

Dependence of mast cell IgE-mediated cytokine production on nuclear factor- κ B activity

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Background: The transcription factor nuclear factor- κ B (NF- κ B) has been implicated in the regulation of a number of inflammatory cytokines and has been the proposed target for anti-inflammatory therapeutics.

Objective: Our purpose was to explore the role of NF- κ B in the regulation of allergic inflammation.

Methods: To determine whether NF- κ B is activated during IgE-mediated reactions and what types of mediators it regulates, a mutant form of I κ B was used to block the ability of NF- κ B to translocate to the nucleus and promote the transcription of selected genes.

Results: Mouse bone marrow–derived mast cells stimulated by IgE receptor cross-linking exhibited an activation of NF- κ B as assessed by electrophoretic mobility shift assays. Transfected mast cells expressing the mutant I κ B showed very little NF- κ B activation. Both control and transfected cells released β -hexosaminidase after specific antigen challenge, and this release could be potentiated by exogenous adenosine. Transfected mast cells that failed to develop NF- κ B activation did not produce IL-6 messenger RNA or protein after IgE-mediated stimulation, but these cells retained the ability to produce transcripts for IL-4 and IL-5 in spite of the suppression of NF- κ B activity. **Conclusions:** It appears that NF- κ B is activated during IgE-mediated allergic inflammation and that this activity is necessary for the production of IL-6, but not IL-4 or IL-5. When considering the use of agents that target NF- κ B to reduce inflammatory processes, it is important to know precisely which cytokines are under its control. (*J Allergy Clin Immunol* 2000;105:500-5.)

Key words: Cytokines, transcription factors, mast cells

Nuclear factor- κ B (NF- κ B) is a ubiquitous protein transcription factor that enhances the transcription of a variety of genes.¹ Many of these genes encode molecules important in inflammatory processes, such as cytokines, adhesion molecules, and growth factors. The human cytokines, TNF- α , IL-1 β , IL-2, IL-6, IL-8, GM-CSF, and

Abbreviations used

DNP:	Deoxyribonucleoprotein
EMSA:	Electrophoretic mobility shift assay
mRNA:	Messenger RNA
NF- κ B:	Nuclear factor- κ B
RT:	Reverse transcription

RANTES, among others, appear to be regulated to some degree by NF- κ B.² NF- κ B itself can be activated by a number of stimuli including viruses, bacterial endotoxin, TNF- α , and IL-1 β .³

A number of approaches have been used to study NF- κ B activation and its role in the inflammatory response. Unstimulated NF- κ B is found in the cytoplasm bound to I κ B. On stimulation, I κ B is phosphorylated on one or more serine residues, ubiquitinated, and degraded, allowing the transport of NF- κ B to the nucleus to bind recognition elements in the upstream promoter region of cytokine DNA.⁴ A mutant form of I κ B with 2 key serine residues, serines 32 and 36, mutated to alanines has been produced.⁵ This mutant I κ B cannot be phosphorylated and degraded, thus inhibiting the activation of NF- κ B.

The role of NF- κ B activation and its regulation of cytokine production in allergic inflammation is poorly understood. Another transcriptional activator, NFAT, has been shown to be induced by the cross-linking of cell surface IgE receptors on RBL-2H3 cells.⁶ This is purported to be involved in the regulation of TNF- α and IL-2 genes in mast cells. A low level of the induction of NF- κ B promoter luciferase by Fc ϵ RI stimulation has been reported recently as well.⁷ The distinct regulation of mast cell–derived cytokines by NF- κ B is yet to be reported. NF- κ B has been demonstrated to be inhibited by glucocorticoids by one or more mechanisms. Glucocorticoids activate the production of I κ B- α , resulting in down-regulation of NF- κ B.⁸ Glucocorticoid receptors may also interact directly with an NF- κ B subunit, Rel A, and inhibit DNA binding.⁹ Glucocorticoids are also potent anti-inflammatory agents with a myriad of effects on mast cell function. Thus, in the studies described here, we investigated the ability of mast cell IgE receptor cross-linking to activate NF- κ B and the importance of its activation in the production of selected mast cell–derived cytokines as well as preformed mast cell mediators.

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METHODS

Preparation of mast cells

Bone marrow from the femurs and tibias of BALB/c mice was cultured in RPMI medium containing 10% FCS, 0.1 mmol/L nonessential amino acids, 50 μ mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 μ g/mL streptomycin, 100 U/mL penicillin, and 15% WEHI-3 supernatant as a source of IL-3. By day 21 of culture with weekly passaging, cells were more than 95% viable pure mast cells.¹⁰

Electrophoretic mobility shift assay

Nuclear or whole cell extract (7–10 μ g of protein) was incubated under conditions detailed elsewhere.¹¹ Reaction mixtures were run on 7% polyacrylamide gels and exposed to x-ray film at -70°C . Band density was quantified with use of a Pharmacia LKB densitometer and NIH Image software. The probe for NF- κ B used in the electrophoretic mobility shift assay (EMSA) was purchased from Promega as part of their Gel Shift Assay System with the following consensus oligo sequence: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. The sense oligonucleotide was labeled with polynucleotide T4 kinase and γ -phosphorus 32-labeled ATP and annealed to unlabeled complementary oligonucleotide.

Transfection of mast cells

The I κ B substitution mutant where serines 32 and 36 are mutated to alanines is described in detail elsewhere.⁵ This plasmid DNA (pI κ B) and a commercially available plasmid to aid in selecting successfully transfected cells magnetically (pHook-2, Invitrogen) were used in mast cell transfections. In 100-mm Petri dishes 1.5 mL of RPMI medium and 36 μ L of DMRIE-C reagent (Gibco BRL), a liposome formulation, were mixed with 30 μ g of pI κ B and 4 μ g of pHook-2 for 30 minutes at room temperature. A total of 10×10^6 mast cells per 0.6 mL were added and incubated for 4 hours in a carbon dioxide incubator at 37°C . Mast cell culture media (6 mL) was added to each dish. After 4 days cells were centrifuged and resuspended in 2 mL of media, and magnetic beads were added as per protocol. After 2 hours at 37°C , cells were selected magnetically. A transfection efficiency of 2% to 4% was commonly seen. Transfection with pHook-2 alone was used for negative control cells.

Assessment of mast cell mediators

Mouse bone marrow-derived mast cells were passively sensitized with antideoxyribonucleoprotein (DNP) IgE (0.5 μ g/ 10^6 cells) at 37°C for 30 minutes in Tyrode's buffer lacking Ca^{++} and Mg^{++} . Cells were washed, resuspended in Tyrode's buffer, and challenged with DNP-BSA antigen (100 ng/ 3×10^5 cells) for 10 minutes. The release of β -hexosaminidase was quantified in cell supernatants and pellets by the hydrolysis of *p*-nitrophenyl- β -D-glucosamide, as detailed elsewhere.¹² Mast cell IL-6 production was assessed in cells sensitized and challenged as described above except that cells were challenged in RPMI medium containing 5% FCS and allowed to remain at 37°C for 16 hours. Cell supernatants were frozen at -70°C for 24 hours or more before assessment of IL-6 production with a commercially available mouse IL-6 ELISA kit (Endogen).¹³

Assessment of cytokine transcripts by reverse transcription-PCR

Mast cells (2.5×10^5 /200 μ L) were challenged for 90 minutes at 37°C and washed with PBS, and pellets were stored at -20°C . RNA was extracted from cells with use of the Invitrogen SNAP kit and half the reaction amount was reverse transcribed with the reagents and conditions in the Invitrogen complementary DNA cycle kit for reverse transcription (RT)-PCR. PCR was performed on the complementary DNA samples with the following primers and protocols: IL-

4 primers, CGA AGA ACA CCA CAG AGA GTG AGC T and GAC TCA TTC ATC ATG GTG CAG CTT ATC G, 30 cycles, annealing temperature 60°C , 180 bp; IL-5 primers, ATG ACT GTG CCT CTG TGC CTG GAG C and AGG CTT CTC CAG TGA CCC TGA TAC, 38 cycles, annealing temperature 63°C , 216 bp; IL-6 primers, GCC AGA GTC CTT CAG AGA GAT ACA G and CCC AAC GAT TCA TAT TGT CAG, 30 cycles, annealing temperature 55°C , 343 bp.

In some experiments primers for actin (molecular weight product = 872) were included in the PCR mixtures to ensure equal amounts of DNA per lane. Products were electrophoresed on 1.4% agarose gels.

Statistical analysis

Statistical significance was assessed with the Student *t* test. Gels were evaluated by densitometric scanning with the NIH Image software with comparisons made to the appropriate β -actin controls. Depicted are means $\pm$ SEM unless otherwise indicated.

RESULTS

Mouse bone marrow-derived mast cell NF- κ B activity was assessed by an EMSA. Control mouse bone marrow-derived mast cells exhibited an activation of NF- κ B within 10 minutes after antigen challenge that was maximal between 20 and 40 minutes later (Fig 1). With use of the 30-minute time point for Fig 2, normal mast cells or those transfected with pHook-2 alone exhibited a 2- to 3-fold increase in radiolabeling with the NF- κ B oligonucleotide probe after sensitization with anti-DNP IgE and challenge with DNP-BSA antigen. Cells transiently transfected with the mutant I κ B construct demonstrated a marked reduction in NF- κ B activation under the same experimental conditions with radiolabeling less than 1.5 times control values after antigen challenge. Thus the release of NF- κ B from its inhibitory subunit appears to be blocked in the cells transfected with the I κ B where selected serines have been mutated to alanines.

To evaluate the importance of NF- κ B activation on early mast cell secretory events, normal cells and mutant I κ B transfected cells were sensitized with anti-DNP IgE and challenged with DNP-BSA for 10 minutes. The release of β -hexosaminidase, a granule-associated mediator released in parallel to histamine, was identical in the 2 cell populations (Fig 3), suggesting that optimal NF- κ B activation is not necessary for mast cell degranulation to occur. Furthermore, the mediator release potentiator, exogenous adenosine,¹⁴ enhanced the release of β -hexosaminidase in both control and I κ B mutant transfected cells ($P < .05$). Thus even nearly maximal mast cell degranulation does not require NF- κ B activation. However, one would be more likely to predict that NF- κ B regulates the later production of cytokines or similar generated mast cell mediators. To address this question, we studied the production of 3 important mast cell derived cytokines, IL-4, IL-5, and IL-6.

Because IL-6 is abundantly produced by stimulated mast cells and has been reported to be at least partially regulated by NF- κ B,¹⁵ we first measured IL-6 transcript production by RT-PCR 90 minutes and 16 hours after antigen challenge. IL-6 RNA levels were strongly increased in activated cells at the 90-minute time point

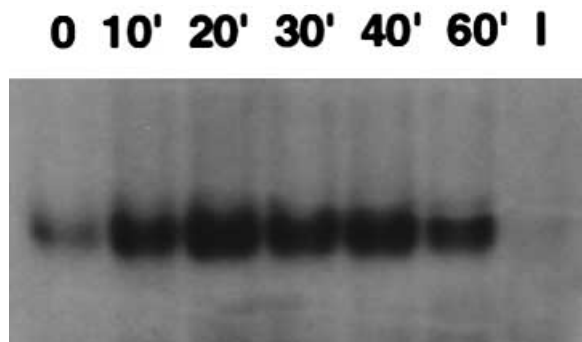


FIG 1. Time course of NF- κ B activation in mouse bone marrow-derived mast cell extracts as assessed by gel shift assays. Time 0, Unstimulated mast cells incubated with ^{32} P-labeled NF- κ B oligo. Times 10 to 60 minutes, times after antigen challenge. I, Addition of unlabeled NF- κ B competitor oligo to reaction mixture at 30-minute time point.

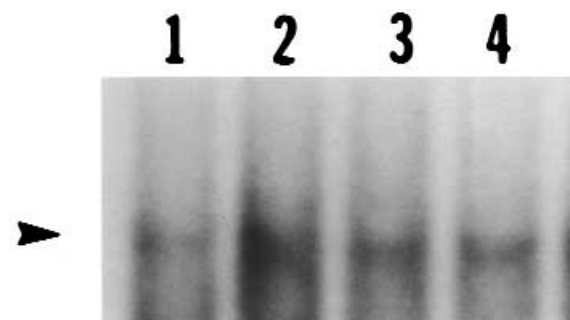


FIG 2. EMSA assay for NF- κ B activity in bone marrow mast cells challenged with DNP antigen. Lane 1, Resting cells; lane 2, cells 30 minutes after antigen challenge; lane 3, resting cells transfected with mutant I κ B; lane 4, cells transfected with mutant I κ B 30 minutes after antigen challenge. With use of densitometric scanning, change in NF- κ B activity between lanes 1 and 2 is statistically significant ($P < .05$) but difference between lanes 3 and 4 is not ($P > .15$), $n = 4$.

and were approaching basal levels by 16 hours (Fig 4). When cells were transfected with the mutant I κ B plasmid and challenged with antigen, IL-6 RNA levels increased only modestly (Fig 5), suggesting that NF- κ B activation was necessary for optimal IL-6 production. To confirm the effect of the mutant I κ B on IL-6 protein production, IL-6 protein levels were measured at a time point 16 hours after mast cell stimulation. IL-6 protein was clearly elevated in antigen-stimulated cells ($P < .0001$) but much less so in those transfected with mutated I κ B (Fig 6).

IL-4 and IL-5 have been shown to be produced by mast cells after high-affinity IgE receptor cross-linking, but the transcriptional regulation of each of these cytokines has not been linked to NF- κ B.¹⁶ Challenge of sensitized mast cells with specific antigen induced an increase in both IL-4 (Fig 7) and IL-5 (Fig 8) transcripts at 90 minutes as assessed by RT-PCR. Cells successfully transfected with the I κ B mutant retained the ability to produce IL-4 and IL-5 transcripts at a level similar to that seen in control cells.

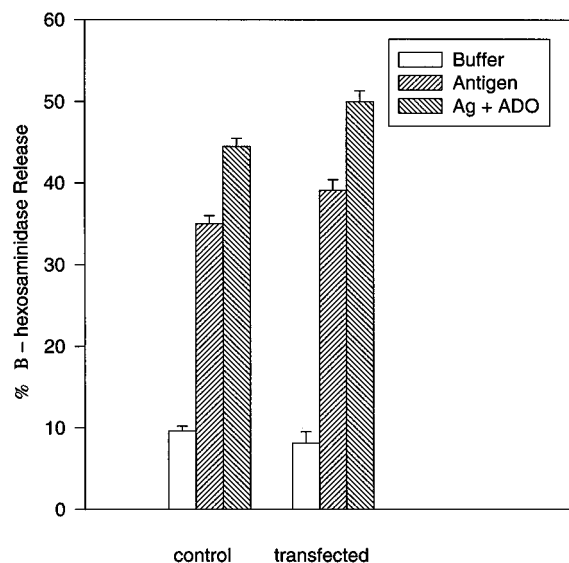


FIG 3. Effect of inhibition of NF- κ B activation on mast cell β -hexosaminidase release. Sensitized bone marrow mast cells transfected with pHook-2 alone or with mutant I κ B plasmid plus pHook-2 were stimulated with specific antigen or antigen (Ag) plus 10 μ M/L adenosine (ADO) and β -hexosaminidase release from mast cell granules was assessed 10 minutes later. Stimulation of β -hexosaminidase release by antigen was significant in both control and transfected cells ($P < .001$ in each case), and potentiation of β -hexosaminidase release by adenosine above antigen-stimulated levels was significant in both groups ($P < .05$ in each case), $n = 3$.

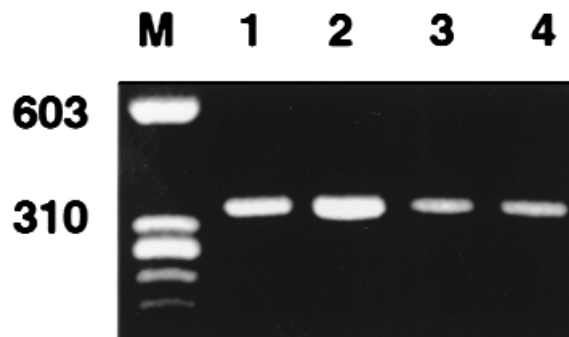


FIG 4. Time course of induction of IL-6 messenger RNA (mRNA) transcript production by IgE-mediated mast cell activation. Sensitized mast cells were challenged with antigen followed 90 minutes or 16 hours later by RT-PCR with IL-6-specific primers. Lane 1, Control cells at 90 minutes; lane 2, controls cells + antigen at 90 minutes; lane 3, control cells at 16 hours; lane 4, control cells + antigen at 16 hours. Antigen-stimulated cells produced more IL-6 mRNA at 90 minutes than control cells did ($P = .008$), $n = 4$.

DISCUSSION

Many stimuli have been shown to activate NF- κ B, including inflammatory cytokines, viruses, and phorbol esters.³ These stimuli appear to directly or indirectly activate protein kinases that lead to the phosphorylation of I κ B.¹⁷ At least 2 of these I κ B kinases have been identified, and these may be future targets for drugs that treat inflammatory or autoimmune diseases. In the realm of allergic inflammation it has been reported that Fc ϵ RI cross-linking on mouse bone marrow-derived mast cells

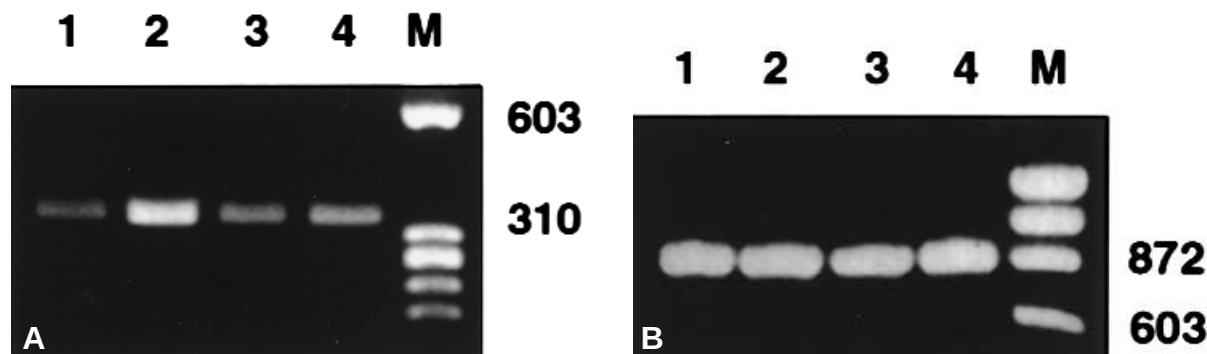


FIG 5. Effect of mutating I κ B on IL-6 mRNA production after antigen challenge. **A**, Control mast cells or cells transfected with mutant I κ B were challenged with antigen, followed 90 minutes later by RT-PCR with IL-6-specific primers. Lane 1, Control cells; lane 2, control cells + antigen; lane 3, plkB transfected cells; lane 4, plkB transfected cells + antigen (n = 4). **B**, RT-PCR with actin-specific primers with cells from same conditions described above in lanes 1 to 4. Antigen induced a 59% $\pm$ 6% increase in IL-6 mRNA ($P < .01$) in control cells and a 14% $\pm$ 9% increase in IL-6 mRNA in I κ B ($P > .10$).

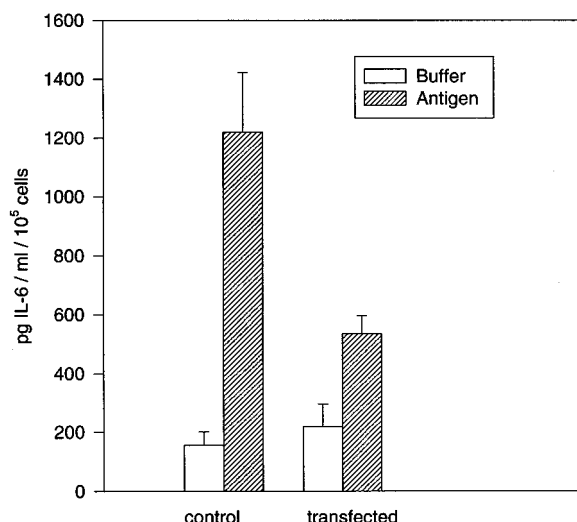


FIG 6. Effect of alteration in NF- κ B activity on mast cell IL-6 protein production. IL-6 production by control and mutant I κ B transfected cells was assessed 16 hours after buffer or antigen challenge. IL-6 protein in cell supernatants was assessed by ELISA. The increase in IL-6 production induced by antigen in control cells was markedly suppressed by transfection with the I κ B mutant ($P < .002$), although transfected cells still exhibited a modest increase in IL-6 production with antigen challenge ($P < .01$), n = 4.

may induce a modest activation of NF- κ B, but the importance of this activation is incompletely understood.⁷ The activation of another transcription factor, NFAT, by Fc ϵ RI cross-linking has been reported as well, and NFAT appears to strongly regulate IL-2 and TNF- α expression in mast cells.⁶ The activity of NFAT may be dependent on Bruton's tyrosine kinase activation, whereas NF- κ B activation in mast cells appears to be relatively independent of Bruton's tyrosine kinase.⁷ NFAT activation may require calcium mobilization and in some rodent mast cells may be blocked by cyclosporin A.¹⁸

There is considerable evidence that transcription factors often act in concert to regulate cytokine, adhesion molecule, or chemokine genes. For example, cooperative binding between NF- κ B and NF-IL6 may be required for

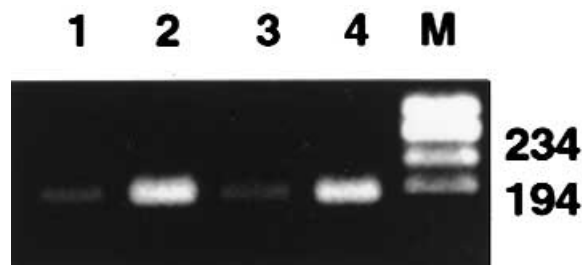


FIG 7. Effect of mutating I κ B on IL-4 mRNA production after antigen challenge. Control mast cells or cells transfected with mutant I κ B were challenged with antigen, followed 90 minutes later by RT-PCR with IL-4-specific primers. Lane 1, Control cells; lane 2, control cells + antigen; lane 3, plkB transfected cells; lane 4, plkB transfected cells + antigen. Control cells stimulated with antigen produced a 40% $\pm$ 3% increase in IL-4 mRNA ($P < .01$); transfected cells stimulated with antigen produced a 33% $\pm$ 4% increase ($P < .025$), n = 3.

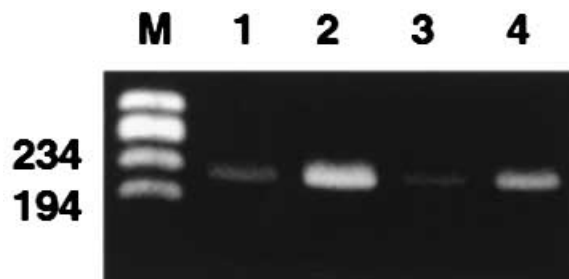


FIG 8. Effect of mutating I κ B on IL-5 mRNA production after antigen challenge. Control mast cells or cells transfected with mutant I κ B were challenged with antigen, followed 90 minutes later by RT-PCR with IL-5-specific primers. Lane 1, Control cells; lane 2, control cells + antigen; lane 3, plkB transfected cells; lane 4, plkB transfected cells + antigen. Control cells stimulated with antigen produced a 74% $\pm$ 4% increase in IL-5 mRNA ($P < .01$); transfected cells stimulated with antigen produced a 58% $\pm$ 4% increase ($P < .01$), n = 4.

the optimal transcriptional activation of IL-6 and IL-8.¹⁹ There is also evidence that some transcription factors interact antagonistically, such as the report that STAT-6 competes with NF- κ B in binding to the E-selectin promoter.²⁰ Therefore it is often difficult to predict the rela-

tive importance of a particular transcription factor in the regulation of a particular gene.

Our initial studies have served to confirm and expand the observation that NF- κ B is activated after an antigen-induced allergic response in the mast cell. This activation does not appear to be necessary for mast cells to undergo degranulation, as evidenced by normal β -hexosaminidase release from mutant I κ B transfected mast cells in the absence or presence of exogenous adenosine. However, NF- κ B activation does appear to be vital for the optimal production of certain mast cell-derived cytokines, particularly IL-6. By use of the dominant positive I κ B mutant where 2 serines are mutated to alanines, we showed that appropriate NF- κ B activation could not take place. In these studies, both IL-6 mRNA and IL-6 protein production were markedly suppressed in the mutant I κ B transfected cells after high-affinity IgE receptor cross-linking. The production of other important mast cell-derived cytokines, IL-4 and IL-5, was unaffected by this manipulation.

Because there is increasing support for the concept that NF κ B plays a pivotal role in orchestrating the inflammatory response,²¹ often in cooperation with other transcription factors, there is a need to understand the importance of NF- κ B in specific inflammatory processes such as immediate hypersensitivity. NF- κ B activation in asthmatic sputum and bronchial biopsy specimens has been reported.²² The fact that NF- κ B does not regulate IL-5 production in mast cells is of interest, particularly because NF- κ B has been postulated to regulate eotaxin,²³ a potent eosinophil-specific chemokine. The exact importance of IL-5 in asthma is very controversial,²⁴ but some have suggested that it is critical for airway eosinophilic inflammation to occur.²⁵ One transcription factor strongly associated with IL-5 production is GATA-3, which may be dysregulated in atopic asthma.²⁶ The fact that NF- κ B does not appear to regulate IL-4 expression is of interest as well in that IL-4 is a key component to the development of IgE production.²⁷ IL-4 also activates STAT-6 (signal transduction and transactivation) in human umbilical vein endothelial cells, and STAT-6 competes with NF- κ B binding to the E-selectin promoter.²⁰ In the human IL-4 promoter NFAT and NF- κ B may bind in a mutually exclusive fashion,²⁸ but NFAT activation is sufficient for maximal IL-4 promoter activity. Therefore the possibility could be entertained that a failure to activate NF- κ B could allow for an increase in IL-4 production because of a loss of promoter competition with NFAT. This was not evident in our studies, probably because this competition is much less apparent in the mouse than the human.

Although the complex interplay of transcription factors and cytokine gene regulation in the mast cell is only beginning to be clarified, the ability to selectively inhibit a particular transcription factor and thus a particular complement of cytokines should provide useful insights into the transcriptional regulation of allergic inflammation. As investigators try to target I κ B kinases and modulate NF- κ B activity, it will be critical to understand the

magnitude of NF- κ B regulation of many types of inflammation to provide useful therapeutic approaches to many common medical problems.²¹

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