

Basophils express a type 2 cytokine profile on exposure to proteases from helminths and house dust mites

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Abstract: The proteolytic activities frequently associated with sources of allergens and parasite secretions have been suggested as important immunomodulators. We have investigated whether the protease activity of the house dust mite allergen Der p1 and the secreted proteases of the hookworm *Necator americanus* are able to directly induce type 2 cytokine production by basophils. Der p1 and the secretions of *N. americanus* induced interleukin (IL)-4, IL-5, and IL-13 but not interferon- γ mRNA in KU812 basophils. Enzyme-linked immunosorbent assay confirmed that IL-4 and IL-13 were secreted. A nonproteolytic antigen failed to induce cytokine expression, and preincubation of Der p1 or *N. americanus* secretions with protease inhibitors inhibited cytokine expression. Data were confirmed using basophils purified from human peripheral blood. We speculate that this innate mechanism may contribute to the development of a cytokine milieu that could promote immunoglobulin E synthesis, eosinophil recruitment, and the development of type 2 T cells. *J. Leukoc. Biol.* 73: 165–171; 2003.

Key Words: hookworm, allergy, *Dermatophagoides pteronyssinus*

INTRODUCTION

Immune responses to environmental allergens and parasitic helminths are often characterized by high levels of immunoglobulin (Ig)E antibodies, eosinophilia, and a dominance of T cells that secrete a type 2 profile of cytokines [1]. However, only a small number of proteins from these organisms consistently sensitize the immune system to make these allergic-immune responses. It has been speculated that certain nonimmunological, biochemical activities associated with environmental allergens and parasite secretions may cause them to act as proallergic adjuvants [2, 3]. Accordingly, we and others have characterized the proteolytic activity of Der p1, the group I allergen of the house dust mite *Dermatophagoides pteronyssinus* [4, 5] and shown that it cleaves the low-affinity IgE receptor (CD23) from human B cells [4, 6] and the α subunit of the interleukin (IL)-2 receptor from T cells [7]. Others have shown that proteases present in house dust mite faecal pellets cause changes in the permeability of epithelial cells [8, 9], Der

p1 in particular, degrading key molecules in tight junctions and desmosomes of epithelial cells at very low concentrations [10].

We have also shown that the excretory-secretory (ES) products of an intestinal parasite of humans, the hookworm *Necator americanus*, are also rich in proteases [11] and may also be causally associated with typically allergic-immune responses to the parasite.

The development and polarization of type 2 T cells and the development of IgE-secreting B cells are strongly driven by IL-4 and IL-13, respectively [12, 13]. The finding that human basophils produced IL-4 and IL-13 after IgE-dependent stimulation [14–18] suggested that these cells may be able to maintain the T helper cell type 2 dominance of established allergic-immune responses. Even in the absence of IgE, a rat basophil line was activated by enzymatically active components of bee venom, house dust mite spent growth medium, and the secretions of *Schistosoma mansoni* [19]. Thus, via the enzymatic activity of these allergens, basophils may influence new and developing T cell responses in individuals without allergen-specific, cytophilic IgE. In this study, we have investigated the hypothesis that human basophils release T cell polarizing cytokines in the presence of proteolytically active allergens from house dust mites and helminths.

MATERIALS AND METHODS

KU812 cell line

KU812 cells (European Collection of Cell Cultures, Salisbury, Wiltshire, UK) were grown in RPMI-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Sigma Chemical Co., Poole, UK) and 1x Glutamax (Life Technologies) in a 5% CO₂, 100% humidity atmosphere at 37°C.

Human peripheral blood basophils

Basophils were immunomagnetically purified using a magnetic cell sorter basophil isolation kit from 100 ml samples of heparinized peripheral blood. Blood was obtained from normal, healthy donors with no history or evidence of allergy to house dust mites. This kit yields “untouched” basophils by depletion

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of B cells, monocytes, natural killer cells, dendritic cells, early erythroid cells, platelets, neutrophils, eosinophils, and T cells (Miltenyi Biotech, Bergisch Gladbach, Germany). In our hands, this kit routinely isolated 8×10^5 $\pm$ 1.9×10^5 basophils from 100 ml peripheral blood with a purity of 80.5% $\pm$ 3.88%, as assessed by staining cytospin preparations of the cells with haematoxylin and eosin. Purified basophils were activated by cross-linking their cytophilic IgE with $1 \mu\text{g/ml}^{-1}$ mouse anti-human IgE.

Der p1

Der p1 was purified as described from the culture medium of *D. pteronyssinus* (donated by Dr. Barbara Hart, Royal Agricultural College, Cirencester, UK) by immunoaffinity chromatography using the anti-group I mite allergen monoclonal antibody 4C1 (Indoor Biotechnologies, Cardiff, UK) [4, 5].

N. americanus

N. americanus were maintained in syngeneic DSN hamsters [20]. Neonatal hamsters were infected percutaneously with 100 L_3 larvae. Adult worms were recovered from the intestine 35 days after infection and were washed for 2 h in sterile RPMI 1640 containing 100 i.u./ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Following a 1-h incubation in RPMI 1640, the medium was changed, and after 24 h of culture, the supernatant containing the worm ES products was collected and $0.2 \mu\text{m}$ -filtered. Adult worms remained viable, as assessed by motility, throughout the washing and culture period.

Cell stimulation

KU812 cells ($1 \times 10^6/\text{ml}$) were incubated in RPMI 1640 and stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co.) and $1 \mu\text{M}$ ionomycin (Sigma Chemical Co.); 10 ng/ml– $1 \mu\text{g/ml}$ Der p1 (full dose response and time course; data not shown); and 100 ng/ml–100 $\mu\text{g/ml}$ *N. americanus* ES or $1 \mu\text{g/ml}$ tetanus toxoid (Evans Medical, Liverpool, UK). Cells were incubated in 24-well plates for 1–4 h at 37°C in a 5% CO_2 , 100% humidity atmosphere at 37°C . In experiments that included the protease inhibitors L-trans-epoxysuccinyl-leucylamide-[4-guanidino]-butane (E64; Novabiochem, Nottingham, UK); 4-[amidino]phenyl-methanesulfonyl fluoride (APMSF; Novabiochem); pepstatin-A (Novabiochem); and 1,10 phenanthroline (Novabiochem), proteases were preincubated with the inhibitors at 37°C for 30 min before adding to the cell cultures.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

KU812 cells were harvested at various time points before and after stimulation. Supernatants were collected and stored at -20°C for enzyme-linked immunosorbent assay (ELISA). RNA was extracted from cells using RNeasy B (Biogenesis, Bournemouth, UK) and was reverse-transcribed by a standard protocol using Superscript RT (Life Technologies). To avoid amplification of any genomic DNA, the PCR primers bridged intron splice sites. PCR reactions were performed using the first-strand cDNA from the equivalent of 5×10^4 cells in a standard protocol with Taq polymerase (Life Technologies) with a negative control in which first-strand cDNA was substituted with water and a positive control of standard cytokine cDNA. Sequences were amplified for 35 cycles [except interferon- γ (IFN- γ), which was amplified for 45 cycles] as follows: 95°C for 45 s; 53.5°C (IL-8), 55°C (β -actin, IL-3, IL-4, IFN- γ), 60°C (IL-5, IL-6), or 66°C (IL-13) for 45 s; and 72°C for 1 min. PCR products ($10 \mu\text{l}$) were examined by agarose gel electrophoresis and photographed under UV transillumination.

The sequences of the primers used were as follows: human β -actin sense primer, 5'-TTCAACTCCATCATGAAGTGTGACGTG-3', antisense primer, 5'-TAAGTCATAGTCCGCTAGAAGCATT-3'; human IL-3 sense primer, 5'-ATGAGCCGCTGCCGCTCCTG-3', antisense primer, 5'-GCGAGGCTCAAAGTCGCTCTGTTG-3'; human IL-4 sense primer, 5'-ATGGGTCTCACCTCCAACTGCT-3', antisense primer, 5'-CGAACACTTTGAATATTTCTCTCAT-3'; human IL-5 sense primer, 5'-GCTTCTGCATTGAGTTTGCTAGCT-3', antisense primer, 5'-TGGCCGTCATATGTTTCTTTATTAAG-3'; human IL-6 sense primer, 5'-ATGAACTCCTTCTCCACAAGCGC-3', antisense primer, 5'-GAAGAGCCCTCAGGCTGGACTG-3'; human IL-8 sense primer, 5'-CTAGCTAGAATGACTTCCAAGCTGGCGG-3', antisense primer, 5'-CCGCTCGAGTTATGAATTCTCAGCCCTC-3'; human IL-13 sense primer, 5'-CCACGGTCATTGCTCTCACTTGCC-3', antisense primer, 5'-CCTTGT-

GCGGGCAGAATCCGCTCA-3'; human IFN- γ sense primer, 5'TGTTACTGC-CAGGACCCATATGTAAAA-3', antisense primer, 5'CATCACTTGGATGAGTTCAT GTATTGC-3'.

Cytokine ELISA

IL-4 and IL-13 were measured in supernatants using a human IL-13 ELISA kit (Endogen, Bradsore Biologicals Ltd., Loughborough, UK) and a human IL-4 ELISA kit (Cytoscreen, Lifescreen Ltd., Hertfordshire, UK). Assays were performed according to the manufacturer's instructions. The assay sensitivities were $<7 \text{ pg/ml}$ and $<3 \text{ pg/ml}$ for IL-13 and IL-4, respectively.

RESULTS

Powerful stimuli induce diverse cytokine mRNA in KU812 cells

To unequivocally distinguish Ig-dependent and Ig-independent triggering of type 2 cytokine production by basophils, we used KU812, a human chronic myelogenous leukaemia cell line that possesses many characteristics of peripheral blood basophils [21, 22].

KU812 was found to express almost no constitutive mRNA for IL-3, IL-4, IL-5, IL-6, and IL-13 and only low levels of mRNA for IL-8 and IFN- γ (Fig. 1, upper panel). After stimulation for 2 h with 10 ng/ml PMA and $1 \mu\text{M}$ ionomycin, the steady-state expression of IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, and IFN- γ mRNA by KU812 was substantially increased (Fig. 1, lower panel). The levels of β -actin mRNA expression were similar before and after activation. Thus, IgE-independent activation of KU812 with powerful stimuli shows the capacity of these cells to make a wide range of type 1 and type 2 cytokines.

Der p1 induces IL-4, IL-5, and IL-13 mRNA expression in KU812 cells

To determine whether the proteolytic activity of Der p1 stimulates cytokine expression in KU812 cells, cells were incubated for various time periods with between 10 ng/ml and 1

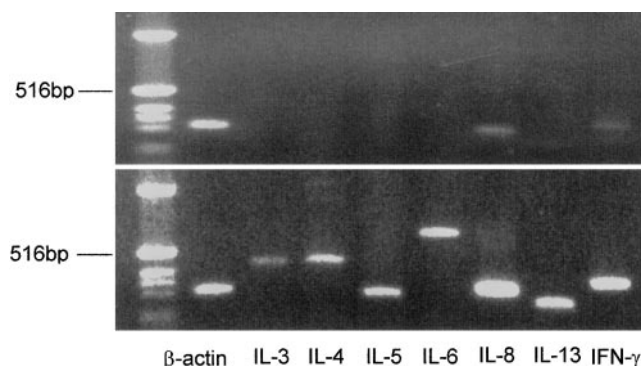


Fig. 1. Analysis of constitutive and induced cytokine mRNA expression by KU812 basophils. RT-PCR analysis of cytokine mRNA expression by unstimulated KU812 cells (upper panel) and after 2 h incubation with 10 ng/ml PMA and $1 \mu\text{M}$ ionomycin stimulation (lower panel). Total RNA was extracted from equal numbers of cells before and after stimulation, and the expression of β -actin, IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, and IFN- γ was analyzed by RT-PCR. A representative example of three independent, replicate experiments is shown.

$\mu\text{g/ml}$ proteolytically active Der p1 or Der p1 that had been inactivated by preincubation with the protease inhibitors, E64 (5 μM) and APMSF (50 μM), for 30 min at 37°C [4]. Unstimulated KU812 cells expressed low levels of cytokine mRNA and when stimulated for 2 h with 1 $\mu\text{g/ml}$ Der p1, expressed substantial amounts of mRNA for the type 2 cytokines IL-4, IL-5, and IL-13 but did not express mRNA for IL-6, IL-8 (data not shown), or the type 1 cytokine IFN- γ mRNA (Fig. 2a). Induction of cytokine mRNA was also observed at other time points (1–4 h) and other concentrations of Der p1 (100 ng/ml–1 $\mu\text{g/ml}$; data not shown). When the protease activity of Der p1 was inhibited with E64 and APMSF, the level of IL-4, IL-5, and IL-13 mRNA expression was similar to the constitutive levels produced by KU812 (Fig. 2a). Cells treated with protease inhibitors but no Der p1 also expressed only constitutive levels of mRNA (Fig. 2a).

To demonstrate that protease inhibitors do not inhibit the expression of cytokine mRNA by KU812, the effect of the inhibitors on PMA and ionomycin-induced IL-13 mRNA expression was investigated. When stimulated with PMA and ionomycin in the presence or absence of E64, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, and 1,10 phenanthroline, the protease inhibitors had no effect on the level of expression of IL-13 mRNA (Fig. 2b). This confirms that inhibition of cytokine mRNA expression observed with Der p1 and protease inhibitors (Fig. 2a) is not a result of the direct inhibition of cytokine mRNA by protease inhibitors.

To further confirm that cytokine mRNA expression by KU812 was induced by proteolytic activity, cells were incubated with a nonproteolytic protein, tetanus toxoid. After 1 and 4 h of culture, tetanus toxoid did not induce the expression of IL-13 mRNA in KU812 (Fig. 2c).

Proteases in *N. americanus* ES products induce expression of type 2 cytokine mRNA in KU812 cells

In a similar manner to Der p1, ES products induced dose-dependent expression of IL-4, IL-5, and IL-13 mRNA in KU812 cells but no detectable IL-6, IL-8 (data not shown), or IFN- γ mRNA (Fig. 3). To determine whether proteases in the ES products of *N. americanus* were responsible for induction of the type 2 cytokine mRNAs, class-specific protease inhibitors were preincubated with the ES products before adding to KU812. ES products treated with E64, pepstatin A, PMSF, and 1,10 phenanthroline failed to induce expression of IL-4, IL-5, and IL-13 mRNAs but had no effect on the expression of β -actin mRNA (Fig. 3). These results suggest that as with Der p1, proteolytic activities associated with *N. americanus* ES are responsible for the induction of cytokine mRNA by KU812.

N. americanus ES proteases and Der p1 stimulate the IL-4 and IL-13 protein production in KU812 cells

IL-4 and IL-13 protein released 24 h after stimulation of KU812 cells with Der p1 or *N. americanus* ES was measured in the supernatants of cultured cells using ELISA (Fig. 4). IL-4 and IL-13 were undetectable in supernatants from unstimulated KU812. In the presence of Der p1, KU812 produced a

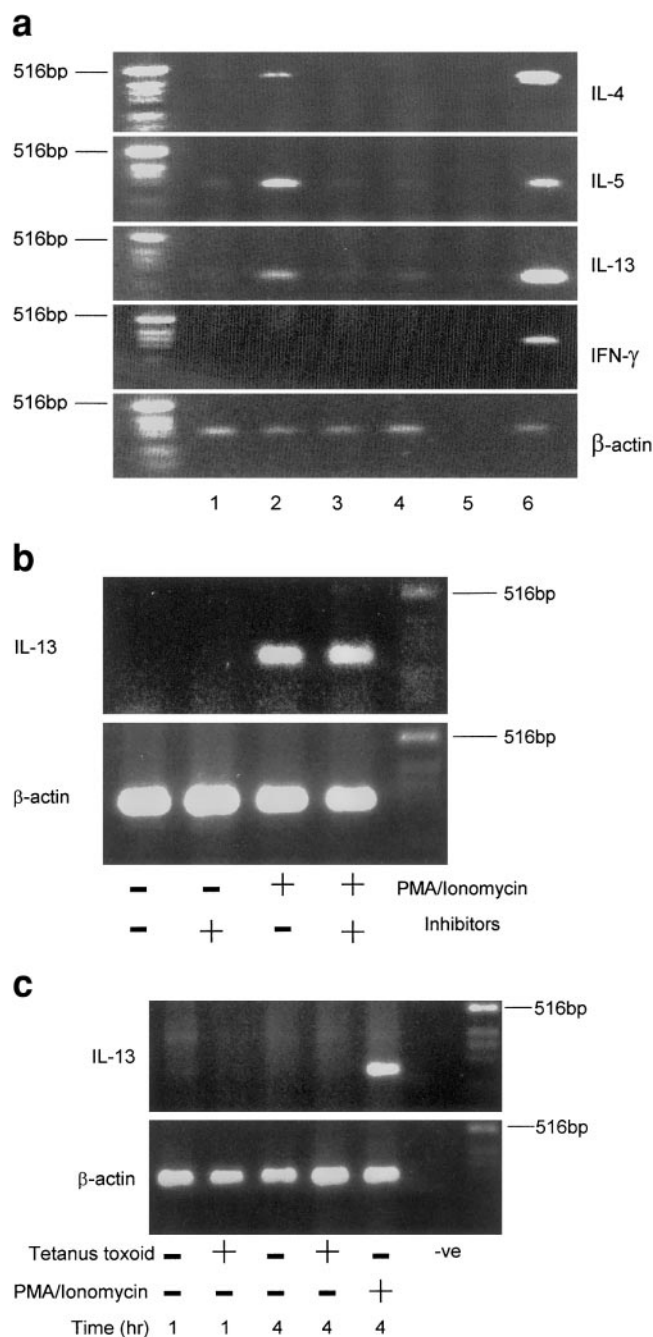
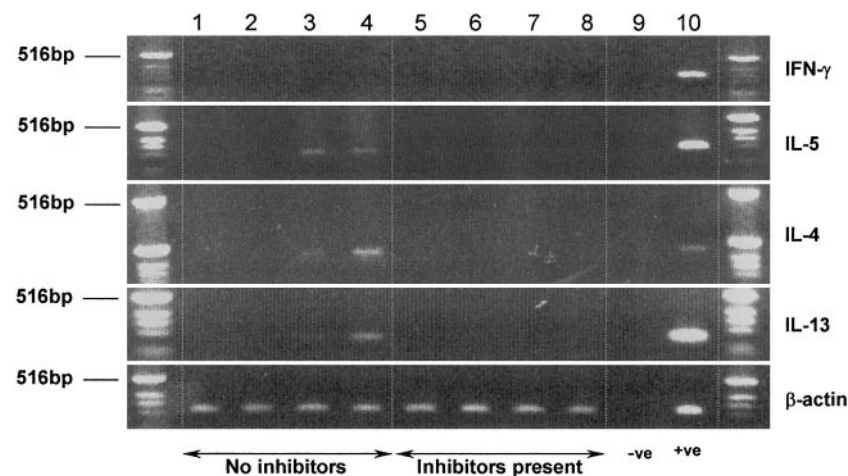


Fig. 2. (a) Der p1 induces the expression of IL-4, IL-5, and IL-13 mRNA but not IFN- γ mRNA in KU812 cells. Lane 1, Unstimulated KU812 cells; lane 2, KU812 incubated with 1 $\mu\text{g/ml}$ Der p1 for 2 h; lane 3, KU812 incubated for 2 h with 1 $\mu\text{g/ml}$ Der p1 that had been inhibited with 5 μM E64 and 50 μM APMSF; lane 4, KU812 incubated for 2 h with 5 μM E64 and 50 μM APMSF and no Der p1; lane 5, negative PCR control in which the template cDNA was substituted with water; lane 6, positive PCR control in which a template cDNA from PMA and ionomycin-stimulated KU812 was used. A representative example of three independent, replicate experiments is shown. (b) Protease inhibitors do not compromise the ability of KU812 to express IL-13 mRNA. KU812 cells were incubated for 1 h in the presence or absence of 10 ng/ml PMA and 1 μM ionomycin and no inhibitors or a cocktail of protease inhibitors containing 1 μM E64, 100 μM PMSF, 1 μM pepstatin A, and 1 mM 1,10 phenanthroline. (c) A nonproteolytic protein antigen, tetanus toxoid, does not induce IL-13 expression by KU812 basophils. KU812 cells were incubated in the presence or absence of 1 $\mu\text{g/ml}$ tetanus toxoid for 1 and 4 h. A negative PCR control (-ve) in which template cDNA was substituted with water was included alongside a positive PCR control consisting of cDNA from PMA and ionomycin-stimulated KU812 cells.

Fig. 3. Proteases in the ES products of *N. americanus* induce the expression of IL-4, IL-5, and IL-13 mRNA but not IFN- γ mRNA in KU812 cells. Lane 1, Unstimulated KU812 cells; lane 2, KU812 cells incubated for 1 h with *N. americanus* ES products at 100 ng/ml; lanes 3 and 4, 200 ng/ml and 1 μ g/ml, respectively; lane 5, unstimulated KU812 cells incubated with 1 μ M E64, 1 μ M pepstatin A, 0.1 mM PMSF, and 1 mM 1,10 phenanthroline; lane 6, KU812 cells incubated for 1 h with *N. americanus* ES products at 100 ng/ml in which proteases had been inhibited with 1 μ M E64, 1 μ M pepstatin A, 0.1 mM PMSF, and 1 mM 1,10 phenanthroline; lane 7, KU812 cells incubated for 1 h with *N. americanus* ES products at 500 ng/ml in which proteases had been inhibited as for lane 6; lane 8, KU812 cells incubated for 1 h with *N. americanus* ES products at 1 μ g/ml in which proteases had been inhibited as for lane 6; lane 9, negative PCR control (-ve) in which template cDNA was substituted with water; lane 10, positive PCR control (+ve) in which a template cDNA from PMA and ionomycin-stimulated KU812 cells was used. A representative example of three independent, replicate experiments is shown.



level of IL-4 that was close to but within the sensitivity limit of the assay (3 pg/ml) and a higher level of IL-13 (14 pg/ml). With *N. americanus* ES products, 4.7 pg/ml and 10 pg/ml IL-4 and IL-13 were produced, respectively, and cells stimulated with PMA and ionomycin as a positive control released higher amounts of IL-4 (7.8 pg/ml) and substantially more IL-13 (85 pg/ml).

To demonstrate that proteolytic activity was responsible for inducing cytokine release under these circumstances, the proteolytic activity of Der p1 and ES products was inhibited before challenge of the KU812 cells. Inhibition of protease activity completely abolished the release of IL-4 and IL-13, confirming that the enzymatic activity of proteases induces the production of proallergic cytokines in KU812 basophils.

Der p1 induces the release and intracellular accumulation of IL-4 in purified human basophils

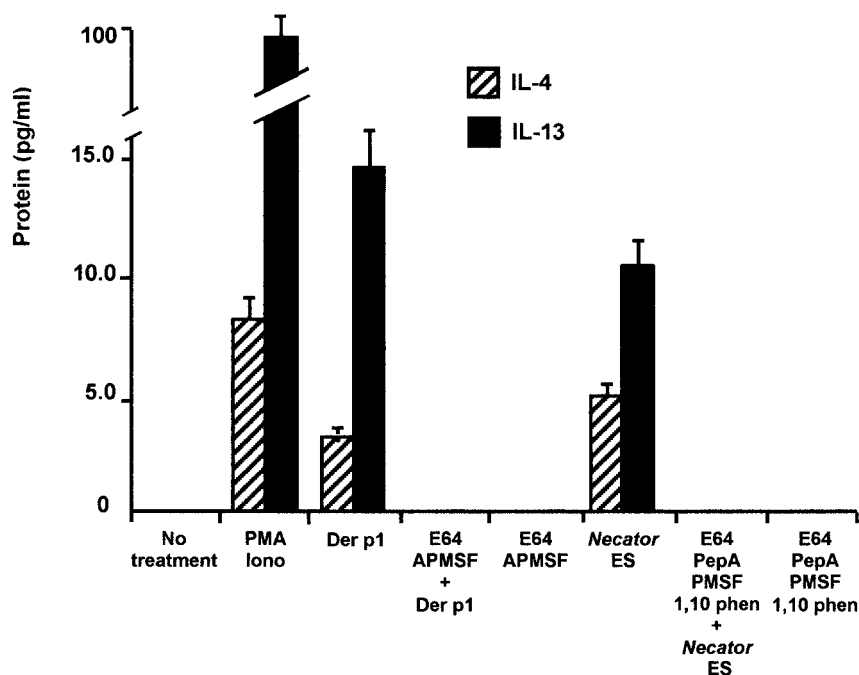
To support our findings with KU812, it was important to show that that purified peripheral blood basophils responded in a

similar manner to the cell line, as well as to demonstrate that the mechanistic class-specific protease inhibitors did not compromise the ability of purified basophils to respond to the physiological stimulus of cross-linked, cytophilic IgE.

Like others, we have found high inter-donor variability in the patterns and levels of IL-4 released into the supernatant of human purified basophil cultures. However, when intracellular and extracellular IL-4 were measured, we found that donors producing high levels of intracellular IL-4 tended to release low levels of extracellular IL-4 and vice versa. Thus, when the levels of total IL-4 (i.e., intracellular+extracellular IL-4) were considered, the pattern of IL-4 responses to challenge with several stimuli was similar between donors (data not shown).

In the presence of anti-human IgE, purified basophils released higher than constitutive levels of total IL-4 that were not reduced by preincubation of the anti-IgE with protease inhibitors (Fig. 5). This confirmed the results reported in Figure 2a, with KU812 showing that the protease inhibitors used have no effect on the activation of cytokine gene expression.

Fig. 4. KU812 basophils release IL-4 and IL-13 in response to Der p1 and proteases in the ES products of *N. americanus*. IL-4 and IL-13 were measured in cell-free supernatants of KU812 cells incubated for 24 h as follows: Unstimulated cells (No treatment); 10 ng/ml PMA and 1 μ M ionomycin (PMA Iono); 1 μ g/ml Der p1 (Der p1); 1 μ g/ml Der p1 inhibited with 5 μ M E64 and 50 μ M APMSF (E64 APMSF+Der p1); 5 μ M E64 and 50 μ M APMSF alone (E64 APMSF); 100 μ g/ml ES products of *N. americanus* (Necator ES); 100 μ g/ml ES products of *N. americanus* in which proteases were inhibited with 1 μ M E64, 1 μ M pepstatin A, 0.1 mM PMSF, and 1 mM 1,10 phenanthroline (E64 PepA PMSF 1,10 phen+Necator ES); or 1 μ M E64, 1 μ M pepstatin A, 0.1 mM PMSF, and 1 mM 1,10 phenanthroline alone (E64 PepA PMSF 1,10 phen). A representative example of three independent, replicate experiments is shown.



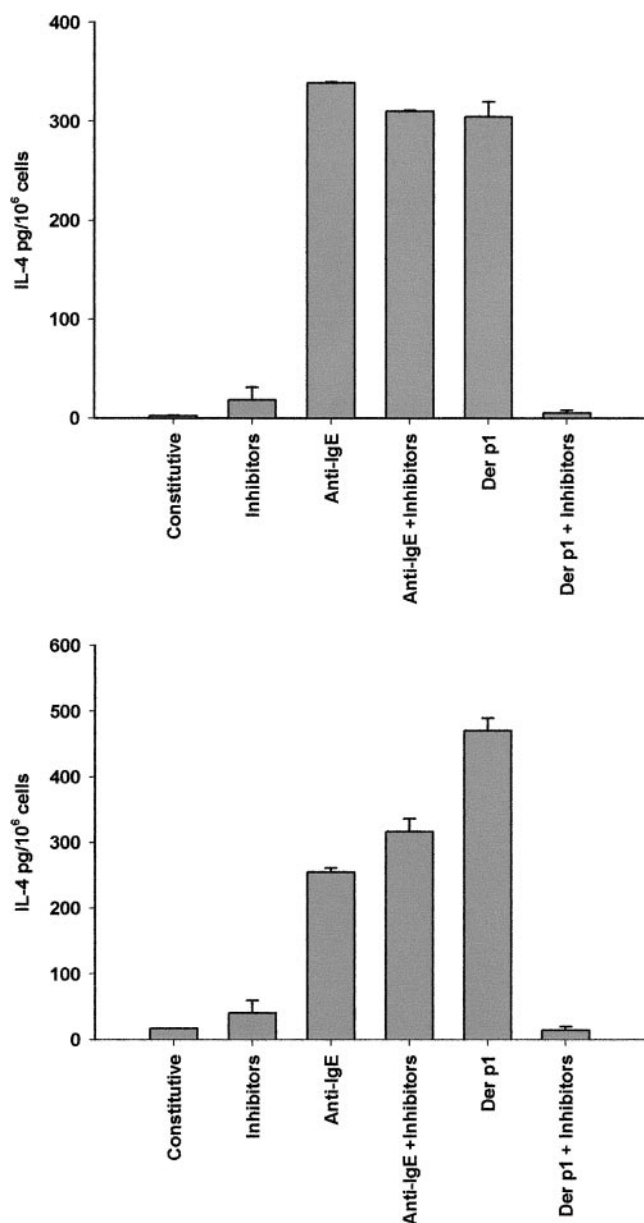


Fig. 5. The proteolytic activity of Der p1 causes purified basophils to produce IL-4. IL-4, in the cell-free supernatant, was measured by ELISA from purified basophils incubated for 24 h as follows: Untreated (Constitutive); treated with 1 μgml^{-1} mouse anti-human IgE (Anti-IgE); 5 μM E64 and 50 μM APMSF (inhibitors); 1 μgml^{-1} mouse anti-human IgE, 5 μM E64, and 50 μM APMSF (Anti-IgE+Inhibitors); 1 μgml^{-1} Der p1 (Der p1); or 1 μgml^{-1} Der p1, 5 μM E64, and 50 μM APMSF (Der p1+Inhibitors). Representative results from two independent, basophil donors are shown.

When purified basophils were incubated with anti-human IgE or Der p1, the patterns of IL-4 released in response to the stimuli were very even, although there was high inter-donor variability of the absolute levels of IL-4. In the representative donors shown in Figure 5, Der p1 and anti-human IgE increased the expression of IL-4 released above constitutive levels. When protease inhibitors alone were incubated with the cells, there was no decrease in the expression of constitutive IL-4 released. When Der p1 was incubated with the inhibitors and was then used to challenge the cells, the level of Der p1-induced IL-4 release was also reduced to background levels

or below (Fig. 5). This suggests, as with the experiments using KU182, that the proteolytic activity of Der p1 induces the expression and release of type 2 cytokines by basophils.

DISCUSSION

Protease activities associated with arthropods and parasites are thought to have a role in the development of allergic-immune responses. In this study, we have investigated whether proteases activate the expression of cytokines involved in the establishment of a milieu that could influence how naïve, responding T cells are polarized toward a type 2 cytokine profile.

In human and animal models, there is evidence that basophils produce substantial amounts of IL-4 and IL-13. This has been recorded when basophils are challenged with antigens to which the basophil donor is sensitized or to substances, such as dietary lectins that interact with basophil cell-surface receptors [23–28].

When challenged with Der p1 or *N. americanus* ES products, KU182 basophils produced IL-13 at a substantially higher level than IL-4. Whether this reflects the consumption of IL-4 by KU182 cells [29] or is an innate characteristic of this cell line is not known. It is known, however, that the relative levels of IL-4 and IL-13 produced by basophils differ substantially depending on the stimulus used [30, 31]. There are also data showing that the regulation of IL-4 and IL-13 production in basophils differs in kinetics and diagnostic sensitivity to immunosuppressive drugs, suggesting that different cell signaling pathways are involved in IL-4 and IL-13 gene expression [30, 31]. This, in many instances, leads to rapid production of IL-4, sometimes within minutes of stimulation, and IL-13 is induced more slowly, peaking at 24 h [23–28].

The finding that proteases induced an increase in the expression of proallergic cytokines by KU812 and purified peripheral basophils was shown by use of mechanistic, class-specific protease inhibitors to be specifically a result of the proteolytic activity of the material used to challenge the basophils. This raises the important question of which of many potential target cell-surface substrates is cleaved to cause activation of basophils to produce cytokines.

There are several reports showing that proteolytic allergens cleave immunoregulatory receptors such as CD23 and CD25 [4, 7]. These studies and those of others speculate that disturbance of immune regulation caused by receptor cleavage or changes in intercellular adhesion may be important factors in the maintenance of allergic immunity [32]. However, the regulatory activities of proteases are not confined to the immune system, and physiologically important protease targets are found on a wide range of nonimmune cell types. In particular, it has become recognized that many regulatory activities of proteases arise from their ability to directly activate cells via protease-activated receptors (PAR; reviewed in refs. [33, 34]), and we suspect from preliminary experiments using agonist peptides of PAR on KU812 basophils (S. Foster and C. R. A. Hewitt, unpublished) that PAR may provide a link between proteolytic activity and the activation of cytokine release by basophils.

The purity of immunochemically isolated, proteolytically active Der p1 is a contentious subject, and it is arguable that we should have validated our results with recombinant Der p1. Although this viewpoint has its merits, there is no evidence to suggest that the available yeast-derived Der p1 is of higher purity than purified, native material (even commercial sources of recombinant Der p1 contain up to 5% impurities and ~4% of dimeric molecules).

Furthermore, at a time when the effects of carbohydrate on the proteolytic function of native (~24 kDa) Der p1 are unknown, it is known that recombinant Der p1 is hyperglycosylated with up to 16 kDa of N-glycans [35]. This contrasts with native Der p1, which has no detectable N-glycans but appears to have O-glycans that are *not* present on yeast-derived Der p1. Although these differences in glycosylation may account for the lower antigenicity of recombinant Der p1 compared with native Der p1 [35], nothing is known about the effect of heterologous carbohydrates on the proteolytic activity or specificity of the protease.

Perhaps more importantly in the context of cytokine release by basophils, it is also not known whether any microbial carbohydrate recognition molecules on basophils interact with yeast-derived Der p1 in a different manner than that with the native material. Thus, without undertaking extensive steps to modify carbohydrate on recombinant Der p1, the effect of a recombinant protease on cytokine release by basophils will be unpredictable.

Our finding that proteolytic activities associated with substances that provoke allergic type 2 cytokine-dominated immune responses may be significant in explaining why only certain antigens provoke allergies.

In other systems, the innate and early release of IL-4 and IL-13 polarizes subsequent T cell responses toward type 2 cytokine production and thus, influences the development of IgE and eosinophilia [36–38]. Our findings show that the proteolytic activities of substances derived from allergenic organisms also cause an IgE-independent, innate, and early release of IL-4 and IL-13, which we speculate may be involved in the establishment of a type 2 T cell-promoting cytokine milieu that drives the characteristic allergic-immune response to helminth pathogens and house dust mites.

The implications of these findings for host defense or evasion of the immune system by pathogens are unknown; certainly as a noninvasive, noninfectious organism, the mechanism described can have no influence on the pathogenic effects of house dust mites. Nevertheless, it remains to be seen what controls and influences the balance between protease-induced innate immunity and protease-based evasion of immunity by parasites such as *N. americanus* [39].

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REFERENCES

- Pritchard, D., Wilson, R. (1997) Helminth infections. In *Pulmonary Defences* (R. A. Stockley, ed.), London, UK, John Wiley and Sons, 305–345.
- Hewitt, C., Foster, S., Phillips, C., Horton, H., Jones, R., Brown, A. P., Hart, B. J., Pritchard, D. I. (1993) Mite allergens: significance of enzymatic activity. *Allergy* **53**, 60–63.
- Stewart, G., Thompson, P. (1996) The biochemistry of common aeroallergens. *Clin. Exp. Allergy* **26**, 1020–1044.
- Hewitt, C., Brown, A., Hart, B., Pritchard, D. (1995) A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate production by antiproteases. *J. Exp. Med.* **182**, 1537–1544.
- Hewitt, C., Horton, H., Jones, R., Pritchard, D. (1997) Heterogeneous proteolytic specificity and activity of the house dust mite protease allergen Der p1. *Clin. Exp. Allergy* **27**, 201–207.
- Schulz, O., Laing, P., Sewell, H., Shakib, F. (1995) Der p1, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23). *Eur. J. Immunol.* **25**, 3191–3194.
- Schulz, O., Sewell, H., Shakib, F. (1998) Proteolytic cleavage of CD25, the α -subunit of the human T cell interleukin-2 receptor, by Der p1, a major mite allergen with cysteine protease activity. *J. Exp. Med.* **187**, 271–275.
- Wan, H., Winton, H. L., Soeller, C., Tovey, E. R., Gruenert, D. C., Thompson, P. J., Stewart, G. A., Taylor, G. W., Garrod, D. R., Cannell, M. B., Robinson, C. (1999) Der p1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J. Clin. Invest.* **104**, 123–133.
- Wan, H., Winton, H. L., Soeller, C., Taylor, G. W., Gruenert, D. C., Thompson, P. J., Cannell, M. B., Stewart, G. A., Garrod, D. R., Robinson, C. (2001) The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*. *Clin. Exp. Allergy* **31**, 279–294.
- Wan, H., Winton, H. L., Soeller, C., Gruenert, D. C., Thompson, P. J., Cannell, M. B., Stewart, G. A., Garrod, D. R., Robinson, C. (2000) Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p1. *Clin. Exp. Allergy* **30**, 685–698.
- Brown, A., Burleigh, J., Billett, E., Pritchard, D. (1995) An initial characterisation of the proteolytic enzymes secreted by the adult stage of the human hookworm *Necator americanus*. *Parasitology* **110**, 555–563.
- Maggi, E., Parronchi, P., Manetti, R., Simonelli, C., Piccinini, M., Rugiu, F. (1992) Reciprocal regulatory effects of IFN- γ and IL-4 on the in vitro development of Th1 and Th2 clones. *J. Immunol.* **148**, 2142–2147.
- Demeure, C., Chang, Y., Shu, U., Schneider, P., Heusser, C., Yssel, H. (1994) In vitro maturation of human neonatal CD4 T lymphocytes. II. Cytokines present at priming modulate the development of lymphokine production. *J. Immunol.* **152**, 4775–4782.
- Gibbs, B., Haas, H., Falcone, F., Albrecht, C., Vollrath, I., Noll, T., Wolff, H. (1996) Purified human peripheral blood basophils release interleukin-13 and pre-formed interleukin-4 following immunological activation. *Eur. J. Immunol.* **26**, 2493–2498.
- Brunner, T., Heusser, C., Dahinden, C. (1993) Human peripheral blood basophils primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *J. Exp. Med.* **177**, 605–611.
- MacGlashan Jr., D., White, J. M., Huang, S. K., Ono, S. J., Schroeder, J. T., Lichtenstein, L. M. (1994) Secretion of IL-4 from human basophils. The relationship between IL-4 mRNA and protein in resting and stimulated basophils. *J. Immunol.* **152**, 3006–3016.
- Mueller, R., Heusser, C., Rihs, S., Brunner, T., Bullock, G., Dahinden, C. (1994) Immunolocalisation of intracellular interleukin-4 in normal human peripheral blood basophils. *Eur. J. Immunol.* **24**, 2935–2940.
- Li, H., Sim, T., Alam, R. (1996) IL-13 released by and localized in human basophils. *J. Immunol.* **156**, 4833–4838.
- Machado, D., Horton, D., Harrop, R., Peachell, P., Helm, B. (1996) Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitisation with antigen-specific IgE. *Eur. J. Immunol.* **26**, 2972–2980.
- Sen, H., Seth, D. (1967) Complete development of the human hookworm *Necator americanus* in golden hamsters, *Mesocricetus auratus*. *Nature* **214**, 609–611.
- Kishi, K. (1995) A new leukaemia cell line with Philadelphia chromosome characterized as basophil precursors. *Leuk. Res.* **9**, 381–390.
- Agis, H., Fureder, W., Bankl, H., Kundi, M., Sperr, W., Willheim, M. (1996) Comparative immunophenotypic analysis of human mast cells, blood basophils and monocytes. *Immunology* **87**, 535–543.
- Falcone, F. H., Dahinden, C. A., Gibbs, B. F., Noll, T., Amon, U., Hebestreit, H., Abrahamsen, O., Klauke, J., Schlaak, M., Haas, H. (1996)

- Human basophils release interleukin-4 after stimulation with *Schistosoma mansoni* egg antigen. *Eur. J. Immunol.* **26**, 1147–1155.
24. Gibbs, B. F., Haas, H., Falcone, F. H., Albrecht, C., Vollrath, I. B., Noll, T., Wolff, H. H., Amon, U. (1996) Purified human peripheral blood basophils release interleukin-13 and preformed interleukin-4 following immunological activation. *Eur. J. Immunol.* **26**, 2493–2498.
 25. Kasaian, M. T., Clay, M. J., Happ, M. P., Garman, R. D., Hirani, S., Luqman, M. (1996) IL-4 production by allergen-stimulated primary cultures: identification of basophils as the major IL-4-producing cell type. *Int. Immunol.* **8**, 1287–1297.
 26. Li, H., Sim, T. C., Alam, R. (1996) IL-13 released by and localized in human basophils. *J. Immunol.* **156**, 4833–4838.
 27. Ochensberger, B., Daepf, G. C., Rihs, S., Dahinden, C. A. (1996) Human blood basophils produce interleukin-13 in response to IgE-receptor-dependent and -independent activation. *Blood* **88**, 3028–3037.
 28. Haas, H., Falcone, F. H., Schramm, G., Haisch, K., Gibbs, B. F., Klaucke, J., Poppelmann, M., Becker, W. M., Gabius, H. J., Schlaak, M. (1999) Dietary lectins can induce in vitro release of IL-4 and IL-13 from human basophils. *Eur. J. Immunol.* **29**, 918–927.
 29. Hara, T., Yamada, K., Tachibana, H. (1998) Basophilic differentiation of the human leukemia cell line KU812 upon treatment with interleukin-4. *Biochem. Biophys. Res. Commun.* **247**, 542–548.
 30. Dahinden, C. A., Rihs, S., Ochensberger, B. (1997) Regulation of cytokine expression by human blood basophils. *Int. Arch. Allergy Immunol.* **113**, 134–137.
 31. Redrup, A. C., Howard, B. P., MacGlashan Jr., D. W., Kagey-Sobotka, A., Lichtenstein, L. M., Schroeder, J. T. (1998) Differential regulation of IL-4 and IL-13 secretion by human basophils: their relationship to histamine release in mixed leukocyte cultures. *J. Immunol.* **160**, 1957–1964.
 32. Robinson, C., Kalsheker, N. A., Srinivasan, N., King, C. M., Garrod, D. R., Thompson, P. J., Stewart, G. A. (1997) On the potential significance of the enzymatic activity of mite allergens to immunogenicity. Clues to structure and function revealed by molecular characterization. *Clin. Exp. Allergy* **27**, 10–21.
 33. Coughlin, S. R. (2000) Thrombin signalling and protease-activated receptors. *Nature* **407**, 258–264.
 34. Macfarlane, S. R., Seatter, M. J., Kanke, T., Hunter, G. D., Plevin, R. (2001) Proteinase-activated receptors. *Pharmacol. Rev.* **53**, 245–282.
 35. van Oort, E., de Heer, P. G., van Leeuwen, W. A., Derksen, N. I. L., Muller, M., Huveneers, S., Aalberse, R. C., van Ree, R. (2002) Maturation of *Pichia pastoris*-derived recombinant pro-Der p 1 induced by deglycosylation and by the natural cysteine protease Der p 1 from house dust mite. *Eur. J. Biochem.* **269**, 671–679.
 36. Wang, M., Saxon, A., Diaz-Sanchez, D. (1999) Early IL-4 production driving Th2 differentiation in a human in vivo allergic model is mast cell derived. *Clin. Immunol.* **90**, 47–54.
 37. Haas, H., Falcone, F. H., Holland, M. J., Schramm, G., Haisch, K., Gibbs, B. F., Bufer, A., Schlaak, M. (1999) Early interleukin-4: its role in the switch towards a Th2 response and IgE-mediated allergy. *Int. Arch. Allergy Immunol.* **119**, 86–94.
 38. Yamashita, M., Yamaoka, M., Seki, N., Fujii, T. (1996) Non-T cell-derived IL-4 plays an important role in IgE production induced by antigen resensitization and is resistant to FK506. *J. Immunol.* **157**, 714–719.
 39. Culley, F. J., Brown, A., Conroy, D. M., Sabroe, I., Pritchard, D. I., Williams, T. J. (2000) Eotaxin is specifically cleaved by hookworm metalloproteases preventing its action in vitro and in vivo. *J. Immunol.* **165**, 6447–6453.