Activation and Expression of the Nuclear Factors of Activated T Cells, NFATp and NFATc, in Human Natural Killer Cells: Regulation upon CD16 Ligand Binding

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Summary

The putative factors that couple the signal transduction from surface receptors to the activation of cytokine synthesis in natural killer (NK) cells have not been elucidated. We report here that the nuclear factor of activated T cells (NFATp), a cyclosporin A (CsA)-sensitive factor that regulates the transcription of several cytokines, mediates CD16-induced activation of cytokine genes in human NK cells. CD16 (FcγRIIIA)-induced expression of cytokine mRNA in NK cells occurs via a CsA-sensitive and Ca^{2+}-dependent mechanism. Stimulation of NK cells with CD16 ligands induces NFAT-like DNA binding activity in the nuclear extracts from these cells, as detected in electrophoretic mobility shift assays. This occurs with fast kinetics after stimulation, via a CsA-sensitive and Ca^{2+}-dependent mechanism that does not require de novo protein synthesis. NK cell NFAT is present in the cytosol of nonstimulated cells, migrates to the nucleus upon stimulation, and can associate with AP-1. Two distinct molecules, NFATp and NFATc, have been reported to mediate NFAT activity. The results of supershift assays using NFATp- and NFATc-specific antibodies indicate that NK cell activation early after CD16 ligand binding involves primarily, if not exclusively, NFATp, and Western blot analysis shows that this has the same electrophoretic mobility ($\sim$120 kD) as that of T lymphocytes. NK cells do not express NFATc constitutively, but NFATc mRNA accumulation is induced in these cells within 2 h of stimulation with CD16 ligands. However, supershift assays using the available mAb recognizing the T cell NFATc revealed no detectable NFATc protein in nuclear and cytoplasmic extracts from CD16- or phorbol ester-stimulated cells at any time tested, up to 4 h. These results provide the first direct evidence that both CsA-sensitive transcription factors, NFATp and NFATc, are expressed in human NK cells, and that their activation and/or expression can be regulated in primary cells by a single stimulus that, in the case of CD16 in NK cells, results in early activation of NFATp and subsequently induced expression of NFATc mRNA.

N K cells play an important role in the early phases of an immune response, mediating cytotoxicity against numerous target cells in the absence of previous sensitization, and producing cytokines (IFN-γ, TNF, GM-CSF, and IL-3). Both functions are triggered or enhanced upon stimulation with target cells, immune complexes, and lymphokines, each binding to different receptors (reviewed in references 1–3). The proximal intracellular events induced upon ligand binding to the transmembrane form of the low affinity receptor for

IgG (CD16, FcγRIIIA)[1] on NK cells are being elucidated (4–8), but no information is available on the distal events

and/or factors involved in the receptor-mediated regulation of cytokine genes transcription in these cells.

CD16-induced production of cytokines (e.g., IFN-γ and TNF), but not that of CD25 or other proteins involved in cytotoxicity (8-10), depends on increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) after extracellular Ca^{2+} entrance (8). In T lymphocytes, the TCR-induced increase of $[Ca^{2+}]_i$ is also a requisite for the expression of several lymphokines (reviewed in reference 11), and a pathway has been elucidated which connects the $[Ca^{2+}]_i$ increase to the regulation of cytokine transcription via activation of the cytoplasmic serine/threonine phosphatase calcineurin A and its subsequent effect on the transcription nuclear factor of activated T cells (NFAT) (11-15). NFAT is present in the cytoplasm of non-stimulated cells as a phosphoprotein, NFATp, which, dephosphorylated upon cellular stimulation, migrates into the nucleus, where it binds specific DNA sequences in the promoter/enhancer regions of cytokine genes (reviewed in references 11 and 16-20).

Originally described as a cyclosporin A (CsA)-sensitive factor binding the IL-2 promoter (21-23), NFAT also binds NFAT-specific sites in the promoters of other cytokines (IL-3, IL-4, TNF-α, and GM-CSF) (17, 19, 20, 24, 25), where it may cooperate with other factors, e.g., AP-1 (IL-2, IL-3, and GM-CSF promoters) (12, 17, 25). These observations indicate that its activity is not restricted to the regulation of the IL-2 gene. The distribution of NFAT extends beyond T_H1 lymphocytes: NFATp is transcriptionally active in B cells (26, 27), where it likely regulates expression of TNF (19, 28). At least two related but distinct NFAT proteins exist, NFATp and NFATc, that have identical binding specificity and activation requirements (29, 30), and they are expressed in several hematopoietic lineages and other tissues (11, 30). Unlike NFATp, NFATc is not constitutively expressed in most tissues and cell lines analyzed, but is inducible in lymphocytes by protein kinase C (PKC) activators and/or agents that increase $[Ca^{2+}]_i$ (30). Its distribution in hematopoietic cell subsets, as well as the physiological stimuli that induce its expression, remain to be elucidated.

We have observed that CD16-induced transcription of TNF and GM-CSF mRNA in NK cells is abrogated by CsA. This data, coupled with the reported role of NFAT in the transcriptional regulation of several cytokine genes, led us to analyze its expression and regulation in NK cells. The results presented here indicate that CD16 stimulation in human NK cells sequentially activates NFATp and induces NFATc mRNA accumulation. The former is readily activated from a preformed inactive cytoplasmic pool, whereas the latter must be synthesized de novo: both events occur in a CsA-sensitive manner and require extracellular Ca^{2+}, but not protein synthesis. These data indicate a role for NFATp and, possibly, NFATc in mediating CD16-induced expression of cytokine genes in NK cells.

Materials and Methods

Cell Preparations and Stimulation. The human Jurkat (T lymphoblastoid) and RPMI 8866 (B lymphoblastoid), the monkey kidney COS-7, and the murine mAb-producing hybrid B cell lines were maintained in culture in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 100 μg/ml glutamine. PBL were obtained after density gradient centrifugation of venous peripheral blood from healthy individuals. Homogeneous NK cell preparations (>98% $CD16^+$/$CD56^+$/$CD3^-$ and <1% $CD3^+$ cells by indirect immunofluorescence and flow cytometry) were purified from 10-d cocultures of PBL with 30 Gy irradiated RPMI 8866 B lymphoblastoid cells, as described (31). PHA blasts were obtained after 5 d culture of PBL (5 × 10^5 cells per ml culture medium) with 0.5 μg/ml PHA (PHA-M; Wellcome Diagnostics, Dartford, England).

NK cells (5 × 10^6/ml) were incubated for the indicated times with anti-CD16 mAb or CD16 ligands. The murine mAb used were 3G8 (IgG1, anti-CD16) and B159.5 (IgG1, anti-CD56) (ascitic fluid, final dilution 10^{-3}) plus 10 μg/ml goat anti-mouse Ig as a cross-linking reagent. Immune complexes (rabbit IgG-sensitized bovine erythrocytes, EA) were prepared as described (10); non-sensitized bovine E were used as controls. Phorbol-12, 13-dibutyrate (PDBu; Chemsyn Science Laboratories, Lenexa, KS) and ionomycin (Sigma) were used at a 10^{-7} M and 0.1 μg/ml final concentration, respectively. CsA (gift from Sandoz Pharmaceutics Inc., Vienna, Austria), was used at 100 ng/ml final concentration. In the experiments performed in the presence of the Ca^{2+} chelator EGTA (1 mM; Sigma), 1 mM $MgCl_2$ was added. Inhibitors of RNA (actinomycin D [Act D]; Calbiochem, San Diego, CA) and protein synthesis (emetine; Sigma) were used at a final concentrations of 10 μg/ml and 15 μM, respectively. Cells were preincubated with the inhibitors for 30 min, and stimulation was performed in their presence for the indicated times.

Northern Blot Analysis. Total cellular RNA was extracted from control and stimulated cells (10^7 per sample) using RNAzol (Biotex Laboratories, Houston, TX) (10). Northern blot analysis was performed using cDNA probes specific for human TNF, GM-CSF, and β2 microglobulin (β2M), as described (8, 9). Full-length NFATc cDNA was provided by Dr. G. Crabtree (Howard Hughes Medical Institute, Stanford University, Stanford, CA) and a 2.2-kb Eco-RI fragment (nucleotides +1 to +2,198) was used as a probe. Integrity and amount of RNA loaded per lane were visualized on ethidium bromide-stained gels, and β2M expression was used for normalization. cDNA probes were labeled with [$a^{32}P$]dCTP (specific activity = 3,000 Ci/mmol; NEN, DuPont, Boston, MA) by random hexamer priming (Random primer kit; Amersham Corp., Arlington Heights, IL). Hybridized bands were visualized on the blots exposed to X-AR films (Eastman Kodak, Rochester, NY).

Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA). Nuclear extracts were prepared from control and stimulated cells as described by McCaffrey et al. (32). Briefly, cells (20 × 10^6) were washed twice in PBS and resuspended in 400 μl ice-cold lysis buffer (Dignam buffer A [33]: 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 1 mM PMSF, 2 μg/ml each leupeptin and aprotinin). After 10 min on ice, 25 μl of 10% NP-40 was added, and the cells were vortexed and centrifuged (4°C, 30 s, 9,000 rpm). Pelleted nuclei were washed twice in buffer A and lysed in 50 μl Dignam high salt buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml each leupeptin and aprotinin) (15 min, 4°C). After lysis, nuclear extracts were centrifuged (12,000 rpm, 10 min, 4°C), and the resulting supernatants were diluted (1:1, vol:vol) with Dignam buffer D (20 mM Hepes, pH 7.9, 100 mM KCl, 0.1 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml each leupeptin and aprotinin). Protein concentration was determined using the Bradford colori-

metric method (Bio Rad Laboratories, Hercules, CA). The extracts were used immediately or after storage at −80°C. Hypotonic extracts enriched in cytoplasmic proteins were prepared according to Jain et al. (34). Briefly, cells (2 × 10⁷) were incubated (10 min on ice) in 200 μl of 7.5 mM Tris buffer, pH 7.6, containing 1 mM MgCl₂, 0.5 mM DTT, 0.1 mM EDTA, 2 mM PMSF, 20 μg/ml each leupeptin and aprotinin. They were then subjected to one freeze/thaw cycle (dry ice-cooled with 75% ethanol), centrifuged (12,000 rpm for 10 min at 4°C), and stored at −80°C until use.

EMSA reactions were performed at room temperature in a final 25-μl vol. Nuclear and, when indicated, cytoplasmic hypotonic extracts (3–7 and 7–10 μg per reaction volume, respectively) were incubated for 15 min with 1 μg polyionisine:polycitidilic acid (Boehringer Mannheim Biochemicals, Indianapolis, IN) in binding buffer (final concentrations = 8 mM Hepes, pH 7.9, 84 mM NaCl, 20 mM KCl, 0.3 mM MgCl₂, 9% glycerol, 0.04 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 0.5 μg/ml each leupeptin and aprotinin). After adding 0.5 ng ³²P-labeled probes, the reaction was continued for 15 min, stopped with 2 μl 0.1% (wt/vol) bromophenol blue, and the samples were electrophoresed on 5% polyacrylamide gels in 0.25× Tris-buffered EDTA under nondenaturing conditions. Cold probes used as competitors were added at the beginning of the reaction to identical aliquots of the extracts. The Ab used for supershift (antisera, or ascites from mAb, 1:250 final concentration) were incubated on ice for 20 min with the nuclear extracts, labeled probes were added, and the reaction continued for 15 min at room temperature. Where indicated, cognate and control peptides (0.1 μg) were preincubated for 30 min on ice with the Ab in the reaction mixture without nuclear extracts. Extracts were then added and incubated for 20 min on ice before adding the labeled probe. The anti-NFATp antisera α72 and α67.1 and their cognate and control peptides have been previously described (29, 35), and the anti-NFATc mAb 7A6 (30) was provided by Dr. G. Crabtree (Howard Hughes Medical Institute, Stanford University). The Ab used recognize both human and murine NFAT.

Double-stranded synthetic oligonucleotide DNA probes were synthesized in the Nucleic Acid Facility at the Jefferson Cancer Institute (except for the SP1 and AP-1 probes, purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA) and were end-labeled with [γ³²P]ATP (specific activity = 4,500 Ci/mmol; ICN Biomedicals Inc., Costa Mesa, CA) and T4 polynucleotide kinase (Promega Biotech, Madison, WI) according to the manufacturer's specifications. After labeling, free [γ³²P]ATP was removed by centrifugation on Microspin Sephacryl S-200 HR columns (Pharmacia Fine Chemicals, Piscataway, NJ). The sequences of the oligonucleotide probes used (5′ to 3′, one strand) are: human IL-2 distal NFAT site (NFAT huIL-2) (34), GGAGGAAAAACTGTTTCATACAGAAGG; mouse IL-4 NFAT site (NFAT mIL-4) (20, 24), ATAAAATTTTCCAATGTAAA; consensus AP-1 site (AP-1), CGCTTGATGACTCAGCCGGAA; consensus SP1 site (SP1), ATTCGATCGGTTCGGGGCGAG; human GM-CSF (GM-550, containing an NFAT-binding site) (17), TCTTATTATGACTCTTGCTTTCCTCCTTTC; human TNF (NF-κ3, containing an NFAT-binding site): CAGATGAGCTCATGGGTTTCTCCACC (19) (used in Fig. 3 A), and GAGCTCATGGGTTTCTCCACC (36) (used in Fig. 3 C). The NFAT binding sequences in the relevant probes are underlined.

SDS-PAGE and Western Blot Analysis. Cells (5 × 10⁶) were resuspended in 30 μl lysis buffer (40 mM Tris, pH 7.5, 10 mM EDTA, 60 mM NaPO₄) plus an equal volume of 10% SDS. Lysates were boiled for 20 min. SDS-PAGE (7.5% acrylamide, reducing conditions), Western blot , and enhanced chemiluminescence detection (Amersham) were done according to standard procedures (7).
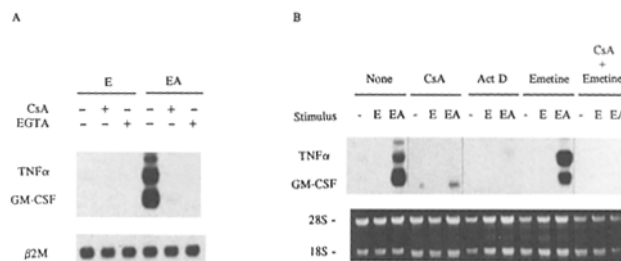

Figure 1. Effect of CsA, Ca²⁺ chelation, and RNA and protein synthesis inhibition on CD16-induced accumulation of TNF and GM-CSF mRNA. NK cells (30 × 10⁶ per sample) were incubated for 30 min in (A) medium without (−) or with (+) 100 ng/ml CsA, or 1 mM EGTA, (B) medium without (*None*) or with CsA, 10 μg/ml Act D, 15 μM emetine, or a combination of CsA and emetine, as indicated. Medium (B, −), E, or EA (0.5% suspension final concentration) was added to each of three identical aliquots from the same samples and the incubation was continued for 2 h. Cells were washed and total RNA was extracted and size fractionated in a 1% agarose denaturing gel. Northern blot analysis was performed using TNF and GM-CSF (*top*) or β2M (A, *bottom*) random primer–labeled cDNA probes, as described in Materials and Methods. Ethiduim bromide staining of the corresponding gel is shown in B.

Results

CsA Sensitivity of CD16-induced Cytokine mRNA Accumulation. TNF and GM-CSF mRNA are induced to accumulate in NK cells stimulated for 2 h with immune complexes (EA) (Fig. 1 A) or anti-CD16 mAb (reference 9 and data not shown). This effect is abolished upon chelation of ex-
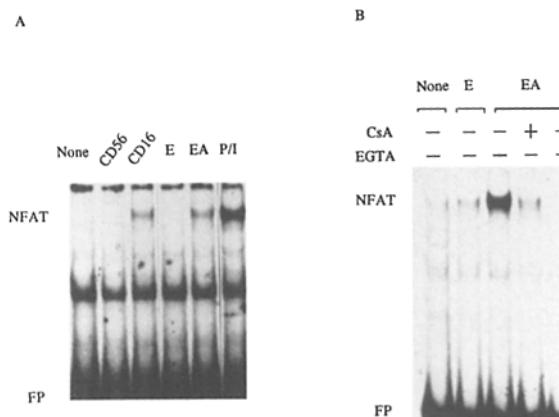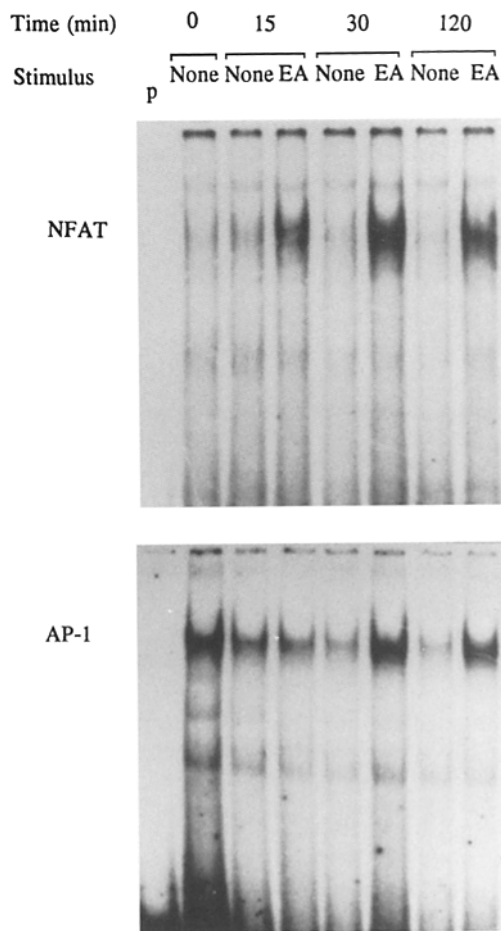

Figure 2. NFAT activation in NK cells upon CD16 stimulation. (A) NK cells (5 × 10⁶/ml, 10⁷ per sample) were incubated for 1 h in medium without (*None*) or with ascites (10⁻³ final dilution) of mAb B159.5 (CD56, control) or 3G8 (CD16), each with added 10 μg/ml goat anti–mouse as cross-linker; E or EA (each 0.5% suspension final concentration); or 10⁻⁷ M PDBu plus 0.1 μg/ml ionomycin (*P/I*), as indicated. (B) Cells were incubated for 30 min in medium without (−) or with (+) CsA or EGTA, as in Fig. 1. Medium (*None*), E, or EA were then added to each of 10⁷ cell aliquots and the incubation was continued for 1 h. Nuclear extracts were prepared and NFAT activation was assessed by EMSA (3 μg protein per lane) in 5% acrylamide nondenaturing gels using a ³²P-labeled dsDNA oligonucleotide probe corresponding to the distal NFAT site of the human IL-2 promotor (NFAT huIL-2), as described in Materials and Methods.

A

B

C
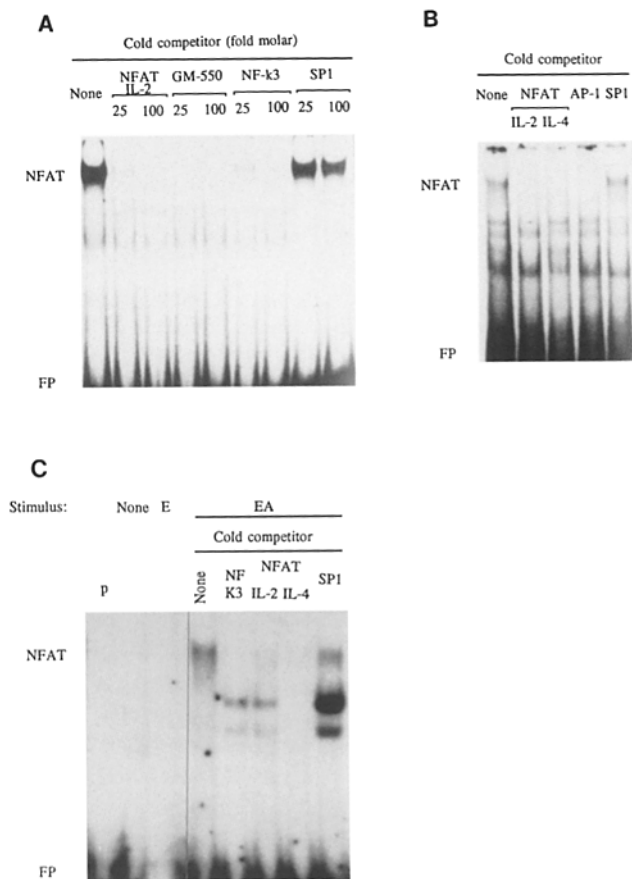
Figure 3. DNA binding specificity of NK cells NFAT. In *A* and *B*, identical aliquots of nuclear extracts (5 µg proteins per lane) from NK cells stimulated for 1 h with EA were incubated without (*None*), with 25- and 100-fold (*A*), or 80-fold molar excess (*B*) of the indicated cold dsDNA oligonucleotides before adding the labeled NFAT huIL-2 probe. (*C*) Identical aliquots of nuclear extracts (3 µg proteins per lane) prepared from NK cells cultured for 1 h in medium alone (*None*), E, or EA added, as indicated, were assayed for binding to the NFAT site in the human TNF promoter using the NF-k3 probe. The sample from EA-stimulated cells was also incubated in the absence (*None*) or presence of 100-fold molar excess of the listed cold double-stranded DNA oligonucleotides before adding the ³²P-labeled NF-κ3 probe. *NFAT*, the shift caused by binding of the factor to the probe; *FP*, the position of the unbound/free probe; *p*, probe alone. The appearance of two lower bands in the presence of both specific and non specific competitors likely represents artefactual binding of proteins in the extracts, when the majority of specific factors has been competed by the excess of the corresponding probe.
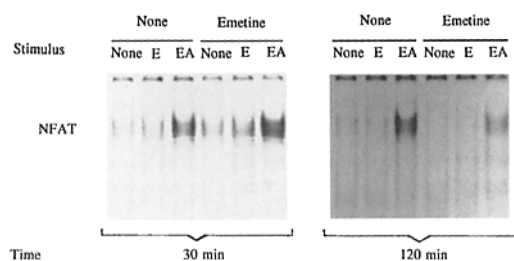

Figure 4. Kinetics of NFAT activation in CD16-stimulated human NK cells. Nuclear extracts were prepared from NK cells incubated for the indicated times in medium without (*None*) or with EA. They were analyzed by EMSA using the NFAT mIL-4 (*top panel*) and the AP-1 probe (*bottom panel*). The shifts due to NFAT and AP-1 binding to the probes are indicated.

tracellular Ca²⁺, in agreement with our previous data (8), or pretreatment of the cells with CsA. Accumulation of GM-CSF mRNA induced upon CD16 stimulation, like that of TNF (8), depends on induced gene transcription and is inhibited in cells pretreated with Act D (Fig. 1 *B*). Pretreatment of the cells with protein synthesis inhibitors resulted in overexpression of CD16-induced TNF and only minimal inhibition of GM-CSF mRNA accumulation under conditions in which the inhibitory effect of CsA was still evident. These results indicate that CD16-induced transcription of these genes involves activation of a CsA-sensitive, Ca²⁺-dependent pathway using elements constitutively present in NK cells.

CD16-induced Activation of NFAT. To determine whether NFAT is expressed in NK cells and is activated upon CD16 stimulation, we performed EMSA with nuclear extracts from CD16-stimulated cells and oligonucleotide probes corresponding to the conserved NFAT distal binding sequences in the human IL-2, the murine IL-4, and the human TNF and GM-CSF promoters. Binding of NFAT to the first probe depends on its association with AP-1 (34, 37), whereas that to the IL-4 probe is independent of other factors (20). Stimulation of NK cells with anti-CD16 mAb, immune complexes, or PDBu/ionomycin for 1 h activated NFAT, detected with the NFAT huIL-2 probe (Fig. 2 *A*). Immune complex– (Fig. 2 *B*) and anti-CD16 mAb– (not shown) induced activation was prevented chelating extracellular Ca²⁺ with EGTA or pretreating the cells with CsA.

The complex activated by CD16 was efficiently and specifically competed (Fig. 3 *A*) by the NFAT huIL-2, by each of two other cold oligonucleotides containing NFAT binding

Figure 5. Effect of protein synthesis inhibition on CD16-induced NFAT activation. NK cells (6 × 10⁷ per sample) were incubated without (*None*) or with emetine for 30 min, after which identical 10⁷ cell aliquots were incubated for the indicated times in medium without (*None*) or with E or EA, as described in Fig. 2. Nuclear extracts were prepared and analyzed by EMSA with the NFAT mIL-4 probe. The shift resulting from NFAT is indicated.

sequences in the promoters of cytokines produced by NK cells (GM-550 from the GM-CSF/IL-3 enhancer [17] and NF-κ3 from the TNF promoter [19]), but not by SP1 (containing an unrelated sequence), and by excess cold oligonucleotides corresponding to an additional NFAT binding sequence (NFAT mIL-4) or to the AP-1 binding sequence (Fig. 3 *B*). In EMSA performed with the labeled NF-κ3 oligonucleotide (Fig. 3 *C*), a shift was detected that was inhibited by all cold oligonucleotides used that contain NFAT binding sites (NF-κ3, NFAT huIL-2, and mIL-4), but not by SP1. Similar results were obtained with the labeled GM-550 oligonucleotide (not shown). Thus, CD16-induced transcription of TNF and GM-CSF correlates with activation of NFAT, the NFAT expressed in NK and T cells have identical DNA binding specificity, and DNA binding activity of both is enhanced upon association with AP-1.

Protein Synthesis Requirement for CD16-induced NFAT Activation. NFAT activation was detected with the NFAT mIL-4 (Fig. 4, *top*) and the NFAT huIL-2, AP-1-dependent, probe (not shown) within 15 min stimulation of CD16, and it was sustained up to 2 h. AP-1 in nuclear extracts from CD16-stimulated NK cells contributed to the observed NFAT binding to the NFAT huIL-2 probe (Fig. 3 *B*); therefore, we analyzed the in vitro AP-1 activity in the same samples. In nuclear extracts from the NK cells used here, AP-1 was detectable by EMSA before addition of any stimulus (time 0). CD16 stimulation induced AP-1 activity higher than background within 30 min and sustained up to 2 h (Fig. 4, *bottom*). These data indicate that NFAT activation is an early step in the CD16 activation pathway and involves two components detectable in vitro: activation of NFAT on its own and increased AP-1 activity.

To test the hypothesis that the factor(s) involved in cytokine gene transcription regulated by CD16 stimulation is (are) directly activated from a preformed pool, we analyzed CD16-induced NFAT activation in the absence of de novo protein synthesis. NFAT activation in NK cells pretreated with emetine (Fig. 5) was sustained throughout the 2-h stimulation with FcγRIII ligands, as assessed in EMSA using the AP-1-independent NFAT mIL-4 probe. NFAT activity detected
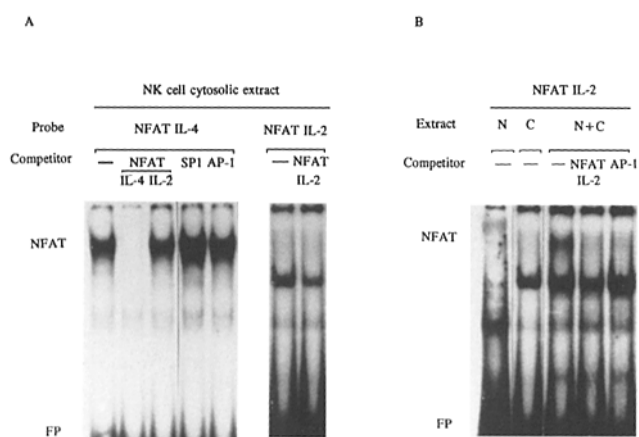

Figure 6. Detection of NFAT in cytoplasmic extracts of nonstimulated NK cells. (*A*) Identical aliquots of cytoplasmic extracts from nonstimulated NK cells (7 μg per sample) were analyzed by EMSA with NFAT mIL-4 and NFAT huIL-2 oligonucleotide probes, as indicated. The specificity of the shift was assessed for each probe by competition with a 100-fold molar excess of NFAT (huIL-2 and mIL-4) cold probes, cold SP1, and AP-1, as indicated; –, no cold oligonucleotide added. (*B*) EMSA were performed with nuclear extracts (*N*, 3 μg per lane) from nonstimulated cells, cytoplasmic extracts (*C*, 7 μg per lane) from the same cells, or a mixture of both (*N+C*). Identical aliquots of the mixture of cytoplasmic and nuclear extracts were incubated without (–) or with a 100-fold molar excess NFAT huIL-2 or AP-1 cold oligonucleotides, as indicated, to assess DNA binding specificity and AP-1 dependence. The shift resulting from NFAT binding to the probes and the position of free probes are indicated.

within 30 min was not affected by the inhibitor, but it was reproducibly decreased compared to controls after 2-h stimulation in the presence of emetine, possibly reflecting the disappearance and lack of replacement of the endogenous NFAT pool in the absence of protein synthesis.
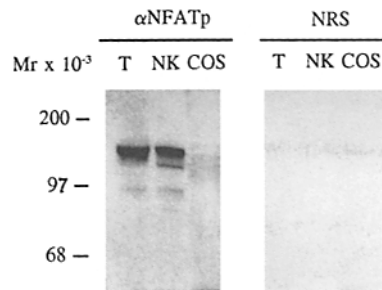
Expression of Cytoplasmic NFAT in Nonstimulated NK Cells. To determine directly whether CD16 stimulation activates a preformed NFAT pool in nonstimulated NK cells and induces its translocation to the nucleus, we analyzed the presence of NFAT in cytosolic extracts of nonstimulated NK cells. The NFAT mIL-4 probe detected NFAT in cytoplasmic extracts from these cells (Fig. 6 *A*), and DNA binding was inhibited by an excess of the same cold probe, but not by the AP-1-dependent NFAT huIL-2 or the AP-1 oligonucleotides. As expected, cytoplasmic NFAT was not detectable using the NFAT huIL-2 probe (Fig. 6 *A*, *right panel*). A band with mobility faster than that of the NFAT complex was observed with this probe in the cytoplasmic extracts, was not competed with excess cold probe and is likely caused by nonspecific binding. NFAT binding to the NFAT huIL-2 probe was reconstituted (Fig. 6 *B*, lane *N+C*) when the cytoplasmic extracts (lane *C*) were combined with nuclear extracts (lane *N*), containing AP-1 (Fig. 2 and data not shown) but not NFAT (Fig. 6 *B*, lane *N*), prepared from the same nonstimulated NK cells. Both cold AP-1 and NFAT huIL-2 oligonucleotides, but not unrelated oligonucleotides (not shown), efficiently inhibited NFAT binding to the probe.

NFATp and NFATc Expression and Activation in NK Cells.
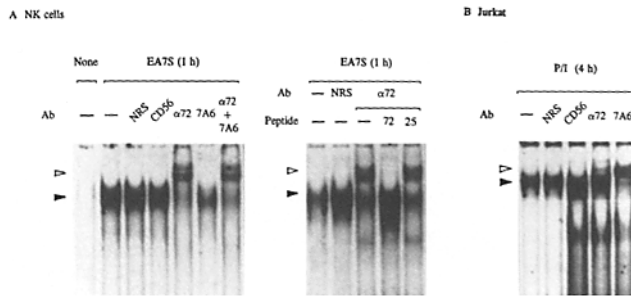
Figure 7. Identification of NFATp in NK cells. (A) Nuclear extracts from NK cells, incubated in medium without (None) or with EA for 1 h, were analyzed for the presence of NFATp and NFATc using specific antibodies and EMSA in supershift assays. Before adding the labeled NFAT mIL-4 probe, identical aliquots of the samples (3 μg per lane) were incubated for 15 min without (−) or with NRS, mAb B159.5, IgG1 (CD56), anti-NFATp rabbit antiserum (α72), anti-NFATc mAb, IgG1 (7A6), or anti-NFATp plus anti-NFATc antibodies (α72 + 7A6). In the right panel, NFATp α72 antiserum, preincubated (30 min, 4°C) in medium alone (−), with its cognate peptide (p72) or an irrelevant peptide (p25), was added to identical aliquots (3 μg per lane) of a nuclear extract from 1-h EA-stimulated NK cells, and the samples were analyzed by EMSA and supershift as described in Materials and Methods. The first two lanes are aliquots from the same nuclear extract preincubated with no antiserum (−) or with a NRS, respectively, and no peptide. (B) Identical aliquots (3 μg per lane) of a nuclear extract from 2 × 10⁷ 4-h PDBu plus ionomycin (P/I)-stimulated Jurkat cells were pretreated with each of the same antibodies as in A and subjected to EMSA. Antibodies were used at a final dilution of 1/250 from the original serum (rabbit) or ascitic fluid (mouse); peptides were used at 4 μg/ml final concentrations. NFAT binding to the NFAT mIL-4 probe and the supershift induced in the presence of the Ab are indicated with closed and open arrowheads, respectively.


Figure 8. Biochemical characteristics of NK cell NFATp. Whole-cell lysates from PHA blasts (T), NK, or COS-7 cells were resolved in SDS-PAGE (7.5% acrylamide, reducing conditions). Western blot analysis was performed with the anti-NFATp rabbit antiserum α67.1 (*left panel*, αNFATp) and a nonimmune rabbit serum (*right panel*, NRS) as control, followed by ECL detection, as described in Materials and Methods. The T and NK cell samples were from 2 × 10⁶ cells, the COS cell sample was from 10⁶ cells; antisera were used at a 2 × 10⁻³ final dilution. Positions of molecular size markers are indicated.

Two related but distinct proteins, NFATp and NFATc, mediate NFAT binding activity (29, 30). To analyze the relative contribution of these elements to the NFAT activity in CD16-stimulated NK cells, we used an antiserum against the murine NFATp peptide 72 (amino acids 206–232) (29), as well as an mAb to the human NFATc (mAb 7A6) (30) in supershift experiments. Most of the NFAT complex detected with the NFAT mIL-4 probe in nuclear extracts from 1-h CD16-stimulated NK cells was supershifted by the anti-NFATp antibody (Fig. 7 A). Similar results were obtained using anti-NFATp antiserum α67.1 (35) and nuclear extracts from 30-min and 2-h CD16-stimulated NK cells (not shown). An irrelevant antiserum (nonimmune rabbit serum [NRS]) and an isotype-matched anti-CD56 mAb did not affect the relative mobility of the complex, and none of the antibodies bound the DNA probe alone (not shown). The supershift induced by the anti-NFATp antibody was specifically prevented by the cognate peptide 72, but not by a different peptide (peptide 25, amino acids 685–703 in the murine NFATp) (29). The anti-NFATc mAb 7A6 induced no detectable supershift (Fig. 7 A), nor did it increase the amount of supershifted complex when combined with the α72 Ab. Consistent with these results, NFATc was detected neither in nuclear extracts from short-term stimulated cells nor in cytosolic preparations of nonstimulated or 4-h stimulated NK cells (not shown). On the contrary (Fig.

7 B), the anti-NFATc mAb efficiently supershifted the NFAT complex in nuclear extracts from Jurkat cells or from PHA lymphoblasts (not shown) stimulated for 4 h with PDBu/ionomycin, conditions in which NFATc is induced and activated in these cells (30). Only a small portion of the complex was supershifted by the anti-NFATp antibody in the Jurkat T cell line. These results confirm that the antibodies do not detect cross-reactive determinants in the two NFATp.

SDS-PAGE of total lysates from non stimulated NK cells followed by Western blot analysis with the anti-NFATp antiserum α67.1 resolved a specific prominent band of ∼120 kD (Fig. 8), similar in size and relative density to that detected in PHA blast lysates, and corresponding to the phosphorylated (cytoplasmic) form of NFATp (35). Bands with lower relative molecular weight detected in both the NK and T cell lysates likely represent partially dephosphorylated NFATp and/or their degradation products.

Consistent with the results from EMSA, NFATc mRNA was undetectable in nonstimulated NK cells (Fig. 9), but it accumulated at levels significantly higher than background within 2-h stimulation with CD16 ligands. Induction of its expression was, as previously reported (30), CsA-sensitive, and it did not depend on de novo protein synthesis, but depended on RNA synthesis.

To determine whether newly synthesized NFATc contributes to the NFAT activity observed at later times after CD16 stimulation, we analyzed its presence in cytoplasmic and nuclear extracts from NK cells by EMSA. NFATc protein was undetectable in nonstimulated cells and in cells stimulated up to 4 h with immune complexes or a combination of phorbol esters and a Ca^{2+} ionophore. The anti-NFATc Ab induced supershift when added to nuclear extracts from phorbol ester and ionomycin-treated PHA lymphoblasts and Jurkat T cells, but not when added to NK cell extracts (not shown).
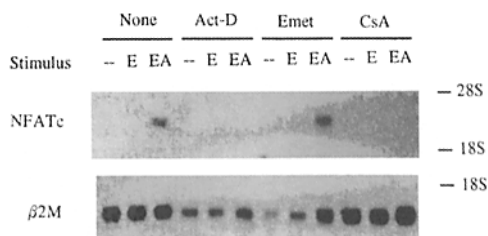
Figure 9. Expression of NFATc mRNA in CD16-stimulated NK cells. NK cells, 4 × 10⁷ per sample, were incubated for 30 min in medium without (*None*) or with Act D, emetine, and CsA, as described in Fig. 1. Medium (−), E, or EA was added to identical 10⁷ cell aliquots of the samples and incubation was continued for 2 h. Total RNA was then extracted, run in 1% agarose denaturing gel, and Northern blot analysis was performed using a human NFATc random primer-labeled cDNA probe (*top panel*). After stripping, the same blot was hybridized to the β2M cDNA (*bottom panel*) to control amounts of RNA loaded per lane. Positions of 18 and 28 S mRNA are indicated.

Discussion

The results presented here demonstrate that NFAT is expressed in NK cells. Stimulation of these cells with immune complexes not only results in activation and nuclear translocation of a preexisting cytoplasmic NFATp component identical to that present in T cells, but also induces de novo expression of NFATc mRNA, and the former NFAT is functionally active in regulating cytokine gene expression. The use of homogeneous NK cell preparations with no detectable T cell contaminants and of stimuli that do not activate T lymphocytes (10) exclude the participation of T cells in the observed effects.

FcγRIIIA ligands and cytokines, e.g., IL-2 and IL-12, induce transcription of cytokine genes in NK cells. The IL-2– and IL-12–induced transcription is Ca²⁺ independent (8); that induced upon FcγR stimulation is Ca²⁺ dependent (8). Our results demonstrate that TNF and GM-CSF (like IFN-γ and c-*myc*, not shown) expression is induced upon CD16 stimulation in a CsA-sensitive fashion, independent of protein synthesis, indicating that the effect of both CD16 stimulation and CsA occur at the level of preexisting elements in NK cells. These data add to previous observations indicating a similarity at the molecular and signal transduction levels between CD16 and the TCR and sIg complexes on T and B lymphocytes (38, 39), where the Ca²⁺-dependent, CsA-sensitive factor NFAT is involved in antigen receptor–mediated gene transcription. In T cells NFAT regulates transcription of IL-2, IL-3, IL-4, GM-CSF, and TNF genes (11, 16, 17, 19, 20, 40). Among these genes, at least TNF appears to be similarly regulated in B cells, where NFAT is transcriptionally active (26–28, 41).

In NK cells, ligation of FcγRIII with specific mAb or Ag–Ab complexes, its physiologic ligand, activates a nuclear factor that has the same DNA binding specificity as NFAT; this activation, like that of the CD16-induced cytokines, is CsA sensitive and Ca²⁺ dependent. Instead, Ca²⁺-independent signals transduced by cytokines (IL-2) that induce TNF and GM-CSF expression do not mediate NFAT activation

(not shown), suggesting that CD16 specifically engages Ca²⁺-dependent, CsA-sensitive elements. Nuclear extracts of CD16-stimulated NK cells contain protein(s) that bind specifically NFAT binding sequences present in the enhancer regions of the promoters of several cytokines produced by NK cells, e.g., TNF, GM-CSF, and IL-3 (16, 17, 19), and these same sequences compete specifically, and as efficiently as those contained in the IL-2 and IL-4 promoters, for NFAT binding to the distal NFAT IL-2 binding site present in the oligonucleotides used in the EMSA. This indicates that NK cell NFAT has DNA binding specificity identical to that of its T and B cell counterparts (11, 26, 27, 42), and it supports a critical role for NFAT in NK cell transcriptional regulation of these cytokines. The same data, however, do not exclude the participation of other as yet not identified components in the transcriptional activation of cytokine genes in NK cells, analogous to the necessary, but not sufficient, role of NFAT in the transcriptional activation of the IL-2 gene promoter in T cells (43).

NK cells NFAT can associate with AP-1, as established using the huIL-2 NFAT site. NFAT binding to this element requires a nuclear component newly synthesized in a PKC-dependent fashion (38, 44, 45). This component contains AP-1, which is formed by various combinations of *jun* or *jun–fos* polypeptides (45, 46). NFATp–AP-1 interaction results in increased NFATp DNA binding and transcriptional capacity at the recognition sites in the IL-2 promoter and in at least one NFAT site (CLEO) of the GM-CSF promoter (11, 25). AP-1 oligonucleotides inhibited binding of NK cell NFATp to the NFAT huIL-2 probe, proving that the factor can associate with AP-1. In the NK cells used here, EMSA revealed significant levels of AP-1 activity, and FcγRIII stimulation increased/maintained this activity for prolonged periods of time. Northern blot analysis revealed expression of c-*jun* and *jun*-D but not *jun*-B or c-*fos* before stimulation (not shown), suggesting that complexes containing c-*jun* and *jun*-D, known to compose AP-1 (45), are preformed in these cells and contribute to the constitutive AP-1 activity. CD16 stimulation also induces c-*fos* mRNA accumulation within 20 min (not shown), which may contribute to the sustained AP-1 levels observed after stimulation. Inhibition of AP-1 synthesis and/or lack of replacement of NFATp with newly synthesized protein might account, at least in part, for the decreased NFAT levels at later times after stimulation in the absence of protein synthesis. Kinetics of induction of the AP-1–dependent NFAT activity in freshly purified NK cells, however, may not correspond to that reported here, where constitutive AP-1 activity likely reflects previous activation of the cells in the culture system used to obtain large numbers of polyclonal NK cell populations. Although phenotypically and functionally equivalent to NK cells freshly separated from peripheral blood (31), the short-term cultured NK cells revert to a resting state after activation/proliferation in culture, and they respond to several stimuli proliferating and/or producing cytokines with faster kinetics than resting, non–previously activated cells (31, 47).

Cytoplasmic extracts from nonstimulated NK cells contain NFAT, but not AP-1 or AP-1–dependent NFAT activity,

as expected since AP-1 is composed of nuclear proteins. Absence of AP-1 in these extracts also serves to exclude possible artefacts due to contamination with nuclear material. NFAT was detected in cytosolic extracts of NK cells using the mIL-4 NFAT probe, to which it can bind in the absence of additional factors. AP-1–dependent NFAT activity detected with the huIL-2 probe was reconstituted when nuclear extracts from nonstimulated cells containing no NFAT activity but expressing AP-1 were combined with cytoplasmic preparations from the same cells, indicating association of cytoplasmic NFAT with nuclear AP-1, and supporting the hypothesis that NFAT activation in NK cells results in its translocation to the nucleus, as reported in T lymphocytes. These data indicate that cytoplasmic NFATp is preexistent in NK cells, as confirmed by its identification in nonstimulated cells as a protein with antigenic and biochemical characteristics indistinguishable from those of the cytoplasmic, phosphorylated form of NFATp (35).

NFATp belongs to a family of factors that include NFATc (30) and the recently cloned NFATx (47), all sharing significant homology, identical DNA binding specificity, and capable of associating with AP-1 (29, 30, 47). NFATx mRNA is expressed preferentially in the thymus and at very low levels in peripheral leukocytes. NFATc expression is inducible by PKC activators in human T cell lines, as well as splenic and thymic cells (30). NFATc mRNA is undetectable in nonstimulated NK cells, but is induced in a CsA-sensitive fashion after a 2-h stimulation with FcγR ligands (Fig. 9) or phorbol esters (not shown). These data for primary NK cells extend results reported using T cell lines. Expression of NFATc mRNA is detectable in emetine-treated cells, but not in the absence of de novo RNA synthesis, suggesting that CD16-induced NFATc expression occurs via a mechanism that includes direct or indirect induction of gene transcription, independent from newly synthesized proteins.

Despite the fact that NFATc mRNA was inducible in NK cells, we did not detect NFATc protein, and the NFATc-specific mAb did not induce supershift in nuclear (Fig. 7) or cytosolic (not shown) extracts from 1-h stimulated NK cells. However, the same mAb supershifted the NFAT detected in the Jurkat cell line and in phorbol ester– and ionomycin-stimulated PHA lymphoblasts (not shown), where the combination of both anti-NFATp and anti-NFATc antibodies supershifted virtually all the complex. Several possibilities may account for the inability of the anti-NFATc mAb to detect this molecule in NK cells. Based on the observations that NFATc isoforms exist (Crabtree, G., personal communication) and may be differentially expressed in distinct hematopoietic cell lineages, and that the supershift induced by the anti-NFATp antibodies

is incomplete, the most likely explanation for these results is that an NFATc isoform or NFAT species different from that detected by the mAb used here is/are expressed in NK cells. Other possibilities include that the epitope detected by the antibody is masked by a protein(s) absent in T cells but present in NK cells, or that the NFATc mRNA transcript induced in NK cells is nonproductive. However, both possibilities are unlikely because (a) the anti-NFATc mAb supershifted the same amount of NFATc in Jurkat cell extracts when tested either separately or mixed with an excess nuclear extract from CD16-stimulated NK cells, where NFATc was undetectable (not shown); and (b) the size of the NFATc mRNA in NK cells is identical to that in T lymphocytes and corresponds to that reported for a productive transcript (30).

Our results indicate that NFATp is the most relevant, if not the only, protein included in the NFAT complexes detected in NK cells at early times after CD16 stimulation. The relative amount of NFATp supershifted varied (60–90%) in experiments repeated with individual nuclear extracts, suggesting that interexperimental variability may account, in part, for the incomplete supershift. However, the presence of residual NFAT not supershifted by the available anti-NFATp and -NFATc antibodies may reflect the participation of additional factors (e.g., NFATx or new family members) with DNA binding specificity overlapping with that of NFATp. Our data support the conclusion that CD16 stimulation in NK cells induces NFATp activation that likely promotes, in a CsA-sensitive and possibly calcineurin A–dependent fashion, translocation to the nucleus of a preexistent NFATp, identical to that predominant in T cells, although formal proof of the latter awaits nucleotide sequence comparison. They confirm that the NFATp cytoplasmic component is not specifically and exclusively expressed in T lymphocytes, and they serve to further generalize this conclusion to all lymphoid and, possibly, most hematopoietic cell types. In this regard, it will be interesting to determine whether Ab-independent target cell recognition by NK cells, which results in Ca^{2+}-dependent activation of cytokine genes via a mechanism at least in part distinct from that induced via FcγRIIIA (49, 50), can activate NFATp, and which physiologic stimuli are involved in NFAT activation in antigen-presenting cells, e. g., monocytes (11). The significance of NFATc mRNA induction in NK cells, and the possibility that new species of the NFAT family are expressed in these cells, awaits further study. Generation of additional antibodies to NFATp and NFATc isoforms and/or, possibly, other NFAT species will help to address the question of the relative contribution of NFAT members to gene expression, not only in NK cells, but also in other immune cell types.

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