

Allergen activates peripheral blood eosinophil nuclear factor- κ B to generate granulocyte macrophage-colony stimulating factor, tumour necrosis factor- α and interleukin-8

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Summary

Background Allergic inflammation is characterized by the influx and activation of eosinophils. Cytokines generated by both resident and infiltrating cells are responsible for the initiation and maintenance of this pathogenesis. This study focuses on allergen-induced activation of eosinophil NF- κ B and generation of granulocyte macrophage-colony stimulating factor (GM-CSF), TNF- α , and IL-8.

Methods Peripheral blood eosinophils were enriched to >99.9% by Percoll gradient sedimentation and negative magnetic affinity chromatography. NF- κ B activation by 10 μ g/mL house dust mite (HDM) extract was demonstrated immunocytochemically using a monoclonal antibody against the active form of NF- κ B (NF- κ B_a). The authenticity of NF- κ B was confirmed by Western blot. Cytokine production was assessed both by immuno-staining of eosinophils and by assay of cytokines in the cell supernatant.

Results Activation of peripheral blood eosinophils from atopic, but not non-atopic, donors induced activation of NF- κ B, which peaked at 4 h and was accompanied by a decline in I κ B- α . The activation of authentic NF- κ B was confirmed in gel shift assays. Supershift assays showed p65 to be the major subunit of eosinophil NF- κ B. Immunofluorescent confocal microscopy demonstrated localization of NF- κ B_a to the nucleus. Following activation, cytokine immunoreactivity was seen in a fraction of the eosinophils and cytokines were released into the supernatant. The NF- κ B inhibitors, calpain inhibitor 1 (10 μ M), pentoxifylline (0.5 mM), pyrrolidine dithiocarbamate (PDTC, 10 μ M) or gliotoxin (1 pg/mL) reduced the generation of GM-CSF, TNF- α and IL-8 in parallel with their inhibition of NF- κ B.

Conclusions HDM allergen activates human eosinophil NF- κ B leading to the production of the cytokines GM-CSF, TNF- α and IL-8. We speculate that a role for eosinophil NF- κ B-dependent cytokines is to act as an autocrine loop augmenting the survival of eosinophils *in vivo*.

Keywords eosinophil, GM-CSF, human, NF- κ B, TNF- α and IL-8

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Introduction

Eosinophilia, both in the blood and the tissues, has long been recognized as a hallmark of allergic disease [1]. Eosinophils are potent pro-inflammatory cells, which release numerous granular cationic proteins, produce reactive oxygen species

and generate lipid mediators, particularly LTC₄ [2]. In addition, eosinophils have the potential to participate within cell networks via the release of cytokines, and thus are able to modulate the resultant inflammatory response. Double immunocytochemical procedures have revealed the co-expression of IL-5 and IL-10, but not of IL-5 and IFN- γ , in human eosinophils [3], which is indicative of the existence of subpopulations of eosinophils expressing type 1 and 2 cytokine profiles (reviewed in [4]).

Nuclear factor kappa B (NF- κ B) is a ubiquitous transcription factor, classically presented as a heterodimer comprising of p50 and p65, each of which contains the 300 amino acid NF- κ B/rel/dorsal domain (NRD) [5]. The N-terminal of the NRD binds to its DNA binding motif [6] while the C-terminal contains the nuclear location signal (NLS) necessary for translocation of NF- κ B across the nuclear membrane [7]. In resting cells, NF- κ B is present in the cytoplasm in an inactive

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form reversibly bound to one of the I κ B family members [8]. Upon activation, I κ B is hyperphosphorylated [9] and its subsequent proteolysis reveals the NLS of NF- κ B. This allows NF- κ B to translocate into the nucleus where it is able to bind to its decameric DNA sequence in the promoter regions of genes, which contain it. The activation of NF- κ B is transient as this event also leads to the transcription of I κ B, which forms complexes with NF- κ B, which are transported back into the cytoplasm [10]. We show here activation of NF- κ B and the subsequent production of TNF- α , granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-8 by house dust mite (HDM) allergen in peripheral blood eosinophils purified from non-atopic and atopic donors.

Materials and methods

Donors

Human peripheral blood was obtained from healthy volunteers both with and without a history of allergy status. To confirm or reject the presence of atopy, individuals were skin prick tested with an aqueous extract of HDM (*Dermatophagoides pteronyssinus*), with histamine (10 mg/mL) and vehicle as positive and negative controls, respectively. Individuals were considered to be atopic if they had a positive skin prick test with a weal diameter at least 3 mm greater than the vehicle control. Blood samples were collected with approval of the Southampton and South West Joint Research Ethics Committee.

Purification of peripheral blood eosinophils

Human peripheral blood eosinophils were purified as described previously [11]. Briefly, fresh venous blood was layered onto a Percoll gradient (1.082–1.084 g/mL) and centrifuged at 400g for 30 min at 20 °C. Red blood cell pellets were lysed in ice-cold ultra-pure quality water for 45 s before adding 1.8% NaCl. Following red cells lysis the granulocytes were washed and incubated at 4 °C for 30 min with anti-CD16 MACS microbeads (Miltenyi Biotec Ltd, Bergisch Gladbach, Germany) and passed down a MACS C column (Miltenyi Biotec Ltd) to remove the neutrophils enabling eosinophils of greater than 99.9% purity to be obtained. Following purification, eosinophils were cultured at 1×10^5 cells/well in 96 tissue culture plates in humidified 95% air with 5% CO₂ at 37 °C in DMEM medium supplemented with 2 mM L-glutamine, 200 U penicillin/200 µg/mL streptomycin and 1% FCS.

Activation of NF- κ B with HDM extract

To investigate the time course of activation of NF- κ B, degradation of I κ B and the immunoreactivity for the cytokines TNF- α , GM-CSF and IL-8, 1×10^5 human peripheral blood eosinophils were incubated in medium alone (control), stimulated with HDM extract (HDM allergen including Der p 1 10.36 mg/vial and Der p 2 11.08 mg/vial kindly donated by Torii Pharmaceutical Co. Ltd, Tokyo, Japan) for 0.25, 0.5, 1, 2, 4, 6, 8, 12 or 24 h. Cells were then centrifuged at 20 g for 7 min onto poly-L-lysine-coated glass

slides and stained with monoclonal antibodies against activated NF- κ B (NF- κ B_a) (antibody 2C7 [12], donated by Pharmacia Upjohn, Kalamazoo, MI, USA), I κ B- α (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and the cytokines TNF- α (donated by Cell Tech, Slough, UK), GM-CSF (Genzyme, Cambridge, UK) and IL-8 (Alexis, Nottingham, UK). Slides were developed using an indirect peroxidase detection system with the substrate 3-amino 9-ethylcarbazole (AEC). Two hundred to three hundred cells were counted, depending upon the quality of the cytopsin, and the percentage of positive cells calculated.

Co-localization of NF- κ B_a to the nuclei of human peripheral blood eosinophils

Two channel confocal microscopy was used to produce a combined image of cytopsin preparations in which activated NF- κ B was stained with monoclonal antibody 2C7 using a biotin and avidin–fluorescein detection system (2C7-biotin-avidin-FITC) and the cell nuclei counterstained with propidium iodide. Co-localization analysis was performed and a multicolour look up table used to show the extent of co-localization of activated NF- κ B to the nucleus.

Electrophoretic mobility shift assay

Nuclear proteins were purified from blood eosinophils using the method of Dignam et al. [13]. Briefly, cell pellets were washed in PBS, cell lysis performed in Dignam buffer A containing 0.2% NP-40, the resultant slurry centrifuged, the cytoplasmic fraction removed and the pellet resuspended in 100 µL of Dignam buffer C. Aliquots of nuclear proteins were incubated with a radiolabelled NF- κ B consensus DNA sequence and the reaction mixture subjected to gel electrophoresis using a non-denaturing polyacrylamide gel. The gel was subjected to autoradiography and the heavier DNA–NF- κ B complexes identified by their slower electrophoretic migration. Supershift assays were performed by preincubating the nuclear extracts with mAbs against subunits, Rel-B, c-Rel, p50, p52 and p65 of NF- κ B (Santa Cruz Biotechnology Inc.).

Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). The difference between two means was tested for significance using Student's *t*-test for paired data. Comparisons of treatments were made by two-way ANOVA. A probability value of $P < 0.05$ was taken as statistically significant.

Results

Activation of NF- κ B

To determine the optimal concentrations of HDM extracts to activate NF- κ B, eosinophils purified from atopic donors were incubated with the allergens for 4 h. HDM extract (0.1–50 µg/mL) caused a concentration related increase in NF- κ B_a from a baseline of $6.2 \pm 1.1\%$ (medium control) to a maximum of $38 \pm 2.1\%$ at 30 µg/mL ($n = 5$). A concentration of 10 µg/mL,

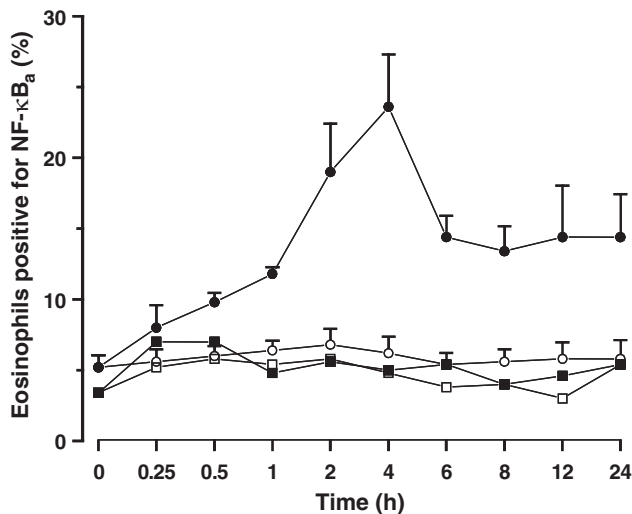


Fig. 1. Kinetic of NF- κ B activation. Eosinophils purified from non-atopic (squares) and atopic donors (circles) were incubated either in medium control (open symbols) or 10 μ g/mL house dust mite extract (closed symbols) for times up to 24 h. At the end of the incubation periods, cytospin preparations were prepared and stained immunocytochemically for NF- κ B_a. Results are expressed as mean $\pm$ SEM from five experiments.

which induced NF- κ B activation in $32.4 \pm 5.6\%$ of cells was used in further experiments.

Kinetics of NF- κ B activation

The kinetics of NF- κ B activation was explored in eosinophil preparations from five non-atopic and five atopic donors. The percentage of cells showing NF- κ B_a immunoreactivity at baseline of $3.4 \pm 0.5\%$ and $5.2 \pm 0.9\%$, respectively, were not significantly different ($P = 0.122$, t -test). When incubated with HDM extract, the percentage of NF- κ B_a immunoreactive eosinophils from non-atopic donors remained close to basal levels throughout the 24 h incubation period (Fig. 1). There was no significant difference between allergen and medium control ($P = 0.106$, ANOVA).

Eosinophils from the five atopic individuals, however, showed NF- κ B activation with a peak at 4 h (Fig. 1). At this time, the percentage of immunoreactive cells, $23.6 \pm 3.7\%$, was significantly greater than baseline ($P = 0.009$, paired t -test). The difference between total response in the presence and absence of allergen was highly significant ($P < 0.0001$, ANOVA).

I κ B immunoreactivity

The activation of NF- κ B within eosinophils should be accompanied by a fall in its inhibitory protein, I κ B. This was demonstrated in four experiments in which the percentage of eosinophils showing immunoreactivity for I κ B at baseline was $56.5 \pm 8.7\%$. Incubation of these cells with HDM extract caused an inhibition of I κ B immunoreactivity which mirrored the kinetics of the increase in NF- κ B_a immunoreactivity (Fig. 2). The peak fall in the number of I κ B positive eosinophils, to $14.3 \pm 2.2\%$ of total, occurred at 2 h ($P = 0.001$ vs. control, paired t -test). The overall difference between the two groups was highly significant ($P < 0.0001$, ANOVA).

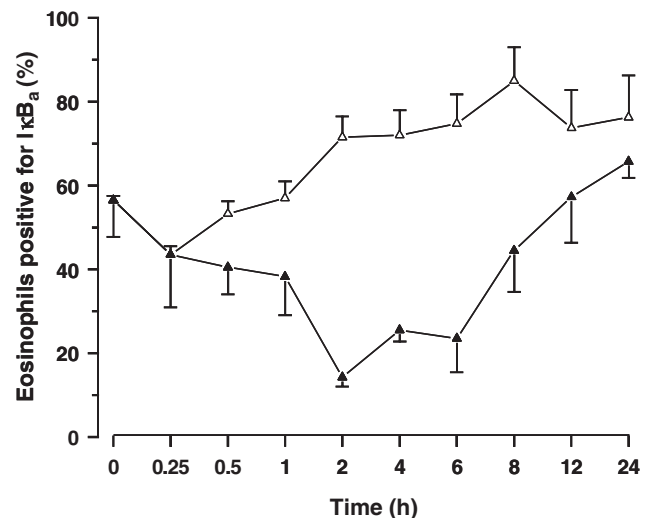


Fig. 2. Kinetics of I κ B degradation. Eosinophils purified from atopic donors were incubated either in medium control (open symbols) or 10 μ g/mL house dust mite extract (closed symbols) for times up to 24 h. At the end of the incubation periods, cytospin preparations were prepared and stained immunocytochemically for I κ B. Results are expressed as mean $\pm$ SEM from five experiments.

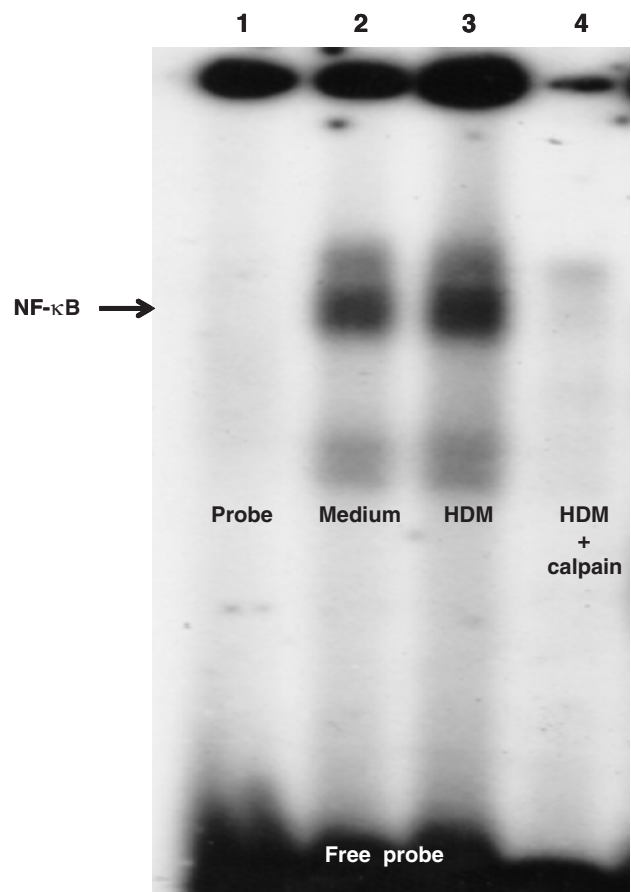


Fig. 3. Gel shift assay for NF- κ B. Eosinophils purified from atopic donors were pre-incubated either in medium or calpain-1 inhibitor (10 mM) for 1 hour before further incubation in medium alone (control) or 10 μ g/mL house dust mite extract for 4 h. Following experimentation nuclear protein was purified and incubated with a radiolabelled NF- κ B oligonucleotide binding motif, and run down a 5% acrylamide gel to determine the extent of NF- κ B activation.

Gel shift assay

To verify NF- κ B activation, a gel shift assay was used (Fig. 3) in which the electrophoretic migration of the heavier DNA–NF- κ B complexes were slower than inactive NF- κ B. Gel shift assays performed on nuclear proteins from peripheral blood atopic eosinophils demonstrated a high basal level of NF- κ B_a suggesting that these cells had already been activated *in vivo*. Activation of these cells for 4 h with HDM extract increased the extent of NF- κ B_a bound to the DNA oligonucleotide binding motif. Pre-incubation of eosinophils for 1 h with 10 μ M of the NF- κ B inhibitor calpain inhibitor 1 prevented the activation of NF- κ B induced by the allergen.

Supershift assay

To gain insight into the composition of NF- κ B_a, a supershift assay was used. Nuclear protein purified from eosinophils which had been activated with HDM extract for 4 h was incubated with antibodies for c-Rel, Rel-B, p50, p52 and p65. The results (Fig. 4) showed a slower electrophoretic migration of heavier DNA–NF- κ B–antibody complexes only with p65 (Rel-A).

Co-localization of NF- κ B to the nucleus

To visualize NF- κ B_a within the nucleus, eosinophils were incubated for 4 h in medium alone or activated with 10 μ g/mL HDM extract. Two-channel confocal microscopy was used to produce a combined image of cytospin preparations stained with 2C7 biotin–avidin–FITC in conjunction with the nuclear stain propidium iodide. Co-localization analysis using multi-colour look up tables demonstrated enhanced levels of NF- κ B_a, clearly localized within the nuclei of eosinophils following activation with allergen compared with controls (Fig. 5).

Production of cytokines following activation

The production of the NF- κ B-dependent cytokines, GM-CSF, TNF- α and IL-8, was examined by assessing both the percentage of cytokine immunoreactive eosinophils in five

experiments (Fig. 6) and cytokine production into the supernatant in six experiments (Fig. 7) following stimulation with HDM extract.

At the beginning of the incubation, $4.4 \pm 1.0\%$ of eosinophils were immunoreactive for GM-CSF. Following

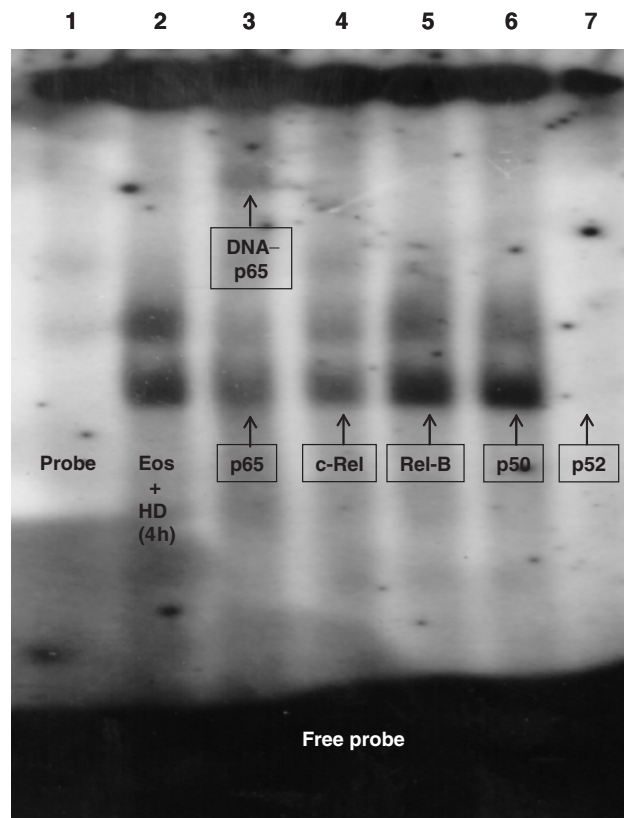


Fig. 4. Supershift assay of NF- κ B. Eosinophils purified from atopic donors were incubated in medium alone or 10 μ g/mL house dust mite extract for 4 h. Nuclear proteins were purified from these cells, incubated with monoclonal antibodies for p65, c-Rel, Rel-B, p50, p52 and then incubated with the radiolabelled NF- κ B oligonucleotide binding motif. Running the protein mAb complex down a 5% acrylamide gel allowed the components of NF- κ B to be determined.

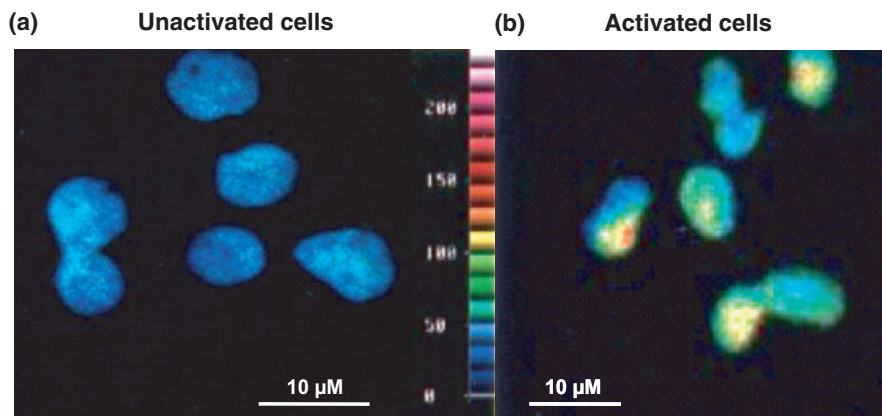


Fig. 5. Colocalization of NF- κ B to the nucleus of human peripheral blood eosinophils. Eosinophils were purified from atopic donors and incubated in medium alone (a) or 10 μ g/mL house dust mite extract for 4 h (b). Two channel confocal microscopy was used to produce a combined image of cytospin preparations stained with the mAb 2C7 in conjunction with fluorescein and the cell nuclei counterstained with propidium iodide (original magnification $\times 400$). Colocalization analysis was then performed; a multicolour look up table was used to show the extent of colocalization between NF- κ B_a and the nucleus. Enhanced levels of NF- κ B_a are clearly evident within the nuclei of those eosinophils activated house dust mite extract.

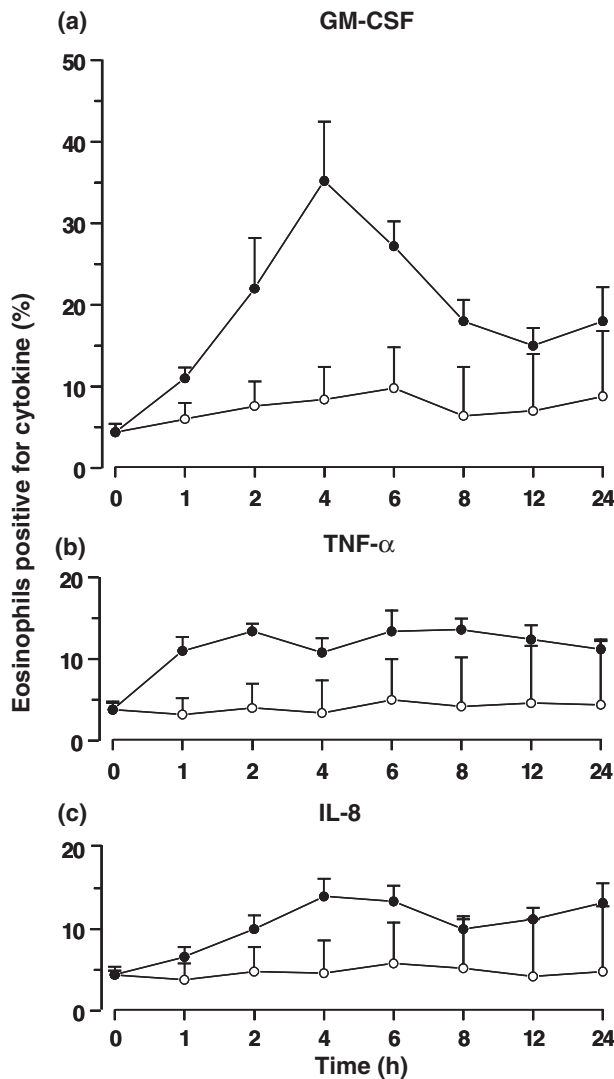


Fig. 6. Cytokine immunoreactivity in human peripheral blood eosinophils. Eosinophils purified from atopic donors were incubated either in medium control (open symbols) or 10 μ g/mL house dust mite extract (closed symbols) for times up to 24 h. At the end of the incubation periods, cytospin preparations were prepared and stained immunocytochemically for (a) GM-CSF, (b) TNF and (c) IL-8. Results are expressed as mean $\pm$ SEM from five experiments.

stimulation, this percentage rose to $35.2 \pm 7.3\%$ ($P < 0.05$, paired t -test) and then waned. In separate experiments, GM-CSF detected in the supernatant rose slowly from 1.8 ± 0.5 to 10.7 ± 3.2 pg/1 $\times 10^6$ eosinophils ($P < 0.05$). With TNF- α , $3.8 \pm 0.8\%$ cells were immunoreactive at the beginning of the experiment. This rose to $13.4 \pm 0.8\%$ ($P < 0.001$, paired t -test) at 2 h after which there were no statistically significant differences ($P = 0.462$, ANOVA). In the culture supernatant, TNF- α concentrations rose from 0.2 ± 0.1 pg/1 $\times 10^6$ eosinophils to a maximum of 16.6 ± 3.6 pg/1 $\times 10^6$ eosinophils at 12 h ($P = 0.008$, paired t -test). There were no statistically significant changes in the percentage of immunoreactive cells after this time ($P = 0.584$, ANOVA). With IL-8, the percentage of immunoreactive eosinophils rose from $4.4 \pm 0.5\%$ to $14.0 \pm 2.2\%$ at 4 h ($P = 0.01$, paired t -test). There was a continual generation of IL-8 into the super-

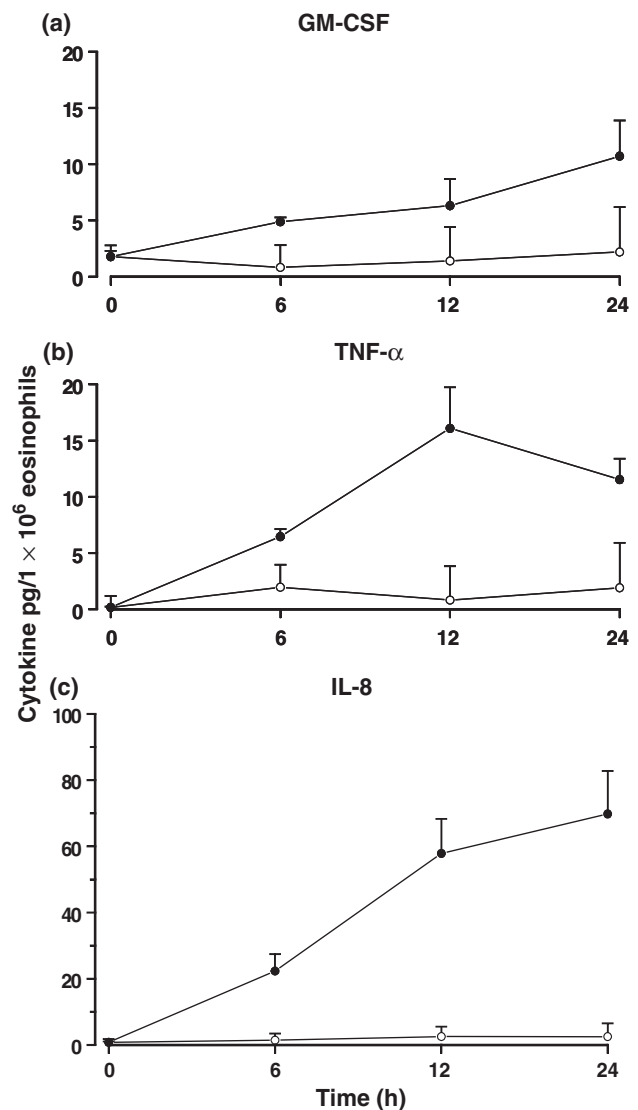


Fig. 7. Production of cytokines from human peripheral blood eosinophils. Eosinophils purified from atopic donors were incubated either in medium control (open symbols) or 10 μ g/mL house dust mite extract (closed symbols) for times up to 24 h. At the end of the incubation periods, supernatants were collected and the concentration of (a) GM-CSF, (b) TNF and (c) IL-8 determined using commercially available ELISA kits. Results are expressed as mean $\pm$ SEM from six experiments.

natant, levels rising from 0.8 ± 0.2 pg/1 $\times 10^6$ eosinophils at baseline to 69.8 ± 13.1 pg/1 $\times 10^6$ eosinophils at 24 h ($P = 0.003$, paired t -test). In all the studies described in this section, the overall difference between the preparations cultured with HDM extract or in control medium was highly significant ($P < 0.0001$, ANOVA).

Effects of NF- κ B inhibitors

In order to demonstrate a functional link between NF- κ B activation and cytokine production, eosinophil preparations were incubated for 1 h before to allergen stimulation with calpain inhibitor 1 (10 μ M), pentoxifylline (0.5 mM), pyrrolidine dithiocarbamate (PDTC, 10 μ M) or gliotoxin (1 pg/mL). The results (Table 1), show that all inhibitors significantly

Table 1. Effects of NF- κ B inhibitors on NF- κ B activation and cytokine expression

	Control	HDM	Calpain	Pentox.	PDTC	Glutoxin
NF-κB						
Positive cells (%)	4.6 $\pm$ 0.5	24.6 $\pm$ 5.3	12.8 $\pm$ 1.8	7.4 $\pm$ 1.5	6.4 $\pm$ 1.3	11.4 $\pm$ 3.3
Inhibition			48%	70%	74%	54%
Significance			$P = 0.049$	$P = 0.033$	$P = 0.043$	$P = 0.020$
GM-CSF						
Positive cells (%)	6.4 $\pm$ 0.5	28.4 $\pm$ 6.3	9.6 $\pm$ 2.5	9.4 $\pm$ 1.5	9.0 $\pm$ 1.2	15.6 $\pm$ 3.1
Inhibition			66%	67%	68%	45%
Significance			$P = 0.030$	$P = 0.052$	$P = 0.049$	$P = 0.185$
TNF-α						
Positive cells (%)	5.2 $\pm$ 0.9	12.8 $\pm$ 2.4	8.4 $\pm$ 2.0	10.8 $\pm$ 1.0	9.4 $\pm$ 1.6	8.4 $\pm$ 1.2
Inhibition			35%	16%	27%	34%
Significance			$P = 0.030$	$P = 0.052$	$P = 0.049$	$P = 0.185$
IL-8						
Positive cells (%)	4.2 $\pm$ 0.7	15.2 $\pm$ 3.2	9.8 $\pm$ 1.6	4.8 $\pm$ 1.2	9.4 $\pm$ 2.0	8.8 $\pm$ 3.0
Inhibition			36%	68%	38%	42%
Significance			$P = 0.130$	$P = 0.018$	$P = 0.154$	$P = 0.002$

Eosinophils from atopic individuals were incubated for 1 h with calpain inhibitor 1 (10 mM), pentoxifylline (Pentox., 0.5 mM), pyrrolidine dithiocarbamate (PDTC, 10 mM), glutoxin (1 μ g/mL) or medium before being incubated for a further 4 h with house dust mite extract (HDM, 10 μ g/mL) or medium alone (control). At the end of incubation, cytospin preparations were made and stained immunocytochemically for activated NF- κ B. Results are expressed as mean percentage immunoreactive cells $\pm$ SEM and percentage inhibition of the HDM response. Statistical significance was calculated using Student's test for paired data.

reduced the number of cells staining for NF- κ B_a (all $P < 0.05$ vs. HDM extract control). The NF- κ B inhibitors also reduced the number of eosinophils immunoreactive for TNF- α , GM-CSF and IL-8.

Discussion

Using a monoclonal antibody directed against NF- κ B_a, we have shown that activation of peripheral blood eosinophils from atopic individuals with HDM allergen induced an activation of NF- κ B, which was accompanied by a decline in I κ B- α . The activation of authentic NF- κ B was confirmed in gel shift assays. The use of FITC immunofluorescent techniques in conjunction with confocal microscopy, enabled the sub-cellular localization of NF- κ B_a to be confirmed in the nucleus. Finally, NF- κ B inhibitors have been shown to reduce the generation of GM-CSF, TNF- α and IL-8 in parallel with their inhibition of NF- κ B.

The observation that allergen activated eosinophils from atopic, but not non-atopic, individuals strongly suggests an interaction with cell surface antibody, both IgE and IgA being able to activate NF- κ B [14–16]. The activation of eosinophils caused a slow activation of NF- κ B, which peaked at around 4 h. The decline of I κ B- α was also slow, not becoming evident until 2 h. In this respect, eosinophils responded more slowly than human lung mast cells in which statistically significant changes in NF- κ B and I κ B- α were observed within 30 min [16]. Interestingly, even at peak response, only around a quarter of the eosinophils were NF- κ B_a immunoreactive. Whether or not this reflects eosinophil heterogeneity, 'hypodense' eosinophils having long been associated with allergy and responsiveness to IgE-dependent stimulation [17, 18] is not known. By comparison, 34% of

human lung mast cells showed NF- κ B_a immunoreactivity following stimulation with anti-IgE.

Western blotting showed that activated NF- κ B was present in unstimulated cells but was increased following incubation with allergen. The observation that pre-incubation of the cells with calpain inhibitor 1, a proteasome inhibitor which stabilizes I κ B- α [19], greatly reduced the amount of NF- κ B on the gel provides strong supportive evidence for its authenticity. Furthermore, supershift assays showed p65 to be the major subunit present. The apparent absence of p50 was a surprise as, using immunostaining, a more sensitive technique, which detects small amounts of protein, this subunit has been reported to be present in human eosinophils [20, 21]. It also contrasts with our studies in human lung mast cells where the dominant subunit was p50 with only small amounts of p65 [16].

The ability of eosinophils to store and generate the cytokines GM-CSF, TNF- α and IL-8 is well established [22–25]. The time course and number of cells immunoreactive for GM-CSF were similar to those of NF- κ B_a, suggesting that the two events are closely linked. However, with TNF- α and IL-8, less than 15% of the eosinophils became immunoreactive following challenge indicating that with these cells other factors, either other transcription factors or post-transcriptional regulatory processes are rate determining.

Cytokine secretion into the supernatant did not reflect the number of immunoreactive cells. Although anti-GM-CSF stained the greatest percentage of cells, secretion of its protein was lowest. In contrast, the percentage of eosinophils immunoreactive for IL-8 was less than half of the GM-CSF percentage and yet IL-8 secretion was almost 7-fold greater.

To gain further insight into the dependency of cytokine production on NF- κ B activation, parallel studies were performed to assess the percentage of cells immunoreactive for NF- κ B_a, GM-CSF, TNF- α and IL-8 cells following incuba-

tion with four NF- κ B inhibitors with different mechanisms of action. Calpain inhibitor 1, proteasome inhibitor [19], pentoxifylline, a Type 5/6/9 phosphodiesterase inhibitor with actions on protein kinase C [26], pyrrolidine dithiocarbamate, an anti-oxidant with possibly multiple intracellular effects [27] and gliotoxin, a proteasome inhibitor of fungal origin [28] were all inhibitory. Although not always correlated quantitatively, probably due to the cross-talk with other transcription factors [29–31], these data further support the role of NF- κ B activation in eosinophil cytokine production.

Although it was not the aim of these experiments to investigate the role of these cytokines in allergic inflammation, not only are GM-CSF, TNF- α and IL-8 generated by eosinophils but they are also involved in the recruitment, priming and the survival of eosinophils [32, 33]. In addition, inhibition of these cytokines reduces the number of activated eosinophils following allergen exposure [34]. Thus, it is tempting to speculate that, as suggested for NF- κ B-dependent cytokines in mast cells [16] a function of NF- κ B-dependent eosinophil cytokines is as an autocrine loop augmenting the survival of eosinophils *in vivo*.

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