

Selective expression of nuclear factor of activated T cells 2/c1 in human basophils: Evidence for involvement in IgE-mediated IL-4 generation

John T. Schroeder, PhD, Katsushi Miura, MD, Hyun-Hee Kim, MD, PhD,
Aytul Sin, MD, Antonella Cianferoni, MD, and Vincenzo Casolaro, MD, PhD
Baltimore, Md

Background: The nuclear factor of activated T cells (NFAT) family of transcription factors plays a key role in rapidly inducing *IL4* gene expression in effector T cells.

Objective: Because human basophils secrete high levels of IL-4, we have examined whether specific NFAT species are expressed in these cells and whether FcεRI-mediated activation affects their subcellular localization and transcriptional function.

Methods: Intracellular NFAT protein was identified by using 2-color flow cytometry; gene expression was done with RT-PCR. Subcellular localization of NFAT was assessed by means of Western blotting. Electrophoretic mobility shift assays assessed NFAT involvement in IL-4 transcription.

Results: Basophils constitutively expressed high levels of NFAT2. In contrast, NFAT1 (NFATp), which is found in most leukocytes, was not seen in basophils. Low-level staining for NFAT4 was detected but was variably expressed among donor cells. Likewise, NFAT2 mRNA was constitutively expressed in basophils, and message for NFAT4 was seen in 3 of 5 preparations, whereas that for NFAT1 was found in only 1 of 5 preparations. NFAT2 protein accumulated in the nuclei of basophils activated for 1 hour with anti-IgE, and this was inhibited with the addition of FK506. A protein-DNA complex was formed with nuclear lysates from basophils and an IL-4 promoter NFAT consensus probe, with greater binding intensities detected in lysates of activated cells. An antibody to NFAT2 reduced the formation of the complex, whereas no effects were seen with antibodies to NFAT1, NFAT4, or unrelated transcription factors.

Conclusions: The selective and specific expression of NFAT2 in basophils is unique among leukocytes. This transcription factor also appears to play a critical role in the FcεRI-mediated production of IL-4 in these cells. (*J Allergy Clin Immunol* 2002;109:507-13.)

Key words: Human, basophil, transcription factor, IgE, cytokine, FcεRI, leukocyte

Abbreviations used

NFAT: Nuclear factor of activated T cells

NF-κB: Nuclear factor κB

PBS-S: PBS containing 0.1% saponin

Crosslinking of the high-affinity receptor for IgE (FcεRI) leads to the release of potent inflammatory mediators, such as histamine and leukotrienes. This mode of activation also produces signals that culminate in the generation and secretion of cytokines previously associated only with activated T_H2 lymphocytes.¹ In human basophils there is evidence that both IL-4 and IL-13 are transcriptionally upregulated because mRNA and protein for these cytokines are generated after activation with either anti-IgE antibody or antigen.²⁻⁵ In fact, the frequency of basophils staining for intracellular IL-4 after antigen stimulation has been estimated to be 10-fold greater than the number of antigen-specific T lymphocytes making this cytokine.⁶ Likewise, basophils were shown to secrete up to 10-fold larger quantities of IL-4 in response to calcium ionophore stimulation than T cells stimulated with the combination of phorbol ester and ionophore.⁷ Given the potent biologic activities of IL-4 (and IL-13), the role of basophils in the pathogenesis of allergic disease takes on a new dimension.

The role of calcium influx in cytokine generation has long been recognized, and mounting evidence has linked the cytosolic increases of this ion to intracellular signals involving the activation of calcineurin A (a serine-threonine phosphatase) and its effects on the nuclear factor of activated T cells (NFAT) family of transcription factors. Presently, there are 4 well-characterized members that constitute this family, including NFAT1 (NFATc2), NFAT2 (NFATc1), NFAT3, and NFAT4 (NFATc3).⁸ A fifth member, NFAT5, has been identified, but less is currently known about this protein.⁹ Whereas NFAT3 expression is generally limited to tissue outside of the immune system, such as testis, heart, and muscle cells, NFAT1, NFAT2, and NFAT4 are expressed in many cell types within the immune system. These factors are known to influence directly, and in concert with other nuclear factors, the transcription of many cytokine genes in T cells, including *IL2*, *TNFα*, *IL3*, *GMCSF*, *IL4*, *IL5*,

From The Johns Hopkins Asthma and Allergy Center, Division of Clinical Immunology, Baltimore.

Supported by grants AI-42221, AI-41463, and AI-07290 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Received for publication August 22, 2001; revised December 18, 2001; accepted for publication December 19, 2001.

Reprint requests: John T. Schroeder, PhD, Unit Office 2, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224.

Copyright © 2002 by Mosby, Inc.

0091-6749/2002 \$35.00 + 0 1/83/122460

doi:10.1067/mai.2002.122460

IFN γ , and *IL13*.¹⁰ In resting lymphocytes NFAT exists as an inactive cytosolic protein that becomes activated and translocates to the nucleus on dephosphorylation catalyzed with calcineurin. Both cyclosporin A and FK506 prevent its nuclear localization by blocking calcineurin-dependent phosphatase activity. Although many studies have additionally identified NFAT-dependent cytokine gene expression in cells other than T cells, including B cells,¹¹ hematopoietic cells,¹² and mast cells,^{13,14} these have been limited primarily to rodent models. Less is known of the role NFAT molecules have in cytokine gene expression in human immune cells, although these transcription factors have been identified in human T cells, thymocytes, natural killer cells,¹⁵ and eosinophils.¹⁶

Previous studies in our laboratory indicate that changes in cytosolic calcium are central to the generation of IL-4 in human basophils.¹⁷ Given the role that NFAT family members have in calcium-dependent transcription of various cytokines in T lymphocytes, particularly IL-4, we have tested for the expression and regulation of these transcription factors in human basophils. Our results show a pattern of NFAT expression that is very unique from that described in other human immune cells. We show that NFAT2 is the predominant species found in resting basophils and that increased immunoreactivity for this protein is found in the nucleus 1 hour after Fc ϵ RI-mediated activation—a time that is consistent with the generation of IL-4. Unlike human T cells and eosinophils, protein and mRNA for the most common NFAT member, NFAT1, could not be identified in resting or activated basophils. In contrast, NFAT4 protein was variably expressed and at much lower levels than those seen for NFAT2. Finally, we show evidence for the involvement of NFAT2 in the generation of IL-4 in basophils.

METHODS

Cell preparation

Venipuncture was performed on nonmedicating consenting adults (age range, 21-55 years) by using guidelines approved by the Johns Hopkins University Institutional Review Board. Although most subjects were free of allergic disease, a few were known to react either to ragweed, dust mite, cat, or a combination of these allergens. Mixed leukocyte suspensions (10%-30% basophils) were rapidly prepared by using a double Percoll (1.072/1.082 g/mL) density centrifugation technique, as previously described.¹⁷ For experiments with Western blotting, RT-PCR, and shift assays, basophils were additionally purified (to >98%) from leukopheresis packs by using the combination of density centrifugation and sorting by means of negative selection (StemCell Technologies Inc, Vancouver, Canada). Briefly, the basophil-enriched suspensions were incubated on ice with a cocktail of IgG₁ mAbs to human leukocyte proteins (CD2, CD3, CD14, CD16, CD24, CD34, CD36, CD45RA, CD56, and glycophorin A). After a 30-minute incubation, magnetic colloid microbeads were added to the cells for an additional 30 minutes. The cell-microbead suspension was then added to a cell-separation system (Miltenyi Corp, Auburn, Calif) consisting of an LS column (primed with column buffer, 10% FCS) inserted into a MidiMACS magnet. Basophils were collected by means of gravity filtration into an iced tube, washed, and counted. Basophil purity and number were determined by using a Spiers-Levy chamber with Alcian blue staining.¹⁸

Detection of intracellular NFAT with 2-color flow cytometry

The expression of intracellular NFAT was determined by using a modification of a 2-color flow cytometric assay for detecting intracellular cytokines.¹⁹ Briefly, basophil-enriched cell suspensions rapidly prepared on double Percoll gradients were washed once with cold PBS and then fixed in 4% buffered paraformaldehyde for 5 minutes at room temperature. After fixing, the cells were washed once with PBS and 0.1% BSA, resuspended in PBS and 10% dimethyl sulfoxide, and frozen at -80°C. For immunostaining, cells were thawed, washed once with cold PBS, and then incubated for 1 hour on ice in PBS containing 0.1% saponin (PBS-S), along with 5% nonfat dry milk and 1 mg/mL human IgG (up to 1×10^6 total cells per 0.05 mL) in 1.5-mL polypropylene microcentrifuge tubes. Without washing, the cell suspensions were placed in aliquots in additional tubes, and an equal volume of PBS-S, 5% milk containing a 1:25 dilution of goat anti-human IgE-FITC (Kirkegaard and Perry Gaithersburg, Md), and monoclonal anti-NFAT1, NFAT2, or NFAT4 was added. Anti-NFAT1 (Transduction Laboratories, Lexington, Ken), anti-NFATc1 (NFAT2; ABR, Golden, Colo), and anti-NFATc3 (NFAT4; Santa Cruz, Santa Cruz, Calif) were all used at a final concentration of 2 μ g/mL. An isotype control (mouse IgG; Coulter, Miami, Fla) was included in all experiments. After 30 minutes on ice, the cells were washed once with 0.5 mL of PBS-S before adding anti-mouse IgG-phycoerythrin (Biosource, Camarillo, Calif) used at 1:100. After 30 minutes' incubation, the cells were washed 3 times with PBS-S. Two-color flow cytometry was done with a FACSCALIBER machine (Becton Dickinson Immunocytometry Systems, San Jose, Calif), with FL1 detecting IgE-bearing basophils and FL2 detecting NFAT staining.

RT-PCR

Basophils were purified to greater than 98%, as described above. Approximately 1 to 2×10^6 cells were pelleted in autoclaved (RNase-free) 1.5-mL polypropylene microcentrifuge tubes in 0.25 mL of Iscove's modified Dulbecco's medium containing 5% heat-inactivated FCS, essential amino acids, and 5 μ g/mL gentamicin (C-Iscove's modified Dulbecco's medium). Total RNA was extracted by using the RNeasy protocol (Tel-test Inc, Friendswood, Tex). After isopropanol precipitation, the RNA was washed with 70% ethanol, semidried under a vacuum, resuspended in 25 μ L of diethylpyrocatechol-treated water, and stored at -80°C. RT-PCR was performed as previously described⁷ with forward and reverse primer sequences published elsewhere for detection of NFAT1, NFAT2, and NFAT4 in eosinophils.¹⁶ Resolution of the cDNA products after amplification was done in 3% agarose gels. Imaging was performed with an Electrophoresis Documentation and Analysis System 290 (Kodak Scientific Imaging Systems, New Haven, Conn).

Western blot analysis

Ultrapure (>98%) basophils were incubated in 1.5-mL microcentrifuge tubes by using culture conditions originally described elsewhere.¹⁷ Briefly, cells (5×10^6 /condition) were stimulated for the indicated times in C-Iscove's modified Dulbecco's medium and in medium containing either anti-IgE antibody (25 ng/mL) or ionomycin (500 ng/mL). In some instances FK506 was also added as indicated. Cytosolic and nuclear extracts from equal numbers of cells were prepared by using the commercially available NE-PER kit (Pierce, Rockford, Ill), according to the manufacturer's protocol. Extracts were diluted with an equal volume of 2 $\times$ loading buffer (125 mmol/L Tris-HCL [pH 6.8], 4% SDS, 0.005% bromophenol blue, and 20% glycerol; NOVEX, San Diego, Calif) containing 5% 2-mercaptoethanol and applied to the wells of 10% Tris glycine gels (NOVEX). After electrophoresis (160 V for 2 hours), gels were then

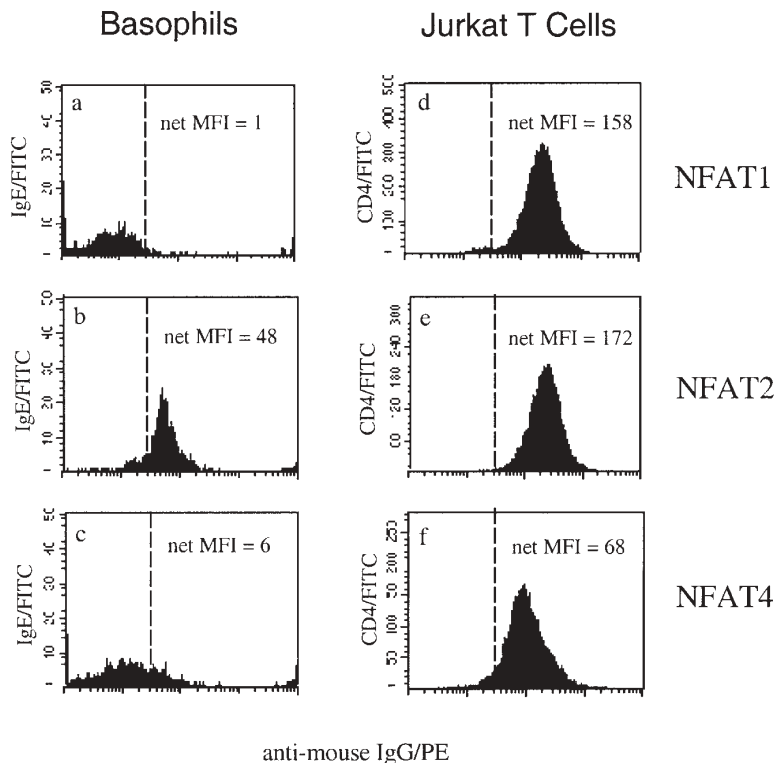


FIG 1. Two-color flow cytometry for intracellular staining of NFAT in human basophils and Jurkat T cells (representative histograms). **a** through **c** show staining for NFAT1, NFAT2, and NFAT4, respectively, in basophils gated with anti-IgE/FITC. For comparison, **d** through **f** show NFAT staining in Jurkat T cells colabeled with anti-CD4/FITC. The net mean fluorescence intensity (net MFI) values are shown for each histogram. PE, Phycoerythrin.

transferred to supported nitrocellulose membranes (OPTITRAN; Schleicher and Schuell, Keene, NH) with a Trans Blot (NOVEX). Western blotting was performed as described elsewhere.²⁰ The monoclonal anti-NFAT antibodies listed above were used for detection of immunoreactive protein, whereas a horseradish peroxidase-conjugated anti-mouse secondary antibody was used for visualization with enhanced chemiluminescence.

Electrophoretic mobility shift assays

Cytosolic and nuclear extracts from purified basophil pellets were prepared as described above. The P1 element of the human IL-4 promoter, which contains a consensus NFAT sequence, was labeled by means of random hexamer priming with the Klenow fragment and α -³²P-dCTP (Amersham Pharmacia Biotech, Piscataway, NJ), as described elsewhere.²¹ Five to 20 fmol of the probe (corresponding to 10,000-30,000 cpm) was incubated (for 30 minutes at 25°C) with each nuclear extract in 10 μ L of 12 mmol/L HEPES (pH 7.9), 50 mmol/L KCL, 0.5 mmol/L MgCl₂, 0.24 mmol/L EDTA, 4 mmol/L dithiothreitol, 12% glycerol, 0.1 mg/mL BSA, and 30 mg/mL poly(dI-dC) (Amersham Pharmacia Biotech). Where indicated, 20 minutes after the addition of probe, the binding reactions were incubated (for 30 minutes at 4°C) with mAbs specific for either NFAT1, NFAT2, NFAT4, or the nuclear factor κ B (NF- κ B) subunit p65 (Santa Cruz Biotechnologies, Santa Cruz, Calif). Free DNA and DNA-protein complexes were resolved by means of electrophoresis on 4% native polyacrylamide gels in 45 mmol/L Tris (pH 8.2), 45 mmol/L boric acid, 1 mmol/L EDTA, and 1% glycerol and then visualized by using autoradiography of fixed and dried gels.

RESULTS

NFAT2 is the predominant NFAT protein expressed in human basophils

To determine what species of NFAT are expressed in basophils, we developed a 2-color flow cytometric assay that rapidly and simultaneously quantified the percentage of IgE-positive basophils expressing a specific NFAT member and the intensity of such staining. Fig 1, A-C, shows the histogram results for NFAT1, NFAT2, and NFAT4 staining, respectively, in basophils from a representative donor. Although little to no staining of NFAT1 and NFAT4 was detected, all of the basophils stained intensely for intracellular NFAT2. The specific mean fluorescence intensities shown represent values above those obtained with an isotype-matched (mouse IgG₁) control antibody. For comparison, Fig 1, D-F, show Jurkat T cells costained for CD4 and all of the NFAT species tested, indicating that the paraformaldehyde fixation and saponin permeabilization steps necessary for this assay did not prevent immunoreactivity to the mAbs. Fig 2 shows the average specific mean fluorescence intensities for NFAT1, NFAT2, and NFAT4 staining in basophils isolated from up to 12 different donors, which were 2 ± 1 , 65 ± 13 , and 8 ± 4 , respectively. No apparent correlations existed with regard to allergic status (data not shown).

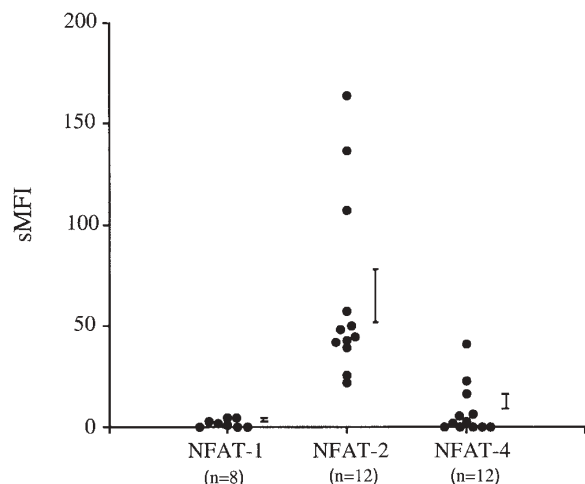


FIG 2. Relative expression of NFAT family members in human basophils. Individual net mean fluorescence intensity values obtained by means of 2-color flow cytometry for intracellular NFAT1, NFAT2, and NFAT4 are shown (filled circles). Respective averages $\pm$ SEM for the expression of each family member are shown for comparison. *sMFI*, Specific mean fluorescence intensity.

NFAT gene expression in human basophils

To further support the evidence that NFAT2 is the principal NFAT species found in basophils, we examined whether mRNA for this transcription factor is constitutively expressed in these cells. Total RNA was isolated from pure basophils (>98% purity) and subjected to RT-PCR for NFAT1, NFAT2, and NFAT4. The primer sets for each of the NFAT members had been previously used to detect mRNA for these transcription factors in human eosinophils.¹⁶ As shown in Fig 3, amplification resulted in detectable product for NFAT2 in 5 of 5 experiments with cells from different donors, which is consistent with the expression of protein for this transcription factor. Interestingly, NFAT4 message was variably expressed, being detectable in 3 of the 5 cell preparations analyzed. In contrast, RNA from cells of only 1 of the 5 donors showed evidence of NFAT1 gene expression.

Increased nuclear localization of NFAT2 after basophil activation

We next investigated the subcellular localization of NFAT2 by means of Western blot analysis to determine whether increased protein localized to the nucleus of activated basophils. Both cytosolic and nuclear extracts were analyzed. Fig 4 shows the results of 3 representative experiments in which several parameters were tested. First, in experiments 1 and 2, an increase in NFAT2 protein was evident in the nuclear extracts of cells activated for 1 hour (Fig 4, *middle lanes*), which was above that detected in the nuclei of resting cells (Fig 4, *first lanes*). In fact, slightly less NFAT2 protein was detected after 2 hours' incubation (Fig 4, *lane 3*, experiment 1), which was consistent with the time course of nuclear localization for this member, as detected with immunohistochemistry (data not shown). Second, it was also apparent

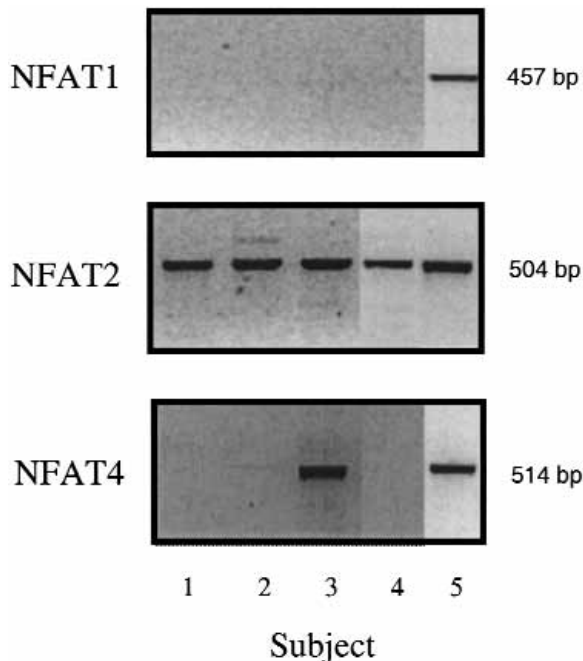


FIG 3. Relative expression of NFAT mRNA in unstimulated human basophils. Total RNA from equal numbers of basophils (>98% purity) was used in qualitative RT-PCR with 35 cycles of amplification. Products are shown in gel images viewed by means of ethidium bromide staining. Viable RNA from cells of each subject are indicated by the constitutive expression of NFAT2.

that FK506 (10 ng/mL) prevented the IgE-mediated subcellular localization of NFAT2 (Fig 4, *lane 3*, experiment 2). Finally, only a modest increase in protein was seen with anti-IgE activation in experiment 3 (Fig 4, *middle lane*). This finding, however, likely reflects the fact that these cells responded poorly to this stimulus, as indicated by a failure to release histamine (data not shown). In contrast, increased NFAT2 was detected in the nuclei of these cells after activation with ionomycin (Fig 4, *lane 3*, experiment 3). For comparison, the results show a general trend toward the subcellular localization of NFAT2 in the cytosolic fractions of cells not activated.

We were unable to identify NFAT4 or NFAT1 by means of Western analysis of lysates isolated from pure basophil suspensions, thus confirming the results obtained with 2-color flow cytometry. However, the same antibodies did identify these species in cell lysates prepared from rat basophilic leukemia cells and Jurkat T cells used as control cells (data not shown).

NFAT2 binding to the IL-4 promoter

FK506 is a potent inhibitor of the IL-4 rapidly generated in basophils after Fc ϵ RI-mediated activation, suggesting the involvement of a calcineurin/NFAT-dependent pathway regulating the generation of this cytokine.²² Therefore we next investigated whether nuclear lysates from resting versus activated basophils contained a protein that would complex with an IL-4 promoter NFAT consensus probe (ie, P element) in electrophoretic mobility shift assays. Fig 5, A, shows the

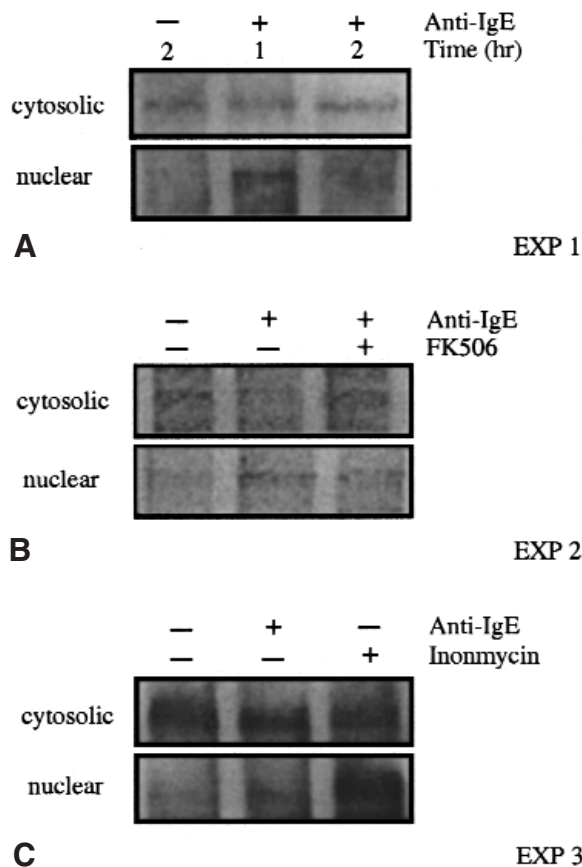


FIG 4. Subcellular localization of NFAT2 protein in activated human basophils. Cytosolic and nuclear lysates were analyzed for NFAT2 protein by means of Western blotting. Lysates were prepared after 1 hour's incubation (unless specified otherwise). Results from 3 experiments (*EXP*) are shown. Anti-IgE was used at 20 ng/mL, FK506 at 10 ng/mL, and ionomycin at 500 ng/mL. Cells used in experiment 3 were from anti-IgE nonresponders. Bands are between the 105- and 165-kD markers (not shown).

results with lysates from 3 different basophil preparations. Two complexes were observed in each experiment, with increased binding detected in the nuclear lysates prepared from cells treated for 1 hour with anti-IgE. The percentage of histamine released by each basophil preparation is shown for comparison in Fig 5, *B*, providing an additional measure of their relative response to anti-IgE stimulation. Evidence to support NFAT2 DNA binding in the formation of these complexes is shown in Fig 6. A reduction in the formation of these complexes was evident when the nuclear lysates of activated basophils, along with the probe, were coincubated with an mAb to NFAT2 (Fig 6, *lane* 3). Monoclonal antibodies to NFAT1, NFAT4, and the unrelated transcription factor NF- κ B did not produce the same effect.

DISCUSSION

We and others have shown that human basophils are a rapid and major source of IL-4, particularly when activated by stimuli that induce prolonged calcium responses,

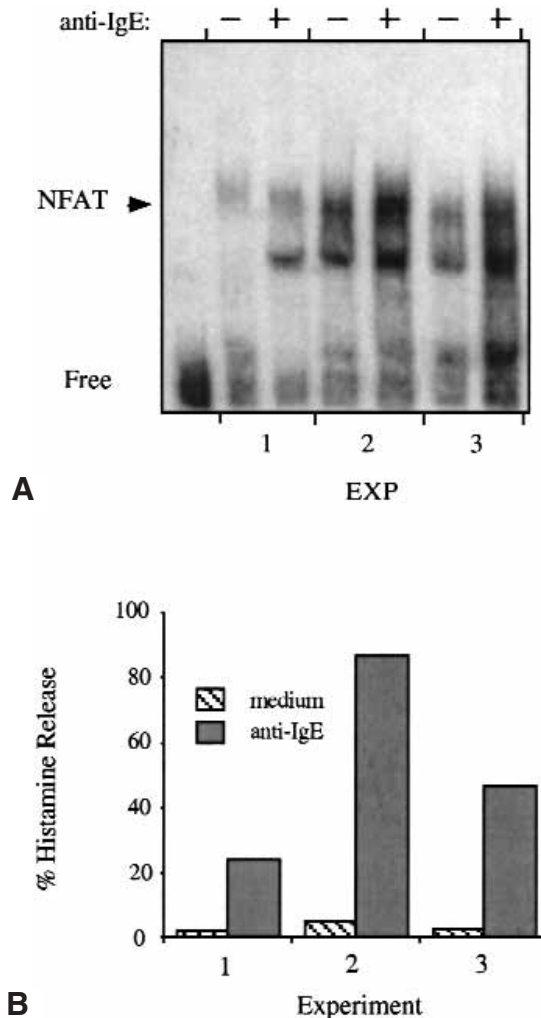


FIG 5. Increased NFAT binding to the IL-4 promoter (P element) after anti-IgE (20 ng/mL) activation. **A**, An image showing the electrophoretic mobility shift assay results from 3 experiments (*EXP*), indicating an increase in the formation of an NFAT-probe complex in the nuclear lysates of basophils activated for 1 hour. **B**, Corresponding percentage of histamine released in the culture supernatants as a measure of the relative response to anti-IgE.

such as those occurring with Fc ϵ RI-mediated activation or with calcium ionophores. In this study we addressed whether human basophils express specific members of the NFAT family of transcription factors, the role of which in cytokine transcription in other leukocytes has been described and the activation of which is very much dependent on the calcium-dependent phosphatase calcineurin. Of the 3 species typically found within cells of the immune system, NFAT2 was, surprisingly, the most abundantly expressed in human basophils. Both protein and mRNA for this transcription factor were constitutively expressed in all basophil preparations examined. In contrast, our findings showed that basophils lacked expression of NFAT1, which is an unusual finding given that most every other leukocyte investigated to date has been shown to constitutively express this transcription factor, including T and B lym-

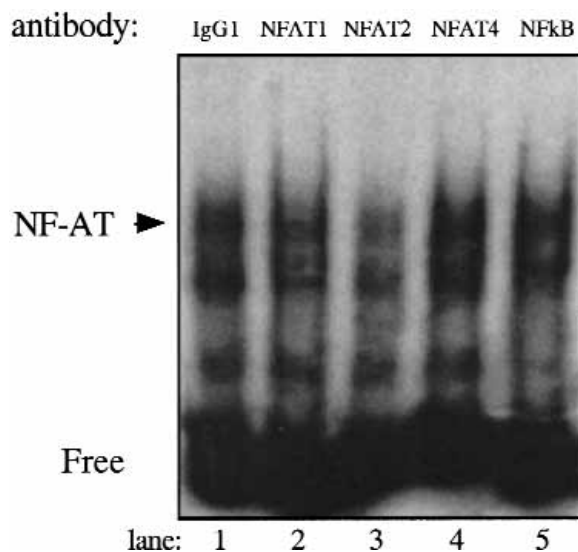


FIG 6. IgE-mediated IL-4 promoter binding is inhibited by monoclonal anti-NFAT2. Pooled nuclear lysates from activated basophils (>98% purity) were incubated with IL-4 probe. Isotype control and mAbs to NFAT1, NFAT2, NFAT4, and NF- κ B were then added to lysate-probe aliquots and incubated for an additional 20 minutes. Electrophoretic mobility shift assay results indicate a qualitative reduction in the formation of the DNA-probe complexes with the anti-NFAT2 antibody.

phocytes, natural killer cells, macrophages, eosinophils, and mast cells. In fact, many reports have shown that NFAT2, particularly its protein, is not constitutively expressed in most leukocytes. Instead, NFAT2 is more commonly shown to be an inducible species of NFAT,^{23,24} particularly in activated human T lymphocytes²⁵ and in certain murine mast cell lines.²⁶ There are conflicting reports as to whether human eosinophils constitutively express NFAT2.^{16,27} However, there is agreement that the eosinophil, which is generally thought to be a close cousin of the basophil, does express NFAT1. Interestingly, we did identify NFAT4 (both protein and mRNA) in the basophils of some individuals, but its expression was not a consistent finding. At this time, it is uncertain as to the significance of this observation because this species of NFAT is found at high levels in thymocytes. We are, however, currently investigating whether this species of NFAT may be differentially expressed among donor populations and whether it is involved in cytokine generation in basophils.

As cytosolic NFAT is dephosphorylated in T cells by calcineurin, its affinity for DNA becomes greater, resulting in its translocation to the nucleus, where it functions as a transcription factor for several cytokine genes, including *IL4*. In this study we found the nuclear localization of NFAT2 in basophils to be consistent with several parameters important for IL-4 generation in these cells. First, increased levels of NFAT2 protein were found in nuclear lysates after IgE-dependent activation of basophils at a time (1 hour) that is consistent with the generation of IL-4. The same was not observed after 3

hours' activation with anti-IgE or at a time when IL-4 generation is known to be essentially complete. Second, the nuclear translocation of NFAT2 was not evident in cells not responding to anti-IgE, the so-called nonreleaser phenotype in which there is reduced secretion of all mediators, including histamine, leukotriene C₄, and cytokines, presumably resulting from a deficiency in the early signaling component, syk.²⁸ In contrast, the calcium ionophore ionomycin which stimulates basophils independently of IgE crosslinking to produce high levels of IL-4,⁷ did cause the nuclear localization of NFAT2. Finally, the nuclear localization of NFAT2, as expected, was inhibited in cells treated with FK506, which, in our hands, is a potent inhibitor of IL-4 secretion by basophils.²²

Both NFAT1 and NFAT2 are reported to bind elements within the IL-4 promoter. The selective expression of NFAT2 and its nuclear localization after IgE-mediated activation in basophils suggested a role for this family member in IL-4 generation. By using an oligonucleotide containing a well-known NFAT consensus sequence found within the IL-4 promoter (ie, the P element), we demonstrated the formation of 2 complexes when used to probe basophil nuclear lysates. Although the observation of 2 complexes is not fully understood at this time, we believe they represent a combination of complexes in which there is coordinate binding of one or more unknown components along with NFAT. As predicted, these complexes were most evident when using the lysates of cells activated for 1 hour with anti-IgE antibody. Furthermore, the results showed that the relative intensities of the complexes formed were very much a function of the responsiveness (ie, histamine release) of the basophil preparation to anti-IgE stimulation. This latter finding is important because we have previously shown a strong correlation between the levels of IL-4 secreted in response to an optimal concentration of anti-IgE and the percentage of histamine released in those cultures.²² Because histamine is released within minutes (approximately 15 minutes) after activation, we were able to test for this mediator as a marker of activation. However, the much longer time required for IL-4 generation (ie, 2-4 hours) made it difficult to analyze both NFAT translocation and cytokine secretion concomitantly because the former comes before the latter. Therefore direct measurements of IL-4 in these cultures were not performed, although our previous work clearly shows that this cytokine is produced in the wake of histamine release after stimulation with anti-IgE. Evidence for NFAT2 involvement in the formation of these complexes was demonstrated by using an mAb specific for this transcription factor. However, rather than observing a so-called supershift in its electrophoretic mobility, which is sometimes observed with polyclonal antibodies, we saw a reduction in the formation of the NFAT-probe complex. Others, in fact, have reported similar findings with the same antibody.²⁹ Nonetheless, mAbs to NFAT1, NFAT4, and the unrelated transcription factor NF- κ B did not produce the same effect.

Finally, there is a body of evidence to suggest that NFAT1 plays a dominant role in regulating the generation of T_H1 -like cytokines, whereas NFAT2 appears more important in inducing T_H2 -like cytokines. For example, mice deficient in NFAT1 have been shown to mount an enhanced type of allergic inflammation compared with that of wild-type mice, as manifested by the accumulation of eosinophils at the site of antigen challenge and increased serum levels of IgE.³⁰ This allergic condition is further marked in mice deficient of both NFAT1 and NFAT4.³¹ Conversely, mutant murine T lymphocytes deficient in NFAT2 (NFATc1) failed to generate IL-4 and IL-6 yet were shown to secrete IL-2.³² Our finding that NFAT2, but not NFAT1, is constitutively expressed in human basophils is consistent with the belief that these transcription factors differentially regulate T_H2 and T_H1 cytokines, respectively. For example, there have been no reports to date of human basophils generating T_H1 -like cytokines. Therefore, assuming that NFAT1 also plays a key role in regulating T_H1 -like cytokines in human leukocytes, it seems appropriate that basophils lack the expression of this transcription factor.

In conclusion, the selective and specific expression of NFAT2 in basophils, compared with the expression of NFAT1 and NFAT4, suggests that these cells are unique among other human leukocytes expressing these transcription factors. Our results further indicate a relationship between NFAT2 subcellular localization and the generation of IL-4, particularly when mediated through FcεRI activation.

We thank Anja Bieneman for her excellent technical assistance. Special recognition goes to the memory of Ellen M. Roche for the invaluable technical help she provided and the genuine enthusiasm she conveyed in the laboratory.

REFERENCES

- Schroeder JT, MacGlashan DW Jr. New concepts: the basophil. *J Allergy Clin Immunol* 1997;99:429-33.
- Brunner T, Heusser CH, Dahinden CA. Human peripheral blood basophils primed by interleukin-3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *J Exp Med* 1993;177:605-11.
- MacGlashan DW, White JM, Huang SK, Ono SJ, Schroeder JT, Lichtenstein LM. Secretion of interleukin-4 from human basophils: the relationship between IL-4 mRNA and protein in resting and stimulated basophils. *J Immunol* 1994;152:3006-16.
- Li H, Sim T, Alam R. IL-13 released by and localized in human basophils. *J Immunol* 1996;156:4833-8.
- Gibbs B, Haas H, Falcone F, Albrecht C, Vollrath I, Noll T, et al. Purified human peripheral blood basophils release interleukin-13 and preformed interleukin-4 following immunological activation. *Eur J Immunol* 1996;26:2493-8.
- Devouassoux G, Foster G, Scott LM, Metcalfe DD, Prussin C. Frequency and characterization of antigen-specific IL-4- and IL-13-producing basophils and T cells in peripheral blood of healthy and asthmatic subjects. *J Allergy Clin Immunol* 1999;104:811-9.
- Schroeder JT, Howard B, Jenkins M, Kagey-Sobotka A, Lichtenstein L, MacGlashan DW Jr. IL-4 secretion and histamine release by human basophils are differentially regulated by protein kinase C activation. *J Leukoc Biol* 1998;63:692-8.
- Rao A, Luo C, Hogan P. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 1997;15:707-47.
- Lopez-Rodriguez C, Aramburu J, Rakeman A, Rao A. NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. *Proc Natl Acad Sci* 1999;96:7214-9.
- Macian F, Lopez-Rodriguez C, Rao A. Partners in transcription: NFAT and AP-1. *Oncogene* 2001;20:2476-89.
- Choi MSK, Brines RD, Holman MJ, Klaus GGB. Induction of NF-AT in normal B lymphocytes by anti-immunoglobulin or CD40 ligand in conjunction with IL-4. *Immunity* 1994;1:179-87.
- Aramburu J, Azzoni L, Rao A, Perussia B. Activation and expression of the nuclear factors of activated T cells, NFATp and NFATc, in human natural killer cells: regulation upon CD16 ligand binding. *J Exp Med* 1995;182:801-10.
- Hutchinson L, McCloskey M. FcεRI-mediated induction of nuclear factor of activated T-cells. *J Biol Chem* 1995;270:16333-8.
- Weiss D, Hural J, Tara D, Timmerman L, Henkel G, Brown M. Nuclear factor of activated T cells is associated with a mast cell interleukin 4 transcription complex. *Mol Cell Biol* 1996;16:228-35.
- Prieschl E, Gouilleux-Gruart V, Walker C, Harrer N, Baumruker T. A nuclear factor of activated T cell-like transcription factor in mast cells is involved in IL-5 gene regulation after IgE plus antigen stimulation. *J Immunol* 1995;154:6112-9.
- Jinquan T, Quan S, Jacobi HH, Reimert CM, Millner A, Hansen JB, et al. Cutting edge: expression of the NF of activated T cells in eosinophils: regulation by IL-4 and IL-5. *J Immunol* 1999;163:21-4.
- Schroeder JT, MacGlashan DW Jr, Kagey-Sobotka A, White JM, Lichtenstein LM. IgE-dependent IL-4 secretion by human basophils: the relationship between cytokine production and histamine release in mixed leukocyte cultures. *J Immunol* 1994;153:1808-18.
- Gilbert HS, Ornstein L. Basophil counting with a new staining method using alcian blue. *Blood* 1975;46:279-86.
- Prussin C, Metcalfe DD. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Methods* 1995;188:117-28.
- Miura K, Schroeder JT, Hubbard W, MacGlashan DW Jr. Extracellular signal-regulated kinases regulate leukotriene C4 generation, but not histamine release or IL-4 production from human basophils. *J Immunol* 1999;162:4198-206.
- Casolaro V, Gaurus S, Song Z, Zubkoff I, Abdulkadir S, Thanos D, et al. Inhibition of NF-AT-dependent transcription by NF-κB: implications for differential gene expression in T helper cell subsets. *Proc Natl Acad Sci U S A* 1995;92:11623-7.
- Redrup A, Howard B, MacGlashan DW Jr, Kagey-Sobotka A, Lichtenstein L, Schroeder JT. Differential regulation of IL-4 and IL-13 secretion by human basophils: their relationship to histamine release in mixed leukocyte cultures. *J Immunol* 1998;160:1957-64.
- Timmerman L, Clipstone N, Ho S, Northrop J, Crabtree G. Rapid shuttling of NF-AT in discrimination of Ca²⁺ signals and immunosuppression. *Nature* 1996;383:837-40.
- Dolmetsch R, Lewis R, Goodnow C, Healy J. Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration. *Nature* 1997;386:855-8.
- Lyakh L, Ghosh P, Rice N. Expression of NFAT-family proteins in normal human T cells. *Mol Cell Biol* 1997;17:2475-84.
- Sherman M, Powell D, Weiss D, Brown M. NF-ATc isoforms are differentially expressed and regulated in murine T and mast cells. *J Immunol* 1999;162:2820-8.
- Seminario M, Guo J, Bochner B, Beck L, Gaurus S. Human eosinophils constitutively express nuclear factor of activated T cells p and c. *J Allergy Clin Immunol* 2001;107:143-52.
- Kepley C, Youssef L, Andrews R, Wilson B, Oliver J. Multiple defects in FcεRI signaling in Syk-deficient nonreleaser basophils and IL-3-induced recovery of syk expression and secretion. *J Immunol* 2000;165:5913-20.
- Wierenga E, Walchner M, Kick G, Kapsenberg M, Weiss E, Messer G. Evidence for suppressed activity of the transcription factor NFAT1 at its proximal binding element P0 in the IL-4 promoter associated with enhanced IL-4 gene transcription in T cells of atopic patients. *Int Immunol* 1999;11:297-306.
- Xanthoudakis S, Viola J, Shaw K, Luo C, Wallace J, Bozza P, et al. An enhanced immune response in mice lacking the transcription factor NFAT1. *Science* 1996;272:892-5.
- Ranger A, Oukka M, Rengarajan J, Glimcher L. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity* 1998;9:627-35.
- Yoshida H, Nishina H, Takimoto H, Marengere L, Wakeham AC, Bouchard D, et al. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity* 1998;8:115-24.