

Human eosinophils constitutively express nuclear factor of activated T cells p and c

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Background: Eosinophils are now known to produce a variety of proinflammatory cytokines, although the molecular factors that regulate their production are poorly understood. The expression of almost all of the cytokines produced by eosinophils, including the proallergic cytokine IL-4, is now known to be regulated at the level of transcription by members of the nuclear factor of activated T cells (NFAT) family of transcription factors.

Objective: We sought to characterize the expression of different NFAT proteins in resting and activated eosinophils.

Methods: Nuclear and whole cell extracts were obtained from both peripheral blood eosinophils and those obtained from bronchoalveolar lavage fluid of asthmatic subjects after endobronchial allergen challenge. NFAT expression was determined by using immunoprecipitation and Western blot analysis, DNA-binding assays, and RT-PCR analysis of eosinophil mRNA.

Results: Both peripheral blood and bronchoalveolar lavage fluid eosinophils expressed NFATp and NFATc protein. Unlike activated T cells, which express multiple NFATc isoforms, eosinophils preferentially express the approximately 85-kd isoform. In addition, eosinophils were found to constitutively express NFATc mRNA. A brief incubation with the T_H2 cytokines IL-4 and IL-5 was sufficient to induce the nuclear translocation of NFATc. Eosinophil nuclear extracts contain multiple factors that can specifically recognize the IL-4 promoter P1 NFAT site in DNA-binding assays, including NFATp. **Conclusion:** NFATp and NFATc can regulate the expression of cytokines and other genes in eosinophils but appear to be regulated by a novel signal transduction mechanism in these cells. (*J Allergy Clin Immunol* 2001;107:143-52.)

Key words: Eosinophils, cytokines, transcriptional regulation, nuclear factor of activated T cells, HMGII/Y

Abbreviations used

BAL: Bronchoalveolar lavage
EMSA: Electrophoretic mobility shift assay
GAPDH: Glyceraldehyde-phosphate dehydrogenase
NFAT: Nuclear factor of activated T cells

Eosinophils play a key role in the pathogenesis of allergic diseases through the release of granule cationic proteins and newly synthesized lipid mediators.¹ Recent studies have demonstrated that eosinophils also have the capacity to store and express a wide range of cytokines, chemokines, and growth factors. A review of the current literature indicates that eosinophils are capable of expressing mRNA, protein, or both for IL-2, IL-3, IL-4, IL-5, IL-8, IL-10, IL-11, IL-12, GM-CSF, and RANTES.²⁻¹² The ability of eosinophils to synthesize and store these cytokines suggests a novel mechanism by which eosinophils might contribute to allergic inflammatory responses. In support of this notion, IL-4 protein was recently found to localize to eosinophils within the bronchial mucosa of atopic asthmatic subjects.¹³ In another study the majority of IL-4⁺ cells in nasal polyp tissues were found to be eosinophils.⁵

Although recent reports have established that eosinophils are capable of synthesizing cytokines, little is known about the signal transduction and transcription factor pathways that regulate gene expression in these cells. The expression of almost all of the cytokines produced by eosinophils is now known to be controlled in other cell types by members of the nuclear factor of activated T cells (NFAT) family of transcription factors.¹⁴ There are currently 4 known NFAT family members that appear to differentially regulate immune responses (Table I).¹⁴⁻²¹ Experiments with NFAT-deficient mice found that NFATc is critical for T_H2 differentiation,¹⁷ whereas NFATp and NFAT4 serve to downregulate T_H2 responses.^{15,16} These experiments also revealed an important role for NFATp and NFAT4 in regulating eosinophilia. Deficiency of NFATp alone resulted in enhanced antigen-driven eosinophil recruitment,¹⁶ whereas combined NFATp- and NFAT4-deficient mice had striking bone marrow and tissue eosinophilia.¹⁵

Although originally detected in T cells, NFAT has been found in multiple other cell types, including mast cells, basophils, B cells, macrophages, natural killer

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TABLE I. Nomenclature, function, and isoform sizes of NFAT proteins

Original name*	Knock-out phenotype†	Molecular weight (kd)‡
NFATp (NFAT1, NFATc2)	Increased T _H 2 responses; increased eosinophil recruitment	130 (100)
NFATc (NFAT2, NFATc1)	Embryonic lethal; CM: decreased T _H 2 responses	140, 110, 86
NFAT3 (NFATc4)		ND
NFATx (NFAT4, NFATc3)	Increased T _H 2 responses	155
NFAT5		

ND, Not detected in eosinophils or T cells.

*Two NFAT nomenclatures have been proposed: NFAT1-4¹⁴ and NFATc1-c4.¹⁹ NFAT5 is described in Lopez-Rodriguez et al.²⁰

†Data compiled from Ranger et al.,¹⁵ Viola et al.,¹⁶ and Ranger et al.¹⁷ CM denotes chimeric mice generated by blastocyst complementation with T cell-deficient precursors.¹⁷

‡Data for NFAT expression in T cells is from Lyakh et al.²¹ Comparable isoform expressed by eosinophils (this article) is indicated in bold. Using immunohistochemistry, Jinquan et al.¹⁸ found that eosinophils expressed NFAT4 but not NFAT3.

cells, and endothelial cells.¹⁴ In a recent report eosinophils were found to express NFATp (NFAT1) and NFAT4.¹⁸ NFAT proteins are constitutively cytoplasmic, and dephosphorylation by the phosphatase calcineurin is sufficient to induce their rapid nuclear translocation.¹⁴ In T cells and mast cells NFAT activation is mediated by calcium signals emanating from their respective antigen receptors, namely the T-cell receptor²² and FcεRI.²³ The relevant receptors and signaling pathways that activate NFAT in other cell types have not been well studied.

Multiple additional NFAT isoforms arise because of alternative splicing events, and emerging data suggest that these isoforms can differentially regulate transcription.^{21,24,25} In addition, the function of NFAT proteins can be regulated by other transcription factors. For example, in many cytokine gene promoters, NFAT interacts with a member of the AP-1 family to form a transcriptionally active complex.¹⁹ In contrast, other factors antagonize the ability of NFAT to activate transcription.^{26,27} For example, HMGI/Y has been shown to inhibit the ability of NFAT to bind a high-affinity site in the IL-4 promoter.²⁶ HMGI/Y belongs to a family of nonhistone chromosomal proteins that regulate gene transcription by binding A-T rich stretches in the minor groove of target promoters.²⁸ Thus the ability of NFAT to activate gene transcription depends on its interactions with both positively and negatively acting cofactors.

Here we show, by using immunoprecipitation and Western blot analysis, that both peripheral blood and bronchoalveolar lavage (BAL) eosinophils contain abundant NFATp and NFATc (NFAT2) protein. Unlike T cells, eosinophils express one predominant NFATc isoform with an apparent molecular mass of approximately 85 kd. A brief stimulation with the T_H2 cytokines IL-4 and IL-5 was sufficient to induce NFATc nuclear translocation. We further analyzed the DNA-binding capabilities of eosinophil nuclear proteins by using a radiolabeled oligonucleotide containing the human IL-4 promoter P1 NFAT element in electrophoretic mobility shift assays. Here we report that eosinophil nuclear extracts contain multiple proteins capable of recognizing this element, including NFATp and HMGI/Y.

METHODS

Eosinophil purification and stimulation

Eosinophils were isolated from peripheral blood obtained from 6 atopic donors and from BAL fluid collected 20 hours after segmental allergen challenge of allergic asthmatic subjects (n = 2). Peripheral blood cells were purified, as previously described, by using Percoll density centrifugation (specific gravity, 1.090) followed by negative selection with anti-CD16 antibodies to remove neutrophils.²⁹ BAL fluid was also enriched for eosinophils by using Percoll density centrifugation, as previously described.³⁰ Eosinophil purity in both preparations was always greater than 98%, with neutrophils being the only contaminating cells. Cells were either used fresh or stimulated with A23187 (0.5 μmol/L, Calbiochem), IL-4 (50 ng/mL, Peprotech), or IL-5 (10 ng/mL, R&D Systems) as indicated. Neutrophils (>95% pure) were isolated from peripheral blood by using single Percoll density centrifugation and hypotonic lysis.

Cell culture

The Jurkat T cell lymphoma cell line (ATCC) was maintained in RPMI-1640 media supplemented with 5% FBS and 1% penicillin-streptomycin. Jurkat cells were passaged every 3 to 4 days. Nuclear extracts were obtained from both unstimulated cells (Fig 1) or after cell stimulation with A23187 (0.5 μmol/L, Calbiochem) plus phorbol myristate acetate (20 ng/mL, Calbiochem) for 4 hours (Fig 2).

Nuclear extraction

Nuclear extracts were obtained from peripheral blood eosinophils by using the method of Schreiber et al.³¹ with the following modifications: 600 μL of buffer A for 20 minutes on ice, followed by vigorous vortexing for 30 seconds in a final concentration of 1% NP-40 before centrifugation and resuspension in cold buffer C. All buffers were adjusted to contain higher concentrations of dithiothreitol and the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and aprotinin (Sigma Chemicals).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed with 5 μg of cell extracts in a final volume of 20 μL containing 84 mmol/L KCl, 1 mmol/L TRIS, 1 mmol/L EDTA, 20 μg of BSA (Sigma), and [³²P] end-labeled probe. Free probes and protein-DNA complexes were resolved by using 5% PAGE with 0.5× Tris borate EDTA. Oligonucleotides corresponding to the high-affinity P1 NFAT/AP-1 site from the human IL-4 promoter and the Box II HMGI/Y-binding site from the mouse IL-4 promoter were synthesized as follows: NFAT, 5'-TGAGTTTACATTGGAAATTTTCGT-

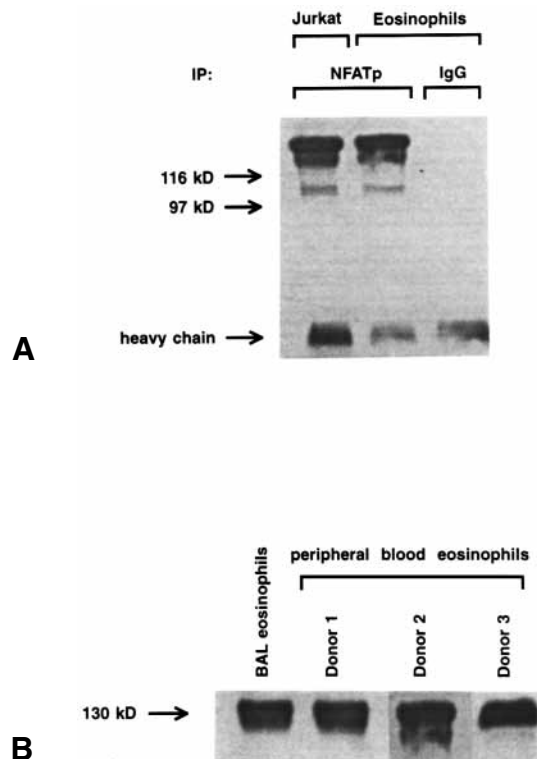


FIG 1. Detection of NFATp isoforms by using immunoprecipitation and Western blot analysis of peripheral blood and BAL eosinophils. **A**, A representative experiment with Jurkat T cells, which are known to contain NFATp as a positive control (*lane 1*), and extracts obtained from peripheral blood eosinophils (*lane 2*). Similar bands were detected in both the Jurkat and the eosinophil immunoprecipitates, including a major isoform of approximately 130 kD. The relative migration of standards of known molecular weight is indicated (*arrows*), as is the Ig heavy chain. Isotype-matched IgG was used as negative control for immunoprecipitation (*lane 3*). A minor band of approximately 105 kD was also detected in some eosinophils and the T-cell line, which may represent an NFATp splice variant. Similar results were obtained in 4 additional experiments. **B**, A distinct band of approximately 130 kD appeared in lysates obtained from peripheral blood eosinophils from 3 separate donors, as well as from BAL eosinophils from an asthmatic subject. A smaller band was also detected in cells obtained from donor 2 (see text for further discussion).

TACACCA-3'; Box II, 5'-AGATTTAAAAAAAAGGGGGGGG-GGA-3'.

Consensus AP-1, AP-2, and SP-1 oligonucleotides were purchased (Santa Cruz). Monoclonal anti-NFATp and anti-NFATc (7A6) were the kind gift of Dr G. Crabtree.

Immunoprecipitation and Western blot analysis

Cells were lysed for 20 minutes on ice with Triton lysis buffer (20 mmol/L TRIS [pH 7.4], 100 mmol/L NaCl, 10 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 2 mmol/L EDTA, 50 mmol/L NaF, 1% Triton X-100, 200 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaVO_4 , and 1 mmol/L each of leupeptin, aprotinin, and pepstatin A). Insoluble cell debris was removed by using centrifugation (15,000g for 5 minutes at 4°C). For Western blot analysis of whole cell extracts, lysates from 2×10^6 cells were boiled for 5 minutes in SDS sample buffer (2% SDS, 50 mmol/L TRIS [pH 6.8], 100 mmol/L dithiothreitol, 0.1% bromophenol blue, and 10% glycerol). For immunoprecipitation experiments, whole cell extracts from 5×10^6 cells were incubated for 2 hours at 4°C with either polyclonal goat anti-NFATp (Santa Cruz) or monoclonal mouse anti-NFATc (Affinity BioReagents) as indicated bound to Protein A-Sepharose beads (Pharmacia Biotech). The beads were washed 4 to 5 times in lysis buffer, 20 μL of sample buffer was added, and the samples were boiled for 5

minutes. Lysates or immunoprecipitates were separated by using SDS-PAGE electrophoresis, and proteins were transferred to a polyvinylidene difluoride membrane (BIO-RAD). Membranes were blocked in PBS containing 4% BSA overnight at 4°C. The membranes were immunoblotted with a mouse mAb specific for NFATc (Affinity BioReagents) or an NFATp-specific polyclonal goat antiserum (Santa Cruz Biotechnologies) and then washed 3 times for 10 minutes in TRIS-buffered saline-Tween (0.2%) before incubating with an appropriate horseradish peroxidase-conjugated secondary (Amersham Pharmacia Biotech) for 45 minutes. Membranes were subjected to 3 further washes before proteins were visualized by means of enhanced chemiluminescence (Amersham Corp). Membranes from NFATp immunoprecipitation experiments were stripped with 7 mol/L guanidine-HCl for 20 minutes at room temperature and reprobed for NFATc, as described above.

RNA extraction and RT-PCR

Eosinophils (5×10^6) were cultured for 18 hours in the absence or presence of recombinant IL-5 (20 ng/mL, R&D Systems) followed by extraction of total RNA by using the TRIzol method (Life Technologies) with the addition of carrier tRNA (Sigma). RNA extracted from Jurkat T cells activated for 6 hours with phorbol myristate acetate (20 ng/mL) plus A23187 (0.5 $\mu\text{mol/L}$, both from Calbiochem) were used as a positive control. cDNA was synthesized from equal aliquots of

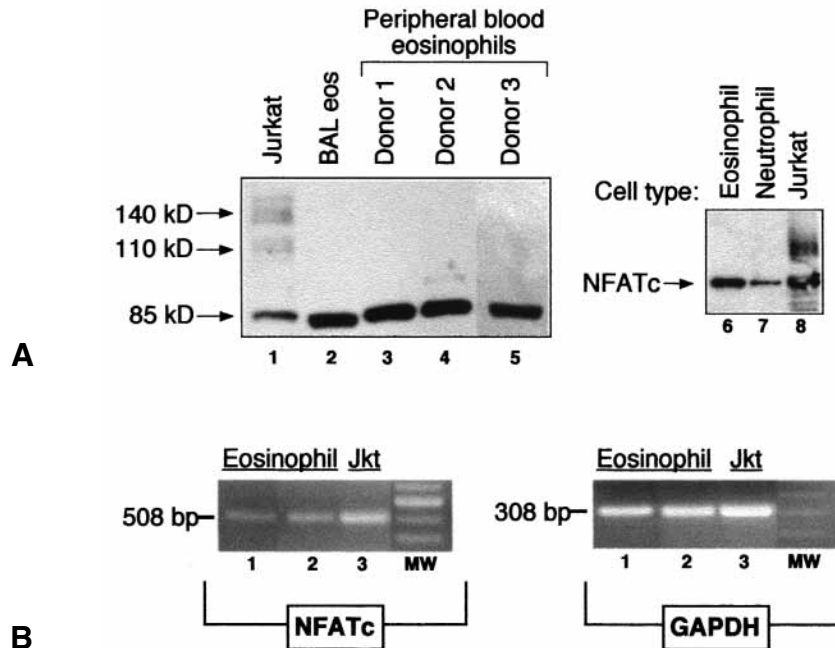


FIG 2. Eosinophils express NFATc protein and mRNA. **A**, Immunoprecipitation and Western blot analysis of peripheral blood and BAL eosinophils. Extracts obtained from peripheral blood and BAL eosinophils (see Fig 1) and from activated Jurkat T cells (see the "Methods" section) were separated by means of SDS-PAGE and immunoblotted with a specific monoclonal NFATc antibody. BAL and peripheral blood eosinophils from 3 donors expressed one major NFATc isoform with an apparent molecular mass of approximately 85 kd, whereas activated T cells contained additional isoforms of greater size, approximately 110 and 140 kd (lanes 1-5). An additional faint NFATc-immunoreactive band of greater molecular weight was also detected in one subject (lane 4). The apparent increase in electrophoretic mobility of the NFATc band in BAL eosinophils was not a consistent finding in other experiments. Whole cell lysates obtained from 2×10^6 eosinophils and neutrophils were analyzed with Jurkat nuclear extracts by means of Western blotting with the NFATc antibody (lanes 6-8). Neutrophils, which contain less NFATc than eosinophils, also preferentially express the low-molecular-weight isoform. **B**, Total RNA was extracted from resting and IL-5-stimulated eosinophils and analyzed by using RT-PCR with primer pairs specific for NFATc or the housekeeping gene *GAPDH* (see the "Methods" section). Eosinophils were found to constitutively express NFATc mRNA (left panel, lane 1), which was not appreciably affected by IL-5 treatment (lane 2).

total RNA (~0.5 mg) by using the Gene Amp RT-PCR kit (Perkin Elmer), according to the manufacturer's instructions, with 50 U of MuLV RT and 0.5 μ mol/L oligo d(T)16 per reaction. The entire cDNA pool was then analyzed by using PCR with the following specific primer pairs (synthesized by Sigma-Genosys): NFATc 5', 5'-GCCGACGACCCCTACCCAG-3'; NFATc 3', 5'-GTTCTTCCTC-CCGATGTCCGTCTCT-3'; glyceraldehyde-phosphate dehydrogenase (*GAPDH*) 5', 5'-TGATGACATCAAGAAGGTGGT-3'; *GAPDH* 3', 5'-CAGTGAGGGTCTCTCTCTTCC-3'.

Each PCR reaction condition contained 2 mmol/L $MgCl_2$, 2 mg/mL primers, and 2.5 U of HotStar Taq (Qiagen) and was subject to 35 cycles at the following temperatures: 94°C, 60°C (*GAPDH* primers) or 72°C (NFATc primers), and 72°C. This was followed by a 10-minute extension cycle at 72°C. One tenth of the final reaction was then analyzed by means of 1% agarose gel electrophoresis with ethidium bromide staining. The expected sizes of the PCR products were 508 (NFATc) and 308 bp (*GAPDH*).

RESULTS

Presence of NFATp and NFATc protein in human eosinophils

To characterize the expression of NFAT proteins in human eosinophils, we first used immunoprecipitation and

Western blot analysis to study the expression of NFATp and NFATc by using specific non-cross-reactive antisera against these factors (see the "Methods" section). In these experiments eosinophil extracts were analyzed in parallel with Jurkat T-cell extracts to allow comparison of different NFAT isoforms expressed by these two cell types. As shown in Fig 1, A, both eosinophils and Jurkat T cells express one major NFATp-specific isoform that migrates with an apparent molecular mass of approximately 130 kd. Similar results were obtained by using unstimulated peripheral blood eosinophils obtained from 6 atopic donors, as well as from BAL fluid after segmental allergen challenge of 2 asthmatic subjects (Fig 1, B, and data not shown). Additional smaller NFATp isoforms were detectable in Jurkat cells and in eosinophils obtained from one donor (Fig 1, donor 2). Whether this reflects post-translational modification (eg, dephosphorylation) or protein degradation is currently not known. No NFATp-immunoreactive bands were detected by using an irrelevant IgG control mAb for immunoprecipitation (Fig 1, A, and data not shown; $n = 5$). To further confirm the specificity of the NFATp antibody, membranes were

stripped and reprobed with an mAb specific for NFATc. No cross-reactivity was found between the 2 antibodies (data not shown).

We next analyzed eosinophils for the expression of NFATc by using similar immunoprecipitation techniques. In these experiments eosinophil lysates were compared with those obtained from activated Jurkat T cells (see the "Methods" section) to detect different isoforms of NFATc.²¹ Unstimulated eosinophils obtained from 3 atopic donors contained abundant immunoreactive NFATc (Fig 2, A) and expressed one major isoform of approximately 85 kd. Similar results were obtained when analyzing BAL fluid eosinophils obtained from an asthmatic subject after segmental allergen challenge (Fig 2, A). In contrast, activated Jurkat T cells expressed 2 additional NFATc isoforms of greater molecular weight, approximately 110 and 140 kd (Fig 2, lane 1), which were not detected in any eosinophil lysates examined. The expression of multiple NFATc isoforms in activated T cells is in keeping with the recent results of Lyakh et al.²¹ We next studied the expression of NFATc in eosinophils and neutrophils by means of Western blotting to determine whether this factor was expressed by other leukocytes. As shown in Fig 2, A (lanes 6-8), neutrophils also preferentially expressed the approximately 85-kd isoform, although to a lesser extent than eosinophils.

Eosinophils express NFATc mRNA

The detection of abundant NFATc protein in eosinophils is a novel finding. Interestingly, in a recent report, Jinquan et al¹⁸ were unable to detect either NFATc protein or mRNA in eosinophils purified from normal donors. To determine whether our findings reflected the long-lived expression of NFATc protein originally synthesized in eosinophil progenitors, we next analyzed total RNA extracted from resting and IL-5-activated eosinophils with NFATc-specific primers by using RT-PCR. As shown in Fig 2, B, constitutive eosinophil NFATc mRNA was readily detected under these conditions and was not appreciably affected by IL-5 treatment.

Eosinophil nuclear proteins present in fresh and stimulated cells associate with an NFAT-binding site

To assess whether eosinophil NFAT proteins were capable of forming competent DNA-binding complexes, we used an oligonucleotide probe containing the high-affinity P1 NFAT/AP1 site from the human IL-4 promoter in EMSAs with nuclear extracts obtained from both resting and stimulated eosinophils. Four complexes were formed with extracts from unstimulated cells, which interestingly were not significantly affected by calcium-ionophore treatment (Fig 3, A, lanes 2 and 3). The formation of the low-mobility complex I was partially inhibited by unlabeled self-oligonucleotides (Fig 3, A, lanes 4 and 5) but not unrelated oligonucleotides. Neither anti-NFAT antisera affected the formation of complex I,

and its identity is currently unknown. Complex II appears to contain NFATp because its formation is inhibited by a specific monoclonal anti-NFATp antibody (Fig 3, A, lane 10) but not control serum (Fig 3, A, lane 8). None of the complexes was altered by antisera directed against NFATc (lane 9). Competition experiments indicated that complex III represents nonspecific protein-DNA interactions because its formation was inhibited by a panel of unrelated oligonucleotides (Fig 3, A and B). A lighter exposure showed that complex IV, which was competed specifically by self-oligonucleotide, is comprised of 2 distinct bands (not shown). Thus at least 3 specific eosinophil nuclear proteins interact with an NFAT-binding site. One complex contains NFATp in both resting and stimulated peripheral blood eosinophils.

Complex IV contains proteins capable of recognizing the Box II HMGI/Y site

In previous studies with extracts from other cell types in EMSAs, we found that a complex with similar mobility as that of complex IV contained members of the HMGI/Y family of transcription factors (unpublished observations). HMGI/Y is a nonhistone chromosomal protein that binds to A-T rich stretches of DNA and can facilitate the assembly of transcription factor complexes on target promoters by inducing structural changes in DNA and through protein-protein contacts.²⁸ Interestingly, HMGI/Y is known to bind to the P1 element and inhibit the binding of NFAT to its cognate site contained therein.^{26,32} To determine whether complex IV contained HMGI/Y or related factors, we used an oligonucleotide corresponding to the high-affinity Box II HMGI/Y site from the mouse IL-4 promoter in additional competition experiments (Fig 3, B). Under these conditions, the formation of complex IV, but that of neither complex I nor complex II (NFATp), was completely inhibited.

T_H2 cytokines IL-4 and IL-5 are sufficient to induce NFATc nuclear translocation

The observation that NFATc does not appreciably participate in complex formation in EMSAs with nuclear extracts from calcium ionophore-treated cells (Fig 3) suggests that this stimulus may not be sufficient to induce its nuclear translocation. In support of this notion, when we analyzed nuclear extracts from calcium ionophore-activated cells for the expression of NFATc by Western blotting, we observed little or no increase above baseline (Fig 4). In addition, this protein remained exclusively cytoplasmic when we analyzed resting and calcium-activated peripheral blood and BAL eosinophils by immunofluorescence with an anti-NFATc antibody (unpublished observations). In contrast, a brief incubation with IL-4 alone or together with IL-5 significantly increased NFATc concentration in the nucleus (Fig 4).

DISCUSSION

During the last few years, our understanding of eosinophil biology has expanded to include the storage

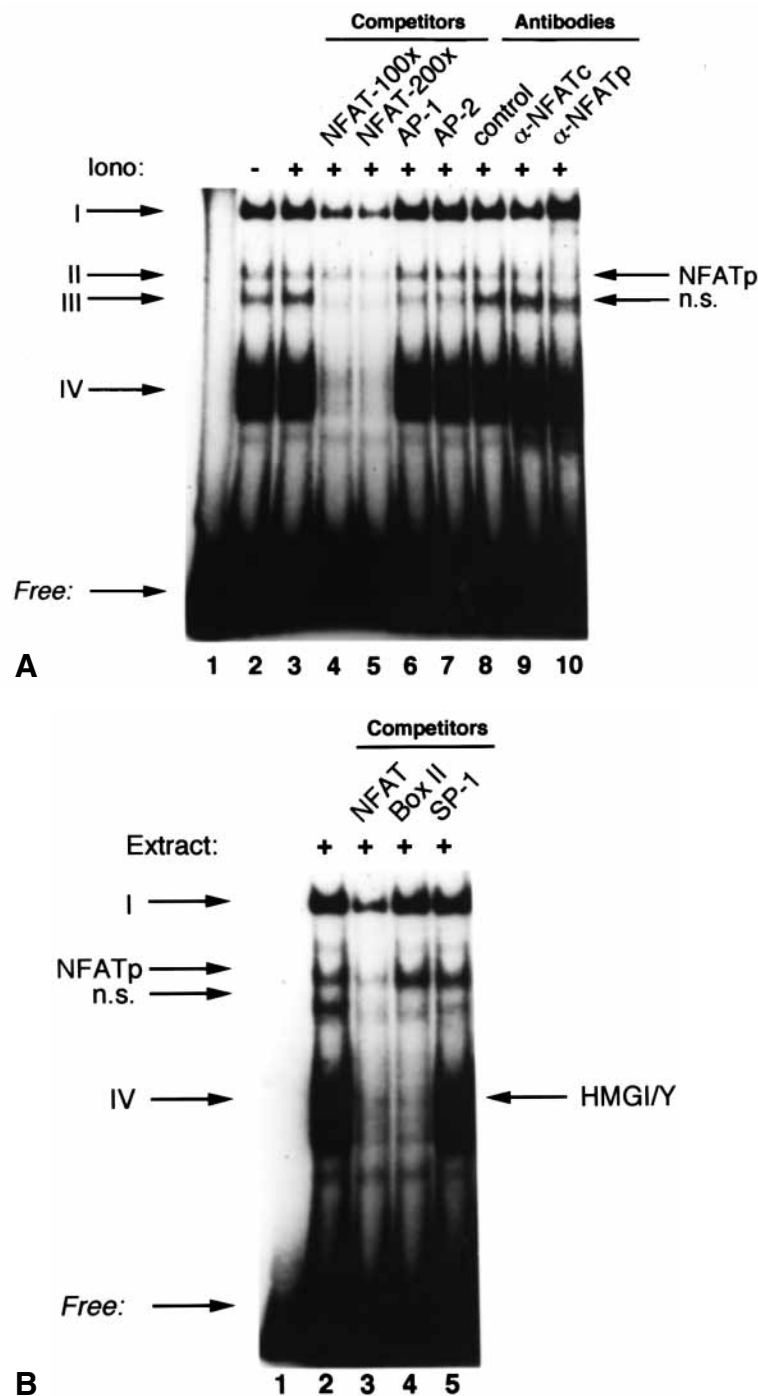


FIG 3. The DNA-binding ability of eosinophil NFAT proteins was analyzed by EMSAs with a radiolabeled oligonucleotide containing the IL-4 promoter P1 NFAT site. **A**, Four complexes formed with nuclear extracts from both unstimulated and calcium ionophore-activated cells (indicated by the *Roman numerals* on the left side of the figure). Complex III appeared to represent nonspecific protein-DNA interactions because its formation was inhibited by a series of unrelated oligonucleotides in competition experiments (*lanes 6 and 7 and B*). Complex II contains NFATp because it was inhibited by unlabeled self oligonucleotide (*lanes 4 and 5*) and by antisera directed against NFATp (*lane 10*) but not control or anti-NFATc antisera (*lanes 8 and 9*). A faint band just below complex I is visible in *lane 10* and likely corresponds to an NFATp supershift. Both complex I and complex IV were inhibited by unlabeled self oligonucleotide but not by unrelated competitors (**B**) or anti-NFAT antisera. The relative migration of the free probe is indicated (*arrow*). *n.s.*, Nonspecific; *lono*, calcium ionophore. **B**, Complex IV contains factors that recognize the Box II HMGI/Y binding site. Eosinophil nuclear extracts were analyzed by EMSAs as in **A**, with additional oligonucleotide competitors as indicated. Note that only the formation of complex IV was inhibited by using an oligonucleotide containing a high-affinity HMGI/Y binding site from the mouse IL-4 promoter (Box II, see the "Methods" section).

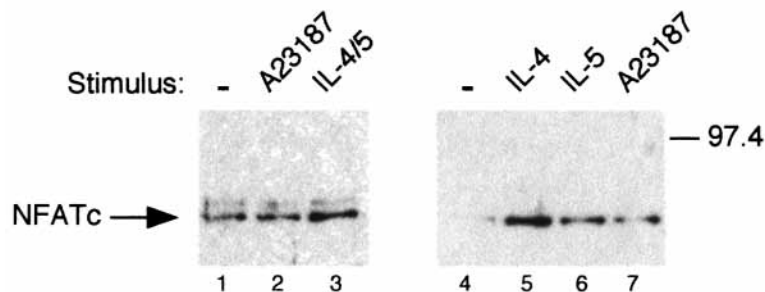


FIG 4. An analysis of the signals that activate NFATc in eosinophils. Eosinophils isolated from 2 donors were stimulated for 1 hour with A23187 (0.5 μ mol/L), IL-4 (50 ng/mL), or IL-5 (10 ng/mL) as indicated (subject 1, lanes 1-3; subject 2, lanes 4-7). Nuclear extracts were then analyzed by means of Western blotting for NFATc expression. Incubation with IL-4 and IL-5 induced greater NFATc nuclear translocation than A23187 alone in both subjects. Equal amounts of protein were loaded per lane.

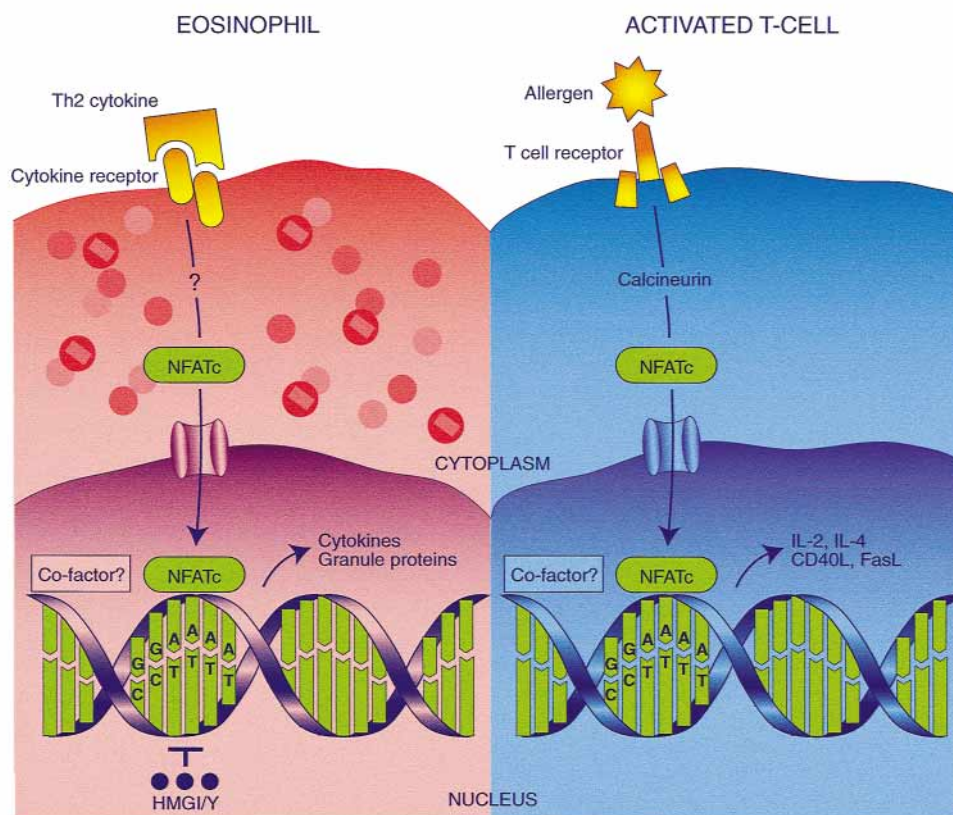


FIG 5. Schematic diagram of the regulation of NFATc in eosinophils and activated T cells. Eosinophils (*left*) constitutively express NFATc, which translocates to the nucleus after cell stimulation with T_H2 cytokines. The precise nature of the signal linking cytokine receptors and NFATc activation is currently not known. In activated T cells (*right*) NFATc is dephosphorylated and thus activated by calcineurin. Nuclear NFATc binds to upstream regulatory elements and induces the transcriptional activation of target genes. Cell type-specific gene expression (eg, granule proteins in eosinophils) could be achieved by interaction with unique cofactors. In addition, the requirement for NFATc to associate with a nuclear cofactor to induce gene transcription likely depends on the promoter context. In eosinophils the abundant expression of HMGI/Y serves to inhibit NFAT DNA-binding (*lower left*).

and production of cytokines by these cells.² Several studies have shown the presence of mRNA and protein and the release of several cytokines either constitutively or in response to different stimuli in both peripheral blood eosinophils and eosinophils from a variety of inflamma-

tory tissues.² Although the quantity of cytokines produced by purified eosinophils is generally low,^{33,34} the significant enrichment of these cells at sites of inflammation suggests that they can contribute to local cytokine generation. Along these lines, eosinophils obtained from

the lungs of asthmatic subjects have been shown to express several immunoregulatory cytokines, including IL-4.^{4,13,35} Little is known about the regulation of cytokine gene expression in eosinophils. In this study we report the constitutive expression of the transcription factors NFATp and NFATc in both peripheral blood and BAL eosinophils. It is noteworthy that these proteins are now known to regulate the expression of many cytokines, including those reportedly produced by eosinophils.¹⁴

On the basis of elegant experiments with NFAT-deficient mice, it appears that different NFAT proteins mediate qualitatively distinct immune responses. Whereas NFATc is essential for the induction of T_H2 responses and cytokine gene expression, NFATp and NFAT4 serve to downregulate this process.¹⁵⁻¹⁷ The combined deletion of NFATp and NFAT4 resulted in pronounced eosinophilia.¹⁵ The precise molecular basis for these opposing effects of NFAT family members is currently not clear, but it likely involves interactions with unique cofactors. This is suggested by the observations that both NFATp and NFATc can transactivate the IL-4 promoter in transient transfection assays.^{27,36} Our results demonstrate that NFAT can influence allergic immune responses by altering gene expression in eosinophils themselves.

Our observation that eosinophils constitutively express abundant NFATc is a novel finding. By using immunoprecipitation and Western blot analysis, we found that eosinophils express quantitatively similar amounts of this factor as activated T cells. Additionally, eosinophils appear to express predominantly a low-molecular-weight (~85-kd) NFATc isoform, whereas activated Jurkat T cells contain additional isoforms of greater molecular weight. This appears to reflect ongoing transcription of the gene encoding NFATc in eosinophils because these cells constitutively express NFATc mRNA. In T cells multiple NFATc isoforms arise because of alternative splicing and polyadenylation of a common gene product, and emerging data suggest that these isoforms can differentially regulate gene transcription (Table I).^{21,24,25} The approximately 85-kd isoform expressed by eosinophils appears to be comparable with the isoform NFATc/A, which is preferentially expressed in effector T cells and possesses the highest transactivation potential for the intact IL-4 promoter.²⁴

T cells and their derived cytokines are now known to be major mediators of allergic diseases.³⁷ As we gain a better understanding of the molecular mechanisms that regulate cytokine gene expression, it is becoming possible to define the role of distinct signaling molecules and transcription factors in allergic inflammation. For example, the expression of proallergic cytokines from the *IL4* gene cluster in T_H2 cells is now known to require the coordinated actions of multiple factors, including NFAT, GATA-3, and Stat-6.^{38,39} Nakamura et al⁴⁰ recently reported that the expression of GATA-3 was increased in the lower airway in allergic asthmatic subjects, as well as in the upper airway after allergen challenge.⁴¹ Interestingly, at least some major basic protein-positive cells expressed GATA-3.⁴⁰ In addition, we recently found that preferential acti-

vation of NFATc correlated with mouse strain susceptibility to allergic inflammation (Steve Georas, MD, In press). These studies link the expression, activation, or both of transcription factors with allergic responses. Studying the regulation of these proallergic transcription factors in T cells and eosinophils should be a productive area for future research.

In T cells nuclear translocation of NFAT proteins is mediated by T-cell receptor-induced calcium signals, which activate the phosphatase calcineurin (Fig 5). Calcineurin dephosphorylates cytoplasmic NFAT, unmasking a nuclear localization sequence.⁴²

Although eosinophils have been reported to be sensitive to cyclosporine A,⁴³ the major target of which is calcineurin in other cell types,⁴⁴ the function and expression of this phosphatase in eosinophils has not been well studied. Interestingly, Jinquan et al¹⁸ recently reported that IL-4 and IL-5 were able to induce the nuclear DNA-binding ability of NFATp (NFAT1) in eosinophils obtained from normal subjects. We also found that these cytokines were sufficient to induce the nuclear translocation of NFATc (Figs 4 and 5). Taken together, our studies have defined a novel mechanism of activating NFAT, but whether this is directly mediated by cytokine receptor signaling remains to be determined. Of note, Jinquan et al¹⁸ did not detect NFATc mRNA or protein in either resting or activated eosinophils. At least 2 possibilities might explain this apparent discrepancy between our two studies. First, this could reflect differences in subject populations because we used eosinophils isolated from mildly atopic donors, whereas Jinquan et al studied eosinophils obtained from normal donors. Second, the oligonucleotide probe used by Jinquan et al corresponded to the IL-2 promoter distal NFAT site and has been shown to bind NFATc with an approximately 5- to 10-fold lower affinity than NFATp, unlike the IL-4 promoter probe used in our studies.⁴⁵ Future experiments will be needed to explore these possibilities.

We detected the formation of 3 specific DNA-binding complexes by using eosinophil nuclear extracts with the high-affinity P1 NFAT-binding site from the human IL-4 promoter in EMSAs. The identity of the low-mobility complex I, which appears to contain a sequence-specific factor, is currently unknown. Because we have not observed a complex of similar mobility with this oligonucleotide in EMSAs with extracts obtained from a variety of cell types, including T cells, B cells, mast cells, and basophils (unpublished observations), it may represent an eosinophil-specific factor. Interestingly, we found that nuclear extracts obtained from unstimulated eosinophils contained NFATp that was capable of binding to the P1 element. Because this element enhances IL-4 promoter activity,⁴⁶ our data provide a plausible molecular explanation for the recent observation that eosinophils constitutively transcribe the *IL4* gene.⁵ Additionally, our finding that stimulation with calcium ionophore did not further increase NFAT binding or nuclear translocation is consistent with the observation that ionomycin did not increase eosinophil IL-4 production.⁵

The prominent, high-mobility, DNA-binding complex IV observed by using EMSAs contains HMGI/Y or related factors. This family of nonhistone chromosomal proteins can regulate gene transcription by binding A-T rich stretches in the minor groove of target promoters²⁸ and has not been previously reported in eosinophils. Interestingly, HMGI/Y has been shown to bind the IL-4 P1 element and inhibit the binding of NFAT.²⁶ This likely explains the relatively weak binding of NFATp (and absent binding of NFATc) observed in our experiments.

NFAT can regulate the expression of genes other than cytokines. For example, it is now known that NFAT can regulate apoptosis by inducing the expression of CD95.⁴⁷ Because eosinophils undergo apoptosis in response to CD95 cross-linking,⁴⁸ defective CD95 expression might render eosinophils resistant to apoptosis in NFAT-deficient mice and might help explain the impressive eosinophilia noted in NFATp- and NFAT4-deficient mice.¹⁶ In addition, expression of the genes encoding both eosinophil-derived neurotoxin and eosinophil cationic protein is controlled in part by NFAT sites contained within intronic enhancer elements.⁴⁹ Thus NFAT, in addition to GATA proteins,⁵⁰ can regulate the expression of eosinophil granule proteins. Studying the expression and function of NFAT in eosinophils should enhance our understanding of many aspects of eosinophil biology.

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