend critically on the duration of calcineurin activation. When the activity of calcineurin drops, NFAT proteins are exported from the nucleus, their regulatory domain is rephosphorylated, and NFAT-dependent transcription stops (25, 26). However, the dependence of gene expression on NFAT activation is not fully understood in terms of the length of time and the efficiency of NFAT engagement necessary for optimal activation of gene transcription.

We have previously described a SCID in two male siblings in whom the proliferative response of PBL and T cell lines to mitogens, lectins, and anti-CD3 plus anti-CD28 Abs was markedly reduced to levels ~10% of those in normal controls (27). Furthermore, there was a pronounced impairment in the production of multiple cytokines such as IL-2, IFN-γ, and TNF-α in these patients. This SCID phenotype correlated with the impaired induction of nuclear NFAT DNA-binding activity in their T cells. Here we show that the impairment in NFAT activation is due to impairment of dephosphorylation and nuclear translocation of NFAT. Moreover, the pattern of cytokine expression in these SCID T cells is predictably based on the short duration of nuclear residence of NFAT.

Materials and Methods

Case report

Detailed case reports of the two SCID patients investigated in this study were described previously (27). Whereas the first patient died of gastrointestinal sepsis at the age of 11 mo, the second patient is now 5 years, 9 mo old. At 16 wk of age he received a T cell-depleted haploidentical bone marrow graft donated by his aunt, which he tolerated well. Soon after the bone marrow transplantation (BMT), our patient presented with daily recurrent fever, with a maximum temperature of 39°C, that was more prominent during the summer months without clinically or laboratory indications for an infectious cause. Specifically, titers against rotavirus, CMV, hepatitis A virus, hepatitis B virus, hepatitis C virus, herpes simplex virus, and HIV were negative. Sweat provocation testing revealed a generalized anhidrosis to be the most likely explanation for his fever. Furthermore, the boy was diagnosed with ectodermal dysplasia, as his dental enamel was

Abbreviations used in this paper: FasL, Fas ligand; CN-A, calcineurin A; MIP, macrophage inflammatory protein; CsA, cyclosporin A; BMT, bone marrow transplantation; DBD, DNA binding domain; LMB, leptomycin B; CVIID, common variable immunodeficiency.

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discored and soft. Most prominently, the boy suffers from nonprogressive muscular hypotonia with a normal creatinine kinase and histologically no signs of a primary degenerative, inflammatory, or metabolic myopathy or neurogen- muscular atrophy. Neurological tests, electromyography, and a nuclear magnetic resonance scan of the CNS were not pathological, although clinically our patient shows signs of psychomotor and mental retardation. The muscular hypotonia most likely results from a congenital myopathy in the context of the patient’s immunodeficiency syndrome. Immunologically, the second patient resembles his older brother. Five years and 9 mo after BMT, the patient’s PBL show abnormalities in phenotype and proliferative response, whereas the production of IL-2 and IL-10 was strongly reduced. Conversely, IFN-γ and TNF-α production were normal. Taken together, these results and the fact that our patient is not subject to more frequent or unusual infections indicate that he is immunologically competent after the BMT.

Cell culture conditions and reagents

Continuously growing T cell lines were derived from PBL of the two patients before BMT, their parents, and healthy donors as previously described (27). These T cell lines were used for all experiments described in this study. Stimulation was performed in RPMI plus 10% FCS, which contains an estimated calcium concentration of 0.8 mM. For stimulation, 1–2 × 10^6 cells were treated with 1 μM ionomycin (Calbiochem, La Jolla, CA) alone, 16.2 nM PMA (Calbiochem) plus 1 μM ionomycin, or 10 ng/ml anti-CD3 (PharMingen, San Diego, CA) plus 200 ng/ml anti-CD28 Ab (PharMingen) of anti-CD28 DRB (Frederick, MD) to detect NFAT, followed by Cy-3-conjugated sheep anti-rabbit IgG (Sigma). Nuclear counterstaining was performed with 1 μg/ml DAPI (Molecular Probes, Eugene, OR) for 1 min. Where indicated, nuclear translocation of NFAT was inhibited by preincubation for 30 min with 1 μM CsA. Nuclear export was inhibited by incubation with 200 nM leptomycin B (LMB; gift from B. Wolff, Novartis, Vienna, Austria) for 30 min before or after stimulation.

In vitro dephosphorylation of RII peptide

Cytoplasmic extracts of unstimulated T cells were prepared as described above and by Schreiber (30). In a 50-μl reaction, 3 μg of these extracts were incubated in reaction buffer (100 mM Tris (pH 7.5), 30 mM MgCl₂, 0.5 mg/ml BSA, 1 mM CaCl₂, and 143 mM 2-ME) containing 32P-labeled RII peptide (PMA-driven). The RII peptide corresponding to a sequence in the RII subunit of cAMP-dependent kinase (31) was synthesized at the Tufts New England Medical Center Peptide synthesis facility (Boston, MA). 32P was labeled with the protein kinase A catalytic subunit (Sigma) to ~800 cpm/pmol and added to the reaction for 30 min at 30°C. Where indicated, the reactions were preincubated with 1 μM FK506 (Calbiochem) plus 1 μM FK binding protein (FKBP12; gift from S. Schreiber), 5 mM EGTa, or 500 nM okadaic acid (Calbiochem) for 15 min at 4°C. For positive control reactions, the 32P-labeled RII peptide was incubated with 100 nM bovine calcineurin (Sigma), 1 μM CsA, and 1 μM calmodulin (Sigma). Released 32P was quantified in the supernatant after addition of 50 μl of 0.1% TCA and incubation with 200 μl of a 50% slurry of AG50W resin (Bio-Rad). The indicated values are the means of triplicate determinations, and the percentage of CN-specific phosphatase activity was calculated after subtraction of background activity measured in incubation reactions with 32P-labeled peptide and reaction buffer alone.

RNase protection assay

After in vitro stimulation with PMA (16.2 mM) and ionomycin (1 μM) in RPMI plus 10% FCS, cells were harvested at the indicated time points, and total cellular RNA was extracted with Ultraspec according to the manufacturers’ protocol (Biotex, Houston, TX). Cytokine RNA levels were analyzed by RNase protection assay using the RiboQuant multiprobe kit (PharMingen) according to the manufacturers’ protocol (Briefly, 2 μg of total cellular RNA was hybridized overnight to a 32P-labeled RNA probe that had been synthesized from two different multicytokine template sets (hCK1 and custom-made template set containing probes for FasL, IL-3, TNF-α, GM-CSF, MIP-1α, and Bel-2). After digestion of free probe and ssRNA the protected dsRNAs were purified and resolved on a 5% polyacrylamide gel. Blotting and autoradiography and densitometric scanning of autoradiograms using ImageQuant software (Molecular Dynamics). RNA loading was estimated by measuring the intensities of the protected fragments of the housekeeping genes L32 and GAPDH. For quantification, backgrounds were subtracted from specific bands, and those values were divided by the intensity of the L32 housekeeping transcript band.

RT-PCR and DNA sequencing

cDNA was prepared from total cellular RNA (see RNase protection assay) using Superscript II reverse transcriptase (Life Technologies, Grand Island, NY), and PCR was performed with SuperTag DNA polymerase (Hoffmann-La Roche, Nutley, NJ) using specific primers spanning the Rel homology domain and the N-terminus of human NFAT1, -2, and -4. Commercially available Abs were used for the detection of calcineurin A (CN-A; Upstate Biotechnology, Lake Placid, NY), calcineurin B (CN-B; Affinity BioReagents), and calmodulin (Upstate Biotechnology). To monitor in vitro dephosphorylation of NFAT, cytoplasmic and nuclear extracts of unstimulated cells were prepared as previously described (30) with the above-mentioned concentrations of proteinase inhibitors, aprotinin, leupeptin, and PMSF, present. Lysates were incubated with 100 mM bovine calcineurin (Sigma), 1 μM calmodulin (Sigma), and 0.8 mM CaCl₂ at 30°C for 20 and 60 min, respectively, in reaction buffer containing 1 M Tris (pH 7.5), 1 M MgCl₂, 10 mg/ml BSA, and 0.1 M CaCl₂. Lysates were then treated as described above for Western blotting and separated on 8% SDS polyacrylamide gels. NFAT1 was detected using anti-T2B1 polyclonal antiserum.

Immunocytochemistry

T cells used for immunocytochemistry were stimulated with 1 μM ionomycin or 1 μM thapsigargin in RPMI plus 10% FCS containing 0.8 mM CaCl₂. T cell lines were centrifuged in a cytopsin (Shandon, Pittsburgh, PA) for 3 min at 350 rpm onto poly-L-lysine (Sigma, St. Louis, MO)-coated coverslips (0.01%, w/v) immediately after stimulation, fixed in 3% paraformaldehyde for 20 min at room temperature, and permeabilized by washing three times in wash buffer (1× PBS, 0.5% Nonidet P-40, and 0.01% NaN₃). Nonspecific binding was blocked by incubation with wash buffer plus 10% FCS for 30 min at room temperature. Ab incubation was conducted for 1 h at room temperature using anti-67.1 (1/1000) or anti-T2B1 (1/1000) to detect NFAT1 and polyclonal antiserum 1689 (1/100) gift from B. Wolff, Novartis, Vienna, Austria) for 1 h. Where indicated, nuclear translocation of NFAT was inhibited by preincubation for 30 min with 1 μM CsA. Nuclear export was inhibited by incubation with 200 nM leptomycin B (LMB; gift from B. Wolff, Novartis, Vienna, Austria) for 30 min before or after stimulation.

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2115(as); NFAT4c (L41067): 959(s), 988(as), 1654(s), 1704(as), 2037(s), 2037(as)). Sequencing was performed for the 1 and 2 strands, and several clones from at least two independent cDNAs were analyzed. Critical sequence data were manually reanalyzed.

Results

As we have previously shown, induction of nuclear NFAT-DNA binding activity was strongly reduced in the SCID patients’ T cells, providing a likely explanation for the multiple cytokine production deficiency (27). Here we show the altered regulation of NFAT in the SCID patients’ T cells. Furthermore, the objective of these experiments was to determine whether we could relate the altered pattern of cytokine gene induction in SCID patient T cells to alterations in the functional regulation of NFAT proteins.

SCID T cells express a subset of inducible NFAT-dependent genes

As we have demonstrated previously (27), transcription of IL-2 and IFN-γ genes was not detectable in stimulated patient T cells by Northern blot analysis. We extended these findings by examining transcription of a larger series of inducible genes known or suspected to be regulated by NFAT. We chose RNase protection assays as a sensitive and quantitative method to compare the expression levels of several inducible genes simultaneously. Patient and control T cell lines were left unstimulated (Fig. 1, lanes 1–4) or were stimulated with PMA plus ionomycin (lanes 5–8) or anti-CD3 plus anti-CD28 Abs (data not shown) for 4 h, and total RNA was used for RNase protection assays. Unstimulated control cells showed no detectable cytokine transcription for IL-2, IL-3, IL-4, IL-5, IL-7, IL-10, IFN-γ, FasL, or lymphotactin. Basal transcript levels were observed for IL-13, TNF-α, GM-CSF, M-CSF, MIP-1α, MIP-1β, and RANTES that were comparable in patients and controls. Upon stimulation, control cells showed robust induction of all transcripts, while in the patients’ cells IL-2, IL-3, IL-4, IL-5, IL-7, IL-10, IFN-γ, M-CSF, and lymphotactin transcription was barely detectable. Levels of TNF-α, IL-13, GM-CSF, MIP-1α, MIP-1β, and FasL transcription were significantly reduced, whereas RANTES transcription was almost normal. In the patients’ T cells, the decrease in FasL expression did not lead to a significant defect in the ability of the patients’ T cells to undergo apoptosis, possibly because their cell surface expression of Fas was normal, as shown previously (27). These results indicated that NFAT-dependent gene expression was generally impaired in the patients’ cells, but the degree of impairment was variable, with some genes being more affected than others.

Dephosphorylation of NFAT family members is partially impaired in SCID patients’ T cells

To understand the variability in the expression levels of NFAT-dependent genes, we analyzed the activation of NFAT and the function of its upstream regulator CN. In immunoblotting experiments using Abs specific for four NFAT family members we found that NFAT3 was not expressed, whereas NFAT1, -2, and -4 were expressed at comparable levels in the T lymphocytes of the two patients and in the controls (Fig. 2). No obvious molecular size differences suggesting a major deletion or insertion could be detected when comparing NFAT proteins from resting SCID and control cells (lanes 1, 7, and 13 for NFAT1; -2, and -4 in Fig. 2).
However, activation of all three NFAT proteins was impaired in the patients’ cells. Whereas stimulation of the control cell lines with ionomycin led to the expected complete dephosphorylation, evident as a shift in the apparent m.w. of all NFAT family members within 5 min, only a minor fraction of NFAT was fully dephosphorylated in the patients’ T cells (compare lanes 8 and 14 to lane 2 for NFAT1, -2, and -4 in Fig. 2), and whereas dephosphorylation was sustained for at least 2 h in the control cell lines, the patients’ NFAT proteins were transiently dephosphorylated only during the first 5 min. Fifteen minutes after the beginning of the stimulation all NFAT1, -2, and -4 proteins in the patients’ T cells were again found to be fully phosphorylated (compare lanes 9 and 15 to lane 3 in Fig. 2). As expected (25), preincubation of T cells with 1 μM CsA for 30 min before stimulation resulted in complete inhibition of dephosphorylation in both patients and controls (Fig. 2, lanes 6, 12, and 18).

**SCID T cells show partial nuclear accumulation of NFAT under conditions where nuclear export is inhibited**

Because dephosphorylation leads to nuclear translocation, we tested whether the transient dephosphorylation of NFAT in the patients’ T cells could lead to low level or transient translocation of NFAT in the patients’ cells. For immunocytochemistry experiments, cells were stimulated with 1 μM ionomycin for 30 min in medium containing 0.8 mM calcium, spun onto poly-L-lysine-coated coverslips, and stained with Abs against NFAT1 under the same conditions as those for NFAT1 (Fig. 3, 1 and m). Although we never observed as complete a nuclear translocation as control cells for NFAT4 as for NFAT1 (compare g and k to a and d), the defect in SCID cells is very striking. This difference in translocation was observable over a wide range of stimulation periods from 10 min to 4 h (data not shown). Stimulation with 1 μM thapsigargin, an inhibitor of the endoplasmic reticulum calcium ATPase that initiates capacitative calcium entry by depleting calcium stores, yielded similar results (data not shown). Furthermore, in immunoblotting experiments using nuclear and cytoplasmic fractions of stimulated T cells, we observed that very little NFAT1 and -2 was translocated to the nucleus in the patients’ T cells compared with control cell lines (data not shown).

To detect any low level or transient nuclear translocation of NFAT, we used the nuclear export inhibitor LMB (32) to trap translocated NFAT in the nucleus. T cells were left unstimulated (Fig. 4, a–d), were stimulated with ionomycin for 30 min (e–h), or were preincubated with LMB for 30 min and then stimulated with ionomycin for another 30 min (i–k). In the control cell lines we observed the same complete nuclear translocation as with ionomycin alone (Fig. 4, compare i and k with e and f). In the SCID patients’ T cells, by contrast, ionomycin treatment alone resulted in no nuclear staining (Fig. 4, g and h), whereas treatment with both LMB and ionomycin led to a mixed cytoplasmic and nuclear staining for NFAT1 in ~35% of the cells and to complete nuclear translocation in ~2–5% of the patients’ cells (Fig. 4, i and m). Alternatively, cells were first stimulated with ionomycin for 30 min, and then LMB was added for 30 min (data not shown). Results for both patients and controls were identical whether LMB was added for 30 min before or after stimulation with ionomycin. Furthermore, preincubation for 30 min with LMB alone had no

**FIGURE 3.** Nuclear translocation of NFAT is severely compromised in T cells from SCID patients. T cell lines of patients (P1 and P2) and a control (Co1) were left unstimulated (a–c and g–i) or were stimulated with 1 μM ionomycin for 30 min (NFAT1; d–f) or 60 min (NFAT4; k–m) in the presence of 0.8 mM extracellular calcium. Cells were spun onto poly-L-lysine-coated coverslips and stained with polyclonal antisera against NFAT1 (a–f) or NFAT4 (g–m).

**FIGURE 4.** Low level translocation of NFAT1 in SCID T cell lines in the presence of the nuclear export inhibitor LMB. T cell lines of two controls (Co1 and Co2) and the two SCID patients (P1 and P2) were left unstimulated (a–d) or were stimulated with 1 μM ionomycin for 30 min (e–m) in the presence of 0.8 mM extracellular calcium. LMB (200 nM) was added 30 min before stimulation (i–m). Cells were spun onto poly-L-lysine-coated coverslips, fixed, and stained for NFAT1. Diagrams represent the average percentage of cells with cytoplasmic, cytoplasmic plus nuclear, or nuclear immunofluorescence, corresponding to i–m. One representative experiment of three is shown.
significant effect on nuclear translocation in either control or patient T cell lines (data not shown). However, when we preincubated SCID T cells with LMB for 30 min and stimulated them with PMA plus ionomycin for 1 or 3 h, no significant increase in cytokine transcription in RNase protection assays was observed compared with that in patients’ cells stimulated with PMA plus ionomycin but without LMB preincubation (data not shown).

These results indicate that the patients’ T cells do, in fact, support a transient nuclear translocation of NFAT. Moreover, the results distinguish between the two theoretical possibilities of defective nuclear import vs an altered import/export equilibrium in the SCID T cells, e.g., due to a very effective mechanism for the nuclear export of NFAT. If the only defect in the SCID T cells was an increased nuclear export (i.e., nuclear import was normal), we would have expected comparable amounts of NFAT in the nucleus in T cells from patients and controls after blocking export. Since even in the presence of LMB only a minority of the cytoplasmic NFAT1 is translocated to the nucleus in SCID T cells compared with the controls, the data support a defect in the nuclear import of NFAT in the patients’ cells rather than its rapid export from the nucleus.

No inherent defect of NFAT or CN in the SCID patients’ T cells

To exclude the possibility of mutations in NFAT resulting in its aberrant regulation, we sequenced the cDNAs for NFAT1, -2, and -4. No attempt was made to sequence NFAT3, because we could not detect NFAT3 proteins by immunoblotting, and mRNA expression was absent in immune cells as shown by others (28, 29). The DBD and N-terminus of NFAT1, -2, and -4 of our two SCID patients did not reveal any mutation that would result in an amino acid exchange, frame shift, insertion, deletion, or premature stop codon. Especially the reported nuclear localization signals N-terminal to the DBD and at the C-terminal end of the DBD, the phosphorylation sites in the N-terminus, the CN docking site at the very N-terminus of the regulatory domains, and the DBD itself were intact (for PCR primers used, see Materials and Methods).

Furthermore, we found that NFAT proteins could be properly dephosphorylated when incubated with CN in vitro. Cytoplasmic extracts from patient and control lines were incubated with CN plus calmodulin for 20 and 60 min at 30°C, and NFAT1 was detected by Western blotting (Fig. 5A), revealing the same amount and kinetics of dephosphorylation in control and patient T cells.

These results indicated that the defect in NFAT dephosphorylation, nuclear translocation, and DNA binding in the patients’ T cells was not inherent in the NFAT proteins themselves but, rather, might be due to impaired function of the upstream regulatory phosphatase calcineurin. However, CN-A and CN-B subunits were equally expressed in cytoplasmic extracts of both patient and control cells (Fig. 5B) as was the calcium-binding protein calmodulin that is essential for full activation of CN (Fig. 5B). Moreover, the CN in the patients was fully functional, as judged by in vitro dephosphorylation of the RII peptide by unstimulated cytoplasmic extracts (Fig. 5C). In contrast to the CN-related phosphatases PP1 and PP2A, CN (PP2B) is specifically inhibited by CsA and FK506, whereas PP1 and PP2A are inhibited by okadaic acid, which affects CN activity only at levels of 1 μM or more (Ref. 33 and our own observations). The level of total phosphatase activity represented by 32P release from the radiolabeled RII peptide after 30 min of incubation with untreated cytoplasmic extracts was similar in extracts from SCID T cells and healthy controls (Fig. 5C, left panel). CN activity was defined as calcium dependent and FK506 inhibitable, but okadaic acid-resistant phosphatase activity accounted for 5–15% of the total phosphatase activity in the T cell lines (in accordance with previous findings (34)), while the majority of phosphatase activity originated from phosphatases other than CN (i.e., FK506 and EGTA resistant, but okadaic acid inhibitable; Fig. 5C, middle and right panels). In each of these categories there was no significant difference between control and patient T cells. Finally, sequencing of the catalytic subunit of CN, CN-A, and here the isoform that is predominantly expressed in lymphocytes, namely CN-AβIII, revealed no sequence abnormalities accounting for the impaired NFAT regulation (data not shown).

FIGURE 5. The interaction of NFAT with its upstream regulatory phosphatase CN is not compromised in SCID patients. A, Normal in vitro dephosphorylation of NFAT1 from SCID T cells. Whole cell lysates of T cells of patients (P1 and P2) and a control (Co1) were incubated in vitro with CN and calmodulin (CaM) for 20 and 60 min, respectively, at 30°C. Extracts were separated on a 6% SDS-polyacrylamide gel, and NFAT1 was detected by Western blotting. Arrows indicate the phosphorylated (P) and dephosphorylated (deP) forms of NFAT1. B, Normal levels of CN-A, CN-B, and calmodulin. Cytoplasmic or whole cell extracts from unstimulated cells of patients (P1 and P2) and controls (Co1 and Co2) were separated on 8–13% SDS-polyacrylamide gels, and blots were incubated with Abs against CN-A, CN-B, or calmodulin. For the detection of calmodulin, whole cell extracts were used. Numbers indicate apparent Mr, in kilodaltons. C, Dephosphorylation of RII peptide substrate by cytoplasmic calcineurin. Protein kinase A-phosphorylated [32P]RII peptide was incubated with cytoplasmic extracts from unstimulated cells for 30 min at 30°C in the absence of phosphatase inhibitors for the detection of total phosphatase activity (left panel). Where indicated, the reactions were preincubated for 15 min at 4°C with FK506 (1 μM) plus FKBP12 (1 μM) or EGTA (5 mM), respectively (middle panel), and with okadaic acid (OA; 500 nM) or FK506/FKBP12 plus okadaic acid, respectively (right panel). The values indicated are the mean (counts per minute) of triplicate determinations of 32P released in the supernatant (left panel). For the middle and right panels, the percentages of total phosphatase activity are shown after subtraction of background activity. The black line parallel to the x-axis (left panel) represents background release of radiolabel in the absence of added cell extracts. One representative experiment of six is shown.
The pattern of cytokine production can be mimicked in normal T cells by limiting the duration of nuclear residence of NFAT

It was intriguing to find that genes possessing NFAT binding sites and thought to rely on NFAT binding for their full activation were so differently affected by the NFAT defect in our SCID patients. For instance, IL-3 expression was heavily compromised in SCID T cells, whereas expression of the closely linked GM-CSF gene was only moderately reduced. Similarly, IL-4 mRNA could not be detected, in contrast to a less pronounced reduction in IL-13 transcripts, although both genes are encoded on the same locus only 12.5 kb apart from each other and are thought to be regulated at least partially in a coordinate fashion (19, 35). Because SCID T cells did support a limited activation and nuclear translocation of NFAT, a plausible hypothesis was that the variability of NFAT-dependent gene transcription observed in the SCID T cells was due to each gene having a distinct requirement for the duration of NFAT interaction with the respective gene regulatory elements.

To test this hypothesis, we stimulated T cell lines from healthy donors for 4 h with PMA plus ionomycin alone, preincubated with CsA before stimulation, or added CsA at different time points after the beginning of the stimulation period. Addition of CsA would lead to a fast nuclear export of NFAT, thus allowing us to control the time of NFAT engagement at the inducible gene regulatory elements. Because cytokine expression is dependent on whether T cells are CD4⁺ or CD8⁺, we used predominantly CD4⁺ T cell lines for these experiments, with patient 1 (P1) expressing 84% and 4%, respectively. In unstimulated cells and cells preincubated with CsA for 30 min (lanes 2), or CsA was added at the time points indicated (lanes 3–7), RNase protection assays were performed as described above using hck-1 (A) and custom-made (B) human template sets (PharMingen). C and D, The diagrams show the relative levels of mRNA expression in stimulated SCID T cells. C refers to A, and D refers to B. One representative experiment of four is shown.

From these experiments it is evident that the cytokine genes expressed in control T cells treated with CsA after very short periods of stimulation (0–15 min, lanes 3 and 4) correspond exactly to those detectable over baseline levels in the patients’ cells following stimulation for 4 h (Fig. 6, A and B, lane 10), i.e., GM-CSF, IL-13, TNF-α, and MIP-1α. Conversely, the genes that require longer stimulation periods, such as IL-4 (first transcripts seen at 60 min), IL-2 (60 min), IFN-γ (30–60 min), or IL-3 (60 min), are exactly those whose induction is absent or most strongly reduced in the patients’ cells. Thus, whereas basal transcription levels are comparable in patients’ and control T cells (Fig. 6, A and B, compare lanes 1 and 9), the expression levels of most of the lymphokine genes in the SCID patients following stimulation resemble those of control T cells stimulated with PMA plus ionomycin for 15 min or less.

Discussion

We show here that NFAT1, -2, and -4 proteins were not mutated and were expressed normally in T cells from the SCID patients investigated in this study. However, regulation of this transcription factor was impaired, because its dephosphorylation and nuclear translocation occurred only very transiently after stimulation. We
could exclude an impairment in coupling of the TCR to downstream signaling pathways, because the cytokine deficiencies were apparent even when the patients’ cells were stimulated with PMA plus ionomycin, which bypass the TCR. Because we could also exclude an intrinsic defect in NFAT itself, there remained essentially two possibilities for its altered regulation: 1) an increased nuclear phosphorylation and export of the transcription factor, or 2) a primary or secondary defect in CN phosphatase activity leading to impaired nuclear import. Increased nuclear export, e.g., due to the presence of an overactive nuclear NFAT kinase, is unlikely, because we found only a slight nuclear accumulation of NFAT in the SCID T cells after stimulation in the presence of the nuclear export inhibitor LMB. Because LMB is known to effectively inhibit the nuclear export of NFAT by interfering with Crm1, an export receptor that recognizes the leucine-rich class of nuclear export signals (36–39), we would have expected a more complete nuclear accumulation of NFAT if the T cells possessed a normal import mechanism but an overactive export mechanism or an overactive kinase. In light of the possibility that specific nuclear kinases exist for each NFAT family member (40, 41), the impaired nuclear localization of all NFAT family members in the SCID patients’ T cells also argues against the existence of an overactive kinase. We have also largely ruled out a primary defect in CN by sequencing the cDNA for CN-A and CN-B proteins, and finding a normal CN-specific phosphatase activity in extracts from our patients’ T cells. Indeed, our preliminary data suggest a secondary defect in the activation of CN due to a defect in capacitative calcium entry (S. Feske, R. Dolmetsch, and A. Rao, unpublished observations).

It was intriguing to find that the expression of the NFAT-dependent genes that we evaluated in this study was so diversely affected by the defect in NFAT activation in the SCID patients. NFAT-dependent gene expression could be observed in the SCID T cells under conditions of very low nuclear translocation. Our results suggest that individual NFAT-dependent target genes show marked differences in their requirements for sustained nuclear residence of NFAT. We were able to generate a cytokine expression pattern similar to, yet not identical with, that in the SCID patients’ T cells when we stimulated control T lymphocytes with PMA plus ionomycin and limited the time of NFAT activation by adding CsA at different time points of stimulation (Fig. 6). In control T cells, mRNA levels comparable with those in the patients’ T cells were observed after stimulation with PMA plus ionomycin for 15 min or less for IL-13, GM-CSF, and TNF-α and for around 30 min for MIP-1α and IFN-γ. In contrast, transcription of IL-2, IL-3, IL-4, and IL-5 in stimulated SCID T cells was never observed above background, i.e., levels in unstimulated control T cells. However, mRNA expression levels for GM-CSF, TNF-α, IL-13, and IFN-γ in the stimulated SCID patients’ T cells were clearly above the low background level of unstimulated T cells, which was comparable in patients and controls. Together, these results provide strong correlative evidence for a short, i.e., ~15 min or less, and low level engagement of NFAT at gene regulatory regions in the SCID T cells. Thus, a transient activation, i.e., dephosphorylation and nuclear translocation, of NFAT seems sufficient to turn on gene transcription, but different genes respond to very different degrees.

One interpretation of these experiments is that although all these inducible genes require NFAT for their full induction (reviewed in Refs. 3 and 42), there are very different threshold levels for NFAT to activate the respective promoters. This threshold, not reached for many lymphokines in the SCID T cells, could be defined by the time of NFAT binding to the promoter or the amount of NFAT in the nucleus. Both parameters are affected in SCID patients, in whom NFAT is only transiently and partially dephosphorylated and translocated to the nucleus (Figs. 2 and 3). The time during which NFAT is resident in the nucleus will affect its ability to cooperate with partner transcription factors (e.g., the different Fos/Jun family proteins) that have different kinetics of activation (43). The quantity of NFAT in the nucleus might influence not only the total number of promoter/enhancer sites occupied by NFAT, but also the extent of tandem occupancy of multiple sites in a single promoter/enhancer region. Clearly, both parameters are important for achieving the long range interactions among transcription factors to create a cooperative “enhanceosome” complex (44). The thresholds for the formation of transcriptionally active enhanceosome complexes will undoubtedly vary for different cytokine genes.

An impaired NFAT-mediated gene expression has not only been shown to be responsible for another SCID patient with multiple cytokine deficiency (45, 46), but might also be involved in a subgroup of patients suffering from the common variable immunodeficiency syndrome (CVID), defined by impaired B cell function and hypogammaglobulinemia. Several reports have described variable degrees of defective cytokine gene expression for IL-2, IFN-γ, IL-9 (47, 48), IL-2, IL-4, and IL-5 (49) depending on the type of stimulus used. Whereas Ag-specific or superantigen stimulation yielded reduced cytokine gene transcription, stimulation with PMA plus ionomycin or anti-CD3 plus anti-CD28 led to normal cytokine expression in the CVID patients’ T cells. Although the role of NFAT in the impaired cytokine transcription was not directly assessed in any of these CVID patients, the varying degrees of defective cytokine production depending on the strength of the stimulus applied support our idea that there is a critical threshold for the expression of each cytokine. In the subgroup of CVID patients with a cytokine deficiency, the genes most affected were also those most impaired in our patients’ T cells. It would be interesting to know whether the genes only moderately impaired in our SCID patients, e.g., TNF-α, IL-13, and GM-CSF, are expressed normally in CVID patients. There are similarities in the characteristics of cytokine gene expression between the SCID T cells described here and T cells that were anergized either with altered peptide ligands (50) or by insufficient stimulation through the TCR. In both SCID T cells and anergic T cells the proliferative response to stimuli is reduced, and IL-2 transcription is strongly compromised. However, there are some differences with regard to other cytokines. In anergic T cells IL-3 and GM-CSF production was reduced with varying decreases in IL-4 production (51, 52), whereas our SCID T cells showed complete absence of IL-4 induction, but only mild reduction in GM-CSF transcription. A more important difference is that anergic T cells showed normal calcium signaling and NFAT activation (53, 54) but poor AP-1-mediated trans-activation (55, 56), while our SCID T cells showed the converse behavior, with impaired NFAT activation but normal binding of AP-1 to its IL-2 promoter elements and normal AP-1-dependent up-regulation of CD25 and CD69 (27). It is conceivable, however, that anergy can be induced through different types of partial signaling in T cells and that the phenotype of our patients’ cells can in part be explained by an anergy-like unresponsiveness.

The features of NFAT-deficient mice did not prove useful in understanding the SCID phenotype in our patients. Mice lacking individual NFAT family members NFAT1, -2, or -4 did not display an immunodeficiency or a dramatic impairment in their cytokine expression, as would have been expected from previous in vitro data. In blastocyst complementation assays, NFAT2−/− or RAG-1−/− mice showed reduced IL-4 and IL-6 levels but normal levels of IL-2, IFN-γ, and TNF-α (57), whereas mice deficient in both NFAT1 and -4 had slightly reduced amounts of IL-2, IFN-γ,
and TNF-α and a significantly increased production of IL-4, IL-5, IL-6, IL-10, and GM-CSF (58). That NFAT is indeed important for the expression of IL-2 in vivo was highlighted by the use of a selective peptide inhibitor of the NFAT-calciulin interaction, that blocks the activation of all NFAT members and leads to decreased IL-2 expression in transfected Jurkat cells (59). Furthermore, none of the dominant features characteristic of these NFAT-deficient mice, such as eosinophilia and increases in serum IgE (60), retarded thymic involution and massive germinal center formation (61), splenomegaly and hyperproliferation of T and B cells for the NFAT1-/- and NFAT1-/-/NFAT4-/- mice (58, 62), or defects in cardiac valve formation as in NFAT2-/- mice (63, 64), were observed in our patients. On the other hand, characteristic phenotypic aspects of our patients, such as muscular hypotonia, dysplastic dental enamel, or early death from infections, were not observed in the NFAT1-/-, -2-, and -4-deficient mice, respectively. Mice lacking only one or two NFAT family member are obviously able to use the remaining NFATs to sustain immune functions and cytokine gene expression. In conclusion, our analysis of the SCID phenotype of our patients has not only uncovered an interesting syndrome of impaired nuclear export of NFAT, but also revealed a predictable relation between cytokine production and duration of NFAT residence in the nucleus. Whereas mice deficient in individual NFAT family members show only subtle alterations of immune function, not consistent with the in vitro data describing the importance of NFAT proteins for the expression of cytokine and other inducible genes, we can here demonstrate in an in vivo disease model the consequences of severely impaired NFAT activation. Furthermore, in light of our results, it would seem worthwhile to analyze the subgroup of CVID patients with cytokine deficiencies for partial impairment in activation of NFAT.

Acknowledgments

We thank Dr. Timothy Hoey (Tularik, CA) for Abs against NFAT3 and -4, Dr. Nancy Cancer (National Cancer Institute, Frederick, MD) for Abs against NFAT2 and -4, Dr. Barbara Wolff (Novartis, Vienna, Austria) for leptomycin B, and Dr. Stuart Schreiber (Boston, MA) for FKB12. We are indebted to Dr. Ian Haidl, Andreas Wuerch, and Dr. Edgar Selrling for valuable discussions, and to Dr. Charlotte Niemyer for providing us with the patient data.

References


CORRECTIONS


Stefan Feske and Anjana Rao wish to correct errors made in the preparation of Figs. 2, 3, 4, and 5. The co-authors (Ruth Draeger, Hans-Hartmut Peter, and Klaus Eichmann), who were not involved in the preparation of the manuscript, bear no responsibility for these mistakes. The integrity of the data and the conclusions of the paper are not affected.

In Fig. 2, no indication was given that bands with “smiles” were straightened and that lanes for the 15-min treatment with cyclosporin A were repositioned from the center to the right side in seven of the nine gel photographs. Two photographs in the upper row (left and right) and two in the middle row (left and middle) were later duplicated as Fig. 1a, A and C in the following article: Feske, S., J. Giltnane, R. Dolmetsch, L. M. Staudt, and A. Rao. 2001. Gene regulation mediated by calcium signals in T lymphocytes. Nat. Immunol. 2: 316–324. For Figs. 2 and 3, please see the corrigendum for the Nature Immunology article acknowledging initial publication of the figures in The Journal of Immunology.

In Fig. 3, photographs d and e represent 15-min, not 30-min time points, and photograph m represents 30 min, not 60 min. Controls for this experiment were from more than one source; therefore, the label should read “Co” not “Co1.” Photographs in Fig. 3, panels a, b, d, and e originally published in The Journal of Immunology were later duplicated as Fig. 1b, panels A, B, C, and D in the Nature Immunology article referenced in the previous paragraph.

In Fig. 4, photograph “I” (L) was from patient 2 (P2), not patient 1 (P1), and does not represent the indicated treatment. The label for the controls should be “Co” because the experiment used sources in addition to “Co1” and “Co2.” The corrected Fig. 4 with panel “I” showing the correct patient and the treatment as described in the figure legend and the corrected figure legend is shown below.

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**FIGURE 4.** Low level translocation of NFAT1 in SCID T cell lines in the presence of the nuclear export inhibitor LMB. Control (Co) T cell lines and T cell lines from the two SCID patients (P1 and P2) were left unstimulated (a–d) or were stimulated with 1 μM ionomycin for 30 min (e–m) in the presence of 0.8 mM extracellular calcium. LMB (200 nM) was added 30 min before stimulation (i–m). Cells were spun onto poly-l-lysine-coated coverslips, fixed, and stained for NFAT1. Diagrams represent the average percentage of cells with cytoplasmic, cytoplasmic plus nuclear, or nuclear immunofluorescence, corresponding to i–m. One representative experiment of three is shown.
In Fig. 5, no indication was given that bands with “smiles” were straightened in panels A and B (bottom). In both the figure and the legend, the time points in A are 70 min, not 60 min. The gel photograph in B (bottom row labeled “CaM”) was separated unnecessarily. The control labeled “Co1” in A and B should be “Co” because the samples were not from the same control. In B, the labels “P1” and “P2” designating patient sources for “CaM” were reversed.

In Results, under the heading No inherent defect of NFAT or CN in the SCID patients’ T cells, the reference to “60 min” in the last sentence of the first paragraph is incorrect. The corrected sentence should read: “Cytoplasmic extracts from patient and control lines were incubated with CN plus calmodulin for 20 and 70 min at 30°C, and NFAT1 was detected by Western blotting (Fig. 5A), revealing the same amount and kinetics of dephosphorylation in control and patient T cells.”


Erin Kelly, Angela Won, and Yosef Refaeli wish to retract Fig. 5B. The retraction involves the part of the paper claiming to show that expression of AKT in Ag-primed T cells leads to up-regulation of Bcl-2 but not cFLIP. This result was used to support the idea that Akt blocks apoptosis of Ag-primed T cells following growth factor withdrawal but not following death receptor activation. Kelly, Won, and Refaeli have no reason to believe that the other results and interpretations in this paper need to be corrected or retracted. This retraction follows an investigation by the Massachusetts Institute of Technology into scientific misconduct by Dr. Luk Van Parijs, the corresponding author of the paper, that found the retracted figure had been falsified or fabricated. The investigation also found that Dr. Van Parijs was solely responsible for the scientific misconduct that resulted in the falsified or fabricated data or conclusions in this paper.


The authors incorrectly stated that the truncated IL-17RA FRET constructs extend from amino acids 1–441 of murine IL-17RA. In fact, these truncated receptors encode residues 1–526 and also incorporate some additional amino acids before the commencement of the CFP or YFP moieties (introduced from cloning). Thus, the final amino acid sequence of the junction is IL-17RA: ... SRYP-HAY-RL ... CFP/YFP, where double underlining indicates IL-17RA sequence, single underlining indicates residues introduced from the vector, and dashed underlining indicates CFP or YFP sequence. This error does not affect any of the conclusions in the paper.


In Fig. 1 and the figure legend there are errors regarding the number of amino acids in a protein. The label “ΔC Splice Variant: 364 aa” should be “ΔC Splice Variant: 442 aa” in Fig. 1. In the legend to Fig. 1, the third sentence should read: “The truncated form lacks almost the entire COOH tail (171 aa, green, red, and short black traits) but bears an extra 18 aa (light blue trait) due to inclusion of the intron between exons 10 and 11 (94).”


In the Introduction, Materials and Methods, Discussion, Fig. 4D, and the Fig. 4 legend, all but one reference made to IRGC should be to IRGM. The sentence on page 7191, repeated on page 7194, “There has been no investigation of the human homolog of LRG-47 (IRGC) in humans” is incorrect. There are two human homologs; the one under investigation in our publication was in fact IRGM. Materials and Methods correctly describe primers to detect the transcript of IRGM,
not IRGC. Furthermore, although the statement on page 7197 “Humans have only one intact p47 GTPase (IRGC) whose expression has been reported from testis but not THP-1 cells” is correct, it has little relevance to our work because we only measured the transcript of IRGM.


The authors apologize for any confusion caused and are grateful to colleagues in the field for bringing the errors to their attention. In particular, the authors wish to acknowledge Dr. Jonathan Howard of the University of Cologne Institute for Genetics (Cologne, Germany) for pointing out these errors and for helpful discussion and advice on preparing the erratum. Overall, the findings with IRGM were modest and do not form a major part of the conclusions, particularly those concerning Cathelicidin LL-37 and its role in resistance to tuberculosis.


In Footnotes, the country listed for grant support is incorrect. The footnote should read: 1 This work was supported by grant 2005-SGR00037 from the Generalitat de Catalunya, Spain.

In Results, under the heading Participation of gray cells in cytotoxic contact reactions, “archaeocytes” is misspelled in the fourth sentence of the first paragraph. The sentence should read: “However, it is likely that this migration of archaeocytes has as a final outcome their differentiation into gray cells (Fig. 2C) rather than a direct action of their own (7).”


In Materials and Methods, under the heading Statistical analysis on page 5979, the phrases “within each individual” and “within individuals” in the last two sentences of the first paragraph are incorrect. The sentences should read: “A two-tailed paired Student’s t test was used to compare response between NK cells expressing different numbers of S-KIR. The paired Student’s t test was also used to compare percentages of KIR-expressing NK subgroups.”


There is an error in the affiliation line for the seventh author due to an incorrect symbol. The correct affiliations for Byoung S. Kwon are: *Department of Biomedicine and ‡Immunomodulation Research Center, University of Ulsan, Ulsan, South Korea.