

Human T cells that have been conditioned by the proteolytic activity of the major dust mite allergen Der p 1 trigger enhanced immunoglobulin E synthesis by B cells

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Summary

Background We have previously demonstrated that the proteolytic activity of Der p 1 selectively cleaves human CD25, the 55 kDa α subunit of the IL-2 receptor. As a result of cleavage of surface CD25, peripheral blood T cells produce less IFN- γ and more IL-4, thereby leading to progressive polarization of the T cells towards a Th2 cytokine profile. Therefore, these observations underline the potential role of the proteolytic activity of Der p 1 in creating a microenvironment conducive for IgE synthesis.

Objective To study the effect of T cells that have been conditioned by the proteolytic activity of Der p 1 on IgE synthesis by B cells.

Methods We have examined this concept in experiments whereby T cells that have been exposed to either proteolytically active or inactive Der p 1 were cocultured with autologous B cells and IgE antibody synthesis was monitored.

Results Here we demonstrate for the first time that coculturing T cells that have been in contact with proteolytically active Der p 1 with autologous B cells leads to augmentation of IgE antibody responses.

Conclusions The proteolytic activity of Der p 1 conditions human T cells, which then become empowered to trigger enhanced IgE synthesis by B cells.

Keywords B cell, Der p 1, IFN- γ , IgE, IL-4, T cell

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Introduction

IgE-mediated hypersensitivity is the underlying mechanism of atopic conditions, such as asthma, that afflict about 20% of the population [1]. Given its high prevalence, there is considerable interest in understanding the mechanisms involved in the triggering and perpetuation of allergic inflammation. Recent work from our laboratory has revealed a previously unrecognized mechanism by which the proteolytic activity of the house dust mite allergen Der p 1, the most significant domestic allergen, biases human Th cell subset development in favour of Th2 [2, 3]. Th2 responses are known to be involved in the pathogenesis of allergic diseases, mainly through the secretion of IL-4/IL-13 and IL-5, which are responsible for stimulating IgE synthesis and eosinophil function, respectively.

We have previously demonstrated that the proteolytic activity of Der p 1 selectively cleaves human CD25, the 55 kDa α subunit of the IL-2 receptor. As a result of cleavage of surface CD25, peripheral blood T cells show markedly diminished proliferation and IFN- γ secretion in response to stimulation by anti-CD3 antibody [2]. It is known that CD4 T-helper cells

undergo a cytokine-driven process of polarization and that IL-2, along with IFN- γ , and IL-4 are considered to be autocrine growth factors for the Th1 and Th2 cells, respectively. The Th1 and Th2 cell populations promote the development of cells of the same subset while suppressing the propagation of those of the other subset [4]. Therefore, Der p 1-induced cleavage of CD25 is likely to lead to impaired growth of cells of the Th1 subset and consequent augmentation of those of the Th2 subset. We have indeed been able to demonstrate that the proteolytic activity of Der p 1 biases human CD4 and CD8 T cells towards a Th2 and a Tc2 cytokine profile, respectively [3]. Therefore, these observations provide compelling evidence for the role of the proteolytic activity of Der p 1 in creating a microenvironment conducive for IgE synthesis.

Given that enhanced IgE synthesis is the ultimate readout for the immunogenic effects of antigens that are known to trigger a Th2 type response, here we demonstrate that coculturing T cells that have been conditioned by the proteolytic activity of Der p 1 with autologous B cells leads to augmentation of IgE antibody responses.

Materials and methods

Antibodies and cytokines

Mouse monoclonal antibodies against human IFN- γ (FITC, clone 45.15), CD3 (FITC and PC5, clone UCHT1), CD19

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(PE, clone J4.119), CD25 (PE, clone B1.49.9), together with mouse IgG1 (FITC, ECD and PE, clone 679.1Mc7) and IgG2b (PC5, clone U7.27) isotype controls, were purchased from Coulter Immunotech (Luton, UK). Normal mouse IgG2a (clone, 20102.1), mouse anti-human IFN- γ (clone, 25718.111), together with rhIL-2 and rhIFN- γ , were purchased from R & D Systems (Oxford, UK). Mouse anti-human CD3 antibody was purified on a Protein G column from the supernatant of a hybridoma cell line (OKT3, ECACC, Porton Down, UK).

Der p 1 preparation

As the recombinant Der p 1 preparations that are currently available are enzymatically inactive, we used pure native Der p 1 whose enzymatic (cysteine protease) activity could unequivocally be switched on and off [5]. Der p 1 was isolated from house dust mite fecal pellets (Allergon, Angelholm, Sweden) using our multistep procedure, involving immunoaffinity chromatography on immobilized monoclonal anti-Der p 1 antibody (clone 4C1, Indoor Biotechnologies, Manchester, UK), removal of contaminating serine proteases on immobilized soybean trypsin inhibitor (Sigma, Poole, UK) and finally FPLC to remove low molecular weight contaminants [5]. The purity of the preparation was confirmed by N-terminal sequencing on an automatic amino acid sequencer (Applied Biosystems, Foster City, CA, USA), SDS-PAGE analysis (15% gel) and by demonstrating that enzymatic activity is completely dependent on pre-activation with cysteine and is totally blocked by the class-specific inhibitor E-64 [L-trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane] [5]. Protein concentration was determined using a BCA microtitre plate assay and confirmed spectrophotometrically using the empirical absorption coefficient value for Der p 1 of $E^{1\%}_{280\text{ nm}} = 16.4$. Der p 1 prepared in this way is proteolytically inactive, as its thiol group becomes oxidized during purification. To activate its proteolytic activity, Der p 1 was treated with 5 mM cysteine (Sigma) to regenerate its thiol group. The catalytic activity of Der p 1 was ascertained in a continuous rate (kinetic) assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC [6].

Cell preparation and sorting

Heparinized whole blood from healthy non-atopic volunteers (obtained with prior consent and ethical committee approval) was used for separation of PBMC on a histopaque density gradient (HISTOPAQUE-1077, Sigma). Cells (30 million/sorting) were stained for CD3 and CD19 surface molecules, and T cells and B cells were isolated, to about 95 to 98% purity, using a Coulter EPICS XL-MCL cell sorter (Beckman Coulter, High Wycombe, UK).

Stimulation of T cells with Der p 1

T cells (4×10^5 cells/500 μL) were suspended in serum free AIM-V medium, supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin (Gibco Life Technologies Limited, Paisley, UK) and 5 mM HEPES (Sigma), and cultured in flat bottom 48-well plates (Corning Costar Corporation, NY, USA) pre-coated with 2 $\mu\text{g/mL}$ of anti-CD3 antibody. Six hours later, Der p 1 (10 $\mu\text{g/mL}$) was added in either proteolytically active (i.e. activated with 5 mM cysteine) or inactive form, and the cultures were continued for a further 24 h. For B and T cell

cocultures, the T cells were harvested, extensively washed and added to B cells (see below). To stain for intracellular IFN- γ , 10 $\mu\text{g/mL}$ of Brefeldin A (Sigma) was added 18 h before harvesting the cells to inhibit intracellular transport of newly produced cytokines. We have previously shown that Der p 1 does not induce cytotoxicity, as judged by determining the number of apoptotic and necrotic cells using the Annexin V/FITC kit [2].

In vitro cultures for IgE synthesis

The experiment was performed as described before [7, 8]. Briefly, sorted B cells (4×10^4 cells/200 μL) were suspended in IMDM medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and 25 mM HEPES (Gibco Life Technologies Limited), and cultured in U-bottom 96-well plates. The cells received 200 IU/mL of recombinant human (rh) IL-4 and 50 $\mu\text{g/mL}$ of human holo-transferrin (Sigma). After 24 h, the T cells (4×10^4 cells/200 μL) that had been treated with either proteolytically active or inactive Der p 1 (see above) were added to B cells and the cultures were continued for another 14 days. On day two, appropriate cocultures received either anti-human IFN- γ (10 $\mu\text{g/mL}$), rhIFN- γ (1000 IU/mL), rhIL-2 (10 IU/mL) or normal mouse IgG2a isotype control.

Staining for cell surface markers

Cells were harvested, washed twice with PBS, 0.1% BSA and azide (PBA) and then stained for cell surface markers for 30 min at 4 °C in the dark. The cells were then washed twice with PBA and fixed in 0.5% formaldehyde in isotonic azide free solution. The cells were either analysed immediately for cell surface markers or kept overnight in 0.5% formaldehyde for intracellular cytokine staining.

Intracellular cytokine staining

Intracellular cytokine staining was performed as previously described [9–11]. Briefly, the fixed cells were washed three times with cold PBA, PBA-0.1% saponin buffer (Sigma) and PBA-0.1% saponin containing 10% FCS, respectively. All washes were done at 4 °C. Labelled anti-cytokine antibodies were added, at concentrations recommended by the manufacturer, to cells directly after the last wash. Cells were incubated for 2 h on ice in the dark and mixed every 15 min. The cells were then washed three times with cold PBA-0.1% saponin buffer and resuspended in 1 mL of 0.5% formaldehyde in azide free isotonic solution.

Flow cytometric analysis

Flow cytometry was performed using a Coulter EPICS XL-MCL (Beckman Coulter, High Wycombe, UK) and 50 000 to 100 000 events were collected for each sample. Dead cells were excluded by forward and side scatter characteristics. The quadrant markers for the bivariate dotplots were set based on the non-stimulated cells stained with specific antibodies and verified with isotype controls.

Detection of IgE

The measurement of IgE concentration in the culture supernatant was performed using a sandwich fluoroenzymeimmunoassay (UniCAP 100; Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). The detection limit of this assay is <2 KU/L.

Statistical analysis

The paired Student *t*-test was used to compare levels of IgE obtained from the various B cell and T cell cocultures; *P*-values less than 0.05 were considered significant.

Results

Peripheral blood B and T cell populations were isolated, to about 95 to 98% purity, using a cell sorter (Fig. 1). As expected

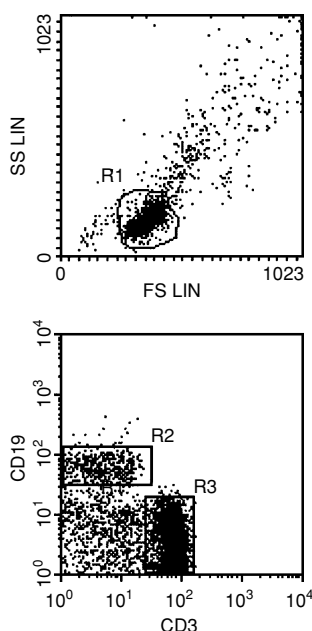


Fig. 1. Representative dotplots showing the purity of the sorted peripheral blood B and T cell populations.

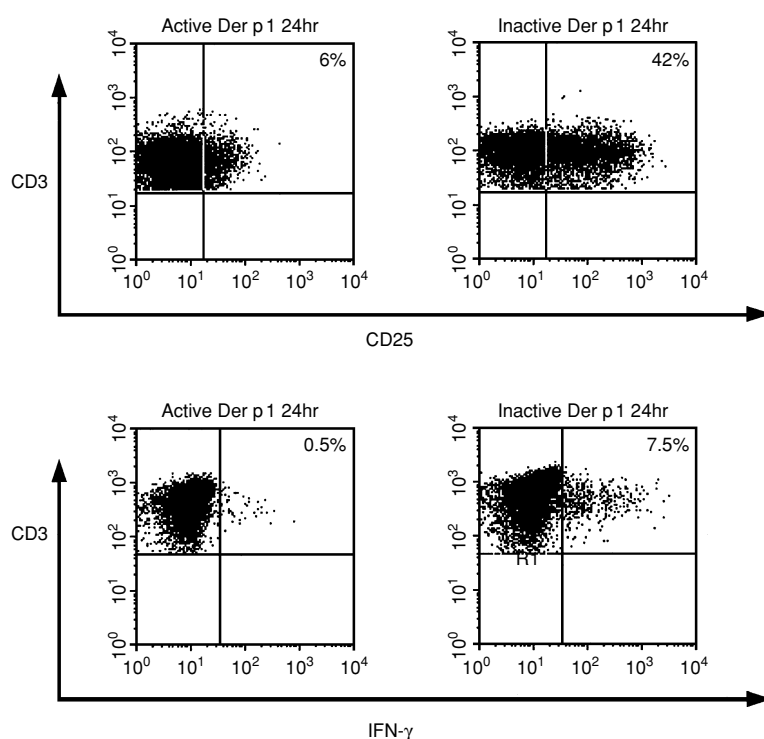


Fig. 2. Representative dotplots showing the suppressive effects of the proteolytic activity of Der p 1 on CD25 expression and IFN- γ production by peripheral blood T cells. At this time-point no difference in IL-4 production was demonstrable. No such effects were observed in control cultures containing 5 mM cysteine, which was used for activating Der p 1 (data not shown).

from our previous studies [2, 3], T cells that have been stimulated with anti-CD3 in the presence of proteolytically active Der p 1 showed very little CD25 expression and IFN- γ production (Fig. 2).

Having ascertained the CD25 and IFN- γ expression status of such T cells, they were harvested at 24 h, extensively washed and added to autologous B-cell cultures supplemented with rhIL-4 and human holo-transferrin. The cocultures were then continued for 2 weeks, at the end of which the supernatants were collected for IgE measurement. This has shown that B cell cultures containing T cells that have been treated by proteolytically active Der p 1 produced significantly more IgE, compared with those containing T cells treated with proteolytically inactive Der p 1 (Fig. 3). This effect was abrogated by exogenously added rhIFN- γ (Fig. 4). The enhanced IgE synthesis observed in cultures containing T cells that have been treated by proteolytically active Der p 1 is therefore most likely to be due to less IFN- γ production by T cells (Fig. 2).

On the other hand, the suppressed IgE synthesis observed in cultures containing T cells that have been treated with proteolytically inactive Der p 1 is most likely to be due to more IFN- γ production by T cells (Fig. 2). Therefore, to investigate the role of IFN- γ in the suppression of IgE synthesis, we conducted further experiments whereby the B cell cultures containing T cells that have been treated with proteolytically inactive Der p 1 received a neutralizing dose of anti-IFN- γ antibody. Such cultures showed only partial restoration of IgE synthesis (Fig. 5), thereby suggesting that the enhanced IgE synthesis observed in B cell cultures containing T cells that have been treated by proteolytically active Der p 1 (Fig. 3) is not solely due to suppressed IFN- γ production.

Interestingly, the addition of exogenous IL-2 to B cell cultures containing T cells that have been treated by proteolytically active Der p 1 resulted in further enhancement of IgE synthesis. On the other hand, the addition of exogenous IL-2 to B cell cultures containing T cells that have been treated with proteolytically inactive Der p 1 resulted in further suppression of IgE synthesis (Fig. 6). These results indicate that exogenously added IL-2 has apparently served to expand T cells that have previously committed themselves to producing either Th1 or Th2 cytokines.

Discussion

In vitro studies have identified a requirement for at least two molecular signals for selective switching to IgE synthesis. The first is IL-4, a Th2 cytokine that directs recombination to the constant region of the ϵ heavy chain. This activity has been shown to be downregulated by IFN- γ [12]. The second signal is CD40L, which is found on activated T cells [13]. In the present study we exploited these interactions with a view to investigating the effect of T cells that have been conditioned by the proteolytic activity of Der p 1 on IgE synthesis by B cells.

This has shown that the proteolytic activity of Der p 1 conditions human T cells, which then become empowered to trigger enhanced IgE synthesis by B cells. This enhancement in IgE synthesis is most likely to be due to suppressed IFN- γ and

increased IL-4 production by T cells. The present observations are in keeping with our recent *in vivo* studies showing that immunization of mice with proteolytically active Der p 1 results in a significant and selective enhancement in IgE synthesis compared with mice immunized with Der p 1 that has been irreversibly blocked with the cysteine protease inhibitor E-64 [5].

However, one of the most intriguing aspects of this work is the demonstration that adding rhIL-2, a Th1 cytokine, leads to further enhancement of IgE synthesis in cultures containing T cells that have been treated with proteolytically active Der p 1 and further suppression in those containing T cells that have been treated with proteolytically inactive Der p 1. The fact that the T cells that have previously been conditioned with proteolytically active Der p 1 have responded to IL-2, by supporting further IgE production, highlights two very important issues, namely that these T cells have re-expressed CD25 and that short-term exposure to proteolytically active Der p 1 biases them towards the Th2 subset.

Clearly, any extrapolation of these effects to the clinical situation would partly depend on the expected concentration of Der p 1 in the target organ, namely the lungs. Der p 1 accounts for 15 to 20% of the total protein content of mite fecal pellets, and there is no doubt that chronic exposure would result in the cumulative build-up of sufficient concentrations (nanogram range) to elicit IgE antibody responses. Indeed, nanogram quantities of Der p 1 have been detected in BAL fluid from dust mite-allergic individuals [14]. We have previously shown that

Fig. 3. B cell cultures containing T cells that have been conditioned by proteolytically active Der p 1 produce significantly more IgE, compared with those containing T cells that have been treated with proteolytically inactive Der p 1 ($P < 0.004$). Data shown represent mean $\pm$ SD values obtained from B cell and T cell cocultures of four individuals.

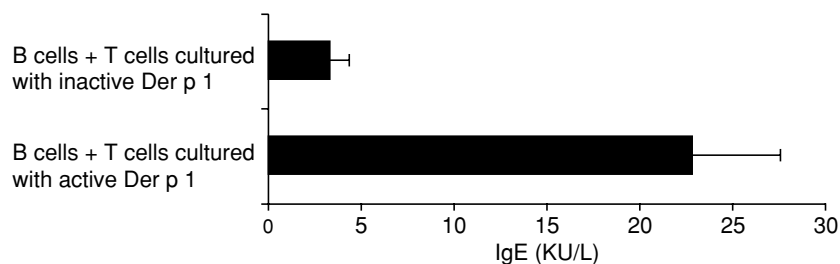


Fig. 4. The enhanced IgE synthesis observed in B cell cultures containing T cells that have been conditioned by proteolytically active Der p 1 is abrogated by exogenously added IFN- γ ($P < 0.006$). Data shown represent mean $\pm$ SD values obtained from B cell and T cell cocultures of three individuals.

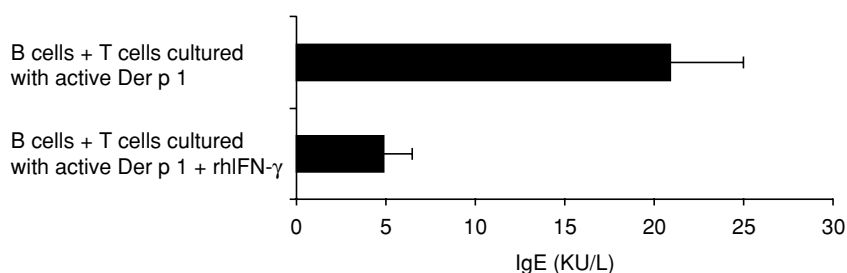
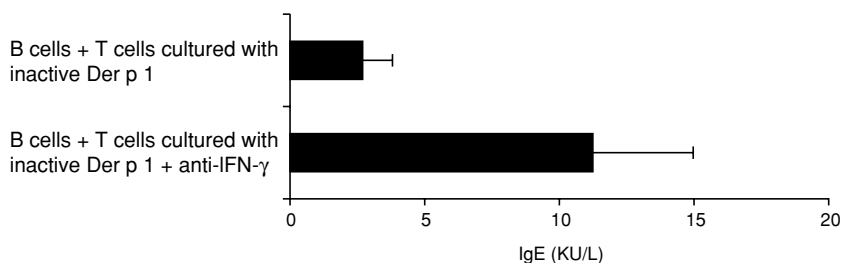


Fig. 5. The suppressed IgE synthesis observed in B cell cultures containing T cells that have been treated with proteolytically inactive Der p 1 is partially restored by adding a neutralizing dose of anti-IFN- γ antibody ($P < 0.05$). No such effect was observed in control cultures that received the isotype control (data not shown). Data shown represent mean $\pm$ SD values obtained from B cell and T cell cocultures of three individuals.



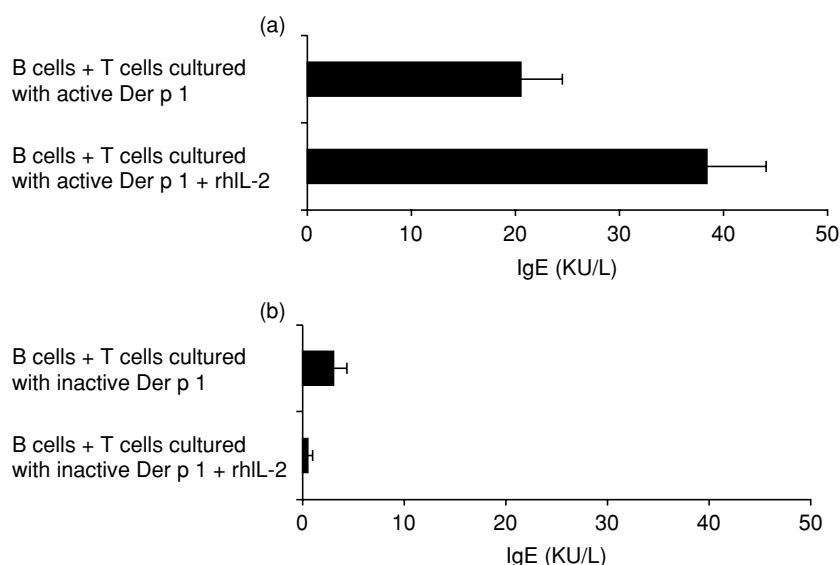


Fig. 6. (a). The addition of exogenous IL-2 to B cell cultures containing T cells that have been conditioned by proteolytically active Der p 1 results in further enhancement of IgE synthesis ($P < 0.01$). (b) On the other hand, the addition of exogenous IL-2 to B cell cultures containing T cells that have been treated with proteolytically inactive Der p 1 resulted in further suppression of IgE synthesis. Data shown represent mean $\pm$ SD values obtained from B cell and T cell cocultures of three individuals.

Der p 1, at a concentration as low as 10 ng/mL, cleaves the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC [6], thereby suggesting that this concentration is sufficient for cleaving *in vivo* protein substrates.

Mouse *in vivo* [5] and human *in vitro* [2, 3] studies have indicated that the proteolytic activity of Der p 1 is a major factor contributing to its allergenicity. Therefore, there is now considerable interest in generating selective inhibitors of its cysteine protease activity [15]. Clearly, the present study provides a sensitive and convenient *in vitro* system for testing such potential inhibitors. The great attraction of this system is that it is based on the synthesis of IgE, the ultimate readout for modulators of the allergic response.

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