

The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level

A. M. Ghaemmamghami, L. Gough, H. F. Sewell and F. Shakib

Division of Molecular & Clinical Immunology, Faculty of Medicine and Health Sciences, University of Nottingham, Queen's Medical Centre, Nottingham, UK

Summary

Background The proteolytic activity of the house dust mite allergen Der p 1 has recently been shown to bias Th cell subset development in favour of Th2. Apart from its direct effect on T cells, it is conceivable that the proteolytic activity of Der p 1 may induce the generation of dendritic cells (DCs) that favour a Th2 response.

Objective To study the effect of the proteolytic activity of Der p 1 on DC functions; namely cell surface phenotype, IL-12 production and ability to favour a Th2 response.

Methods We have generated immature DCs from peripheral blood monocytes, matured them with LPS in the presence of either proteolytically active or inactive Der p 1 and compared their functions using flow cytometric analysis.

Results Here we demonstrate for the first time that DCs that have been matured in the presence of proteolytically active Der p 1 produce significantly less IL-12, compared to DCs that have been matured in the presence of proteolytically inactive Der p 1. The suppression of IL-12 production was due to the cleavage of CD40 by the proteolytic activity of Der p 1, hence rendering the DCs less responsive to stimulation through the CD40L-CD40 pathway. Furthermore, we demonstrate that DCs that have been matured in the presence of proteolytically active Der p 1 induce the production of significantly less IFN- γ and more IL-4 by CD4 T cells, compared to DCs that have been matured in the presence of proteolytically inactive Der p 1.

Conclusions Collectively, our data provide compelling evidence for the role of the proteolytic activity of Der p 1 in directing DCs to induce Th2 subset development.

Keywords Allergen, dendritic cells, Der p 1, Th1/Th2

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Introduction

To mount an immune response, dendritic cells (DCs) have to capture small quantities of antigen at the site of entry and present it to specific T cells in secondary lymphoid organs [1]. CD4 Th cells that have been activated by DCs will follow one of two differentiation pathways leading to the development of two functionally distinct T cell subsets, namely Th1 and Th2. Th1 cells produce IL-2, IFN- γ and TNF- β which promote immunity against intracellular microbial infection, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13 which promote immunity against extracellular pathogens. The reciprocal antagonistic relationship that exists between these Th cell subsets is thought to play a critical role in preventing the development of Th1-mediated autoimmune diseases and Th2-mediated allergic responses [2, 3].

Recent work has demonstrated the existence of two human DC subsets which determine the differentiation of either Th1 or Th2 cells [4]. Thus, monocyte-derived DCs (myeloid-like cells or DC1) were found to induce Th1 differentiation by secretion of IL-12, whereas DCs derived from CD4⁺CD3⁺CD11c⁺ plasmacytoid cells (lymphoid-like cells or DC2) induce Th2 differentiation by a mechanism which apparently does not involve IL-4 or IL-12 secretion. Other authors have argued that myeloid DCs are good inducers of both Th1 and Th2 cytokines in naïve T cells, and that what determines Th cell subset development is not the ability to secrete IL-12 *per se*, but rather the level of IL-12 produced [5]. The amount of IL-12 produced by DCs can be up-regulated or down-regulated by the microenvironmental factors they mature in [6]. Collectively, these data underline the crucial role of IL-12 concentrations in the differential priming of naïve Th cells [7].

The above findings raise the important question of whether DC maturation and function are in any way influenced by the nature of the antigen it encounters. The immune system is exposed to a countless number of potentially antigenic proteins, yet only some of those proteins elicit Th2 responses, hence referred to as allergens. This therefore suggests the possibility

Correspondence: Dr Farouk Shakib, Division of Molecular & Clinical Immunology, Faculty of Medicine & Health Sciences, Queen's Medical Centre, Nottingham NG7 2UH, UK. E-mail: farouk.shakib@nottingham.ac.uk

that allergens may have an intrinsic property (e.g. proteolytic activity) which enables them to condition DCs to produce low levels of IL-12, thereby leading to powerful Th2 responses and a consequent bias towards IgE-mediated allergic disease.

The house dust mite *Dermatophagoides pteronyssinus* group 1 allergen Der p 1 has long been recognized as the most immunodominant allergen involved in the expression of mite-specific IgE-mediated hypersensitivity [8]. The reason for this potent IgE-eliciting property of Der p 1 is likely to be due to its cysteine protease activity, which we have shown to cleave both human [9, 10] and mouse [11] CD25, the 55 kDa α subunit of the T cell IL-2 receptor. As a result, these T cells show markedly diminished proliferation and IFN- γ secretion, and a consequent bias towards Th2 subset development [9, 10]. These *in vitro* findings are in line with our recent *in vivo* work which showed that immunization of CBA/J mice with proteolytically active Der p 1 results in a selective enhancement in total IgE and Der p 1-specific IgE synthesis compared to animals immunized with Der p 1 that has been irreversibly blocked with the cysteine protease inhibitor L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64) [11].

Given its powerful IgE-eliciting property, it is conceivable that the proteolytic activity of Der p 1 enhances Th2 subset development by also targeting immunological events that are upstream from T cells, namely those orchestrated by DCs. Here we demonstrate that Der p 1, in its proteolytically active form, does indeed condition DCs to produce less IL-12 upon subsequent stimulation through CD40, and that such DCs induce naïve CD4 T cells to produce more IL-4 and less IFN- γ .

Materials and methods

Antibody reagents

Mouse anti-human IFN- γ (FITC, clone 45.15), IL-4 (PE, clone 4D9), CD1a (PE, clone BL6), CD3 (ECD and PC5, clone UCHT1), CD4 (ECD, clone SFC1 12 T4 D11), CD11c (PE, clone BU15), CD40 (PE, clone MAB89), CD45RA (PE, clone ALB11), CD80 (FITC, clone MAB104), CD86 (PE, clone HA5.2B7) and HLA-DR (PC5, clone IMMU-357) antibodies, together with mouse IgG1 (FITC, ECD, PE and PC5, clone 679.1Mc7) and IgG2a (PC5 and PE, clone U7.27), were purchased from Coulter Immunotech (Luton, UK). Mouse anti-human IL-12 (p70) (PE, clone 20C2), CD40 (clone 5C3) and CD83 (FITC, clone HB15e) antibodies were purchased from Pharmingen (San Diego, CA, USA). Mouse anti-human CD28 antibody (clone CD28.2) was purchased from Coulter Immunotech. 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). Mouse anti-human IFN- γ receptor (CD119) antibody (FITC, clone BB1E2) was purchased from Serotec (Oxford, UK).

Der p 1 preparation

Since 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 [11]. 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 fast protein liquid chromatography (FPLC) to remove low molecular weight contaminants [11]. 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 preactivation with cysteine and is totally blocked by the class-specific inhibitor E-64 [11]. 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, since 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 [12].

Generation of mature dendritic cells

Monocytes were purified from peripheral blood of healthy non-atopic donors (with prior consent and Ethical Committee approval) as previously described [13]. Immature CD1a⁺CD83⁻ DCs were generated from monocytes as detailed elsewhere [14]. Briefly, this involved culturing monocytes with recombinant human (rh) GM-CSF (50 ng/mL; R & D Systems, Oxford, UK) and rhIL-4 (250 IU/mL; R & D Systems) in serum-free (AIM V) medium (Gibco Life Technologies, Paisley, UK), in which Der p 1 remains proteolytically active [9]. On day 6, most of the cells in the culture were immature HLA-DR⁺CD1a⁺CD83⁻ DCs. For the following 24 h, the cultures received LPS (200 ng/mL; Sigma) to induce maturation of DCs. To test the effect of the proteolytic activity of Der p 1 during this window of DC maturation, different concentrations (0.2, 1 and 5 μ g/mL) of either proteolytically active (preactivated with 5 mM cysteine) or inactive Der p 1 were added. The control culture was that which received LPS plus 5 mM cysteine (used for activating Der p 1) only.

Staining for cell surface markers

DCs were matured as described above, harvested, washed twice with PBS, 0.1% BSA and azide (PBA) and then stained for CD11c, CD40, CD80, CD83, CD86, CD119 and HLA-DR for 30 min in darkness at 4 °C. The cells were then washed twice with PBA and fixed in 0.5% formaldehyde in isotonic azide free solution.

Measurement of soluble CD40

Soluble CD40 (sCD40) was measured in the culture supernatant of DCs, that have been matured for 24 h with LPS in the presence of different concentrations of proteolytically active or inactive Der p 1, using a sandwich-based ELISA kit (Bender MedSystems, Vienna, Austria).

Stimulation of mature dendritic cells

At the end of the 24-h maturation period, the DCs were harvested, extensively washed and resuspended in fresh medium. The cells (1×10^5) were then stimulated for 24 h with either 10 µg/mL of immobilized anti-CD40 antibody and 10^3 IU/mL rhIFN- γ (R & D Systems) or with 5 µg/mL of an extract of the parasite *Toxoplasma gondii* (soluble tachyzoite antigen or STAg; kindly provided by Dr Alan Sher, NIAID, Bethesda, MD, USA) [15] in 96-well culture plates. Brefeldin A (10 µg/mL; Sigma) was added 4 h after starting the cultures. The cells were then stained intracellularly for IL-12 (p70), the biologically active heterodimer which consists of two disulphide bonded subunits of IL-12 (p35) and IL-12 (p40).

Sorting and priming of naïve T cells

Peripheral blood naïve CD3⁺CD45RA⁺ T cells were isolated, to about 95–98% purity, using a Coulter EPICS XL-MCL cell sorter (Beckman Coulter, Highwycombe, UK). The naïve T cells (3×10^5 cells/500 µL) were cocultured with autologous DCs (6×10^4 cells/500 µL), that had been matured with either proteolytically active or inactive Der p 1 (see above), in 48-well culture plates in the presence of the superantigen *Staphylococcus aureus* enterotoxin B (SEB; 1 ng/mL; Sigma). On day 6, 10 IU/mL of rhIL-2 was added and the cultures were expanded for another 3 days. The cells were then harvested, washed and re-stimulated for 24 h with 2 µg/mL of immobilized anti-CD3 and anti-CD28 antibodies in 96-well culture plates. Brefeldin A (10 µg/mL) was added 4 h after starting the cultures for intracellular measurement of IL-4 and IFN- γ .

Intracellular cytokine staining

Intracellular cytokine staining was performed as previously described [16, 17]. Briefly, cells were harvested and transferred to polystyrene FACS tubes (Falcon Becton Dickinson Labware, Franklin Lakes, NJ, USA) and washed in PBA buffer. Cells were resuspended and kept in 0.5% formaldehyde (Philip

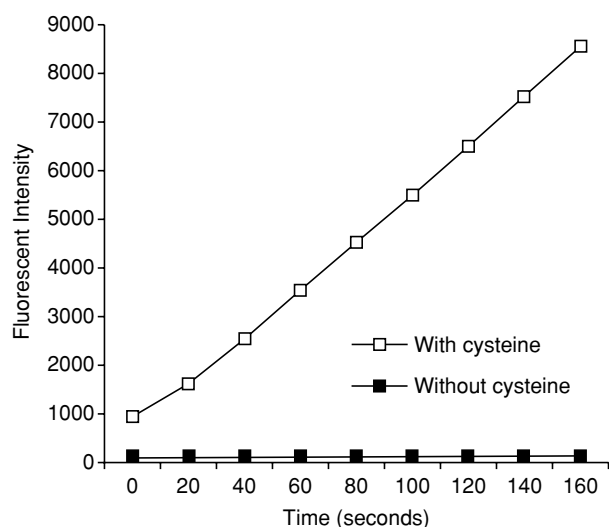


Fig. 1. Progression curves of the catalytic activity of Der p 1, measured in a continuous rate (kinetic) assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC [12], with or without preactivation with cysteine. Data presented are the means of duplicate experiments; standard error was less than 5%.

Harris, Lichfield, UK) in azide free isotonic solution (Beckman Coulter, Highwycombe, UK) and stored overnight at 4 °C. Next day, 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 anticytokine 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 darkness. 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.

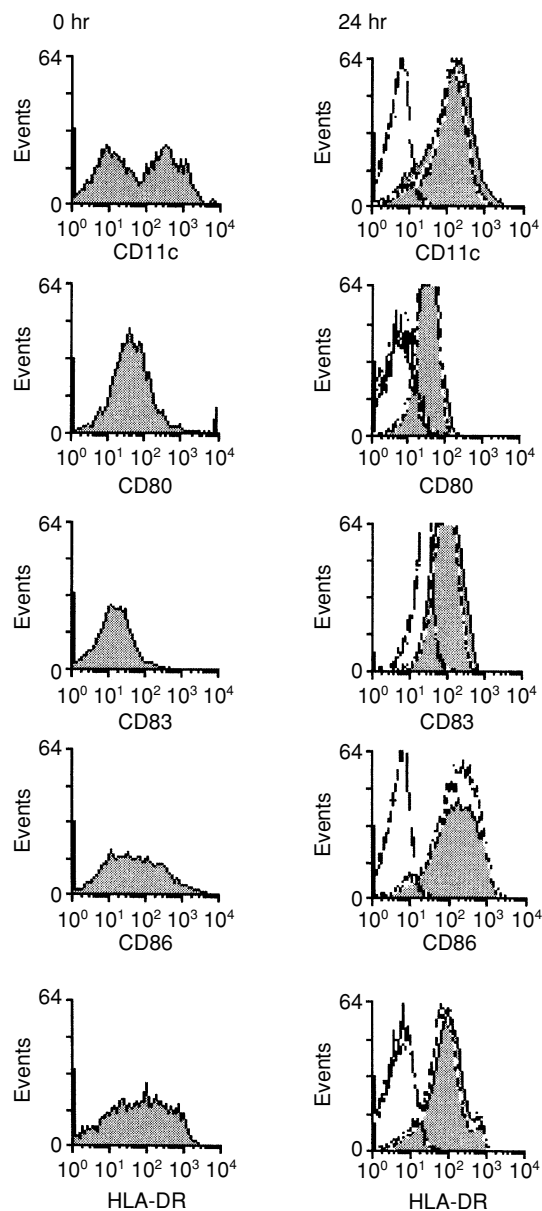


Fig. 2. The effect of the proteolytic activity of Der p 1 on the surface phenotype of DCs at 0 and 24 h of maturation. The histograms show the induction of maturation of DCs that have been matured with LPS in the presence of either proteolytically active or inactive Der p 1. The level of expression of surface markers was the same regardless of whether the DCs were matured in the presence of proteolytically active (solid profile) or inactive (blank profile) Der p 1; the profile closest to the y axis in the 24-h time-point represents the isotype control. Data shown are representative phenotype of DCs obtained from three individuals.

Flow cytometric analysis

Flow cytometry was performed using a Coulter EPICS XL-MCL (Beckman Coulter) and 50 000–100 000 events were collected for each sample. Dead cells were excluded by forward and side scatter characteristics. Isotype-matched irrelevant antibodies were used to verify the staining specificity. The quadrants were set to obtain 1–2% positive cells in the preparation that was stained with the isotype-matched irrelevant antibody.

Statistical analysis

The paired Student *t*-test was used to compare cytokine levels and surface marker expression on cells obtained from cultures that have been treated with either proteolytically active or inactive Der p 1; *P*-values less than 0.05 were considered significant.

Results

The proteolytic activity of Der p 1 depends on preactivation with cysteine

We have purified Der p 1 from fecal pellets using a multistep procedure and confirmed its purity by N-terminal sequencing, SDS-PAGE analysis [11] and by demonstrating that enzymatic activity is completely dependent on preactivation with cysteine (Fig. 1).

The effect of the proteolytic activity of Der p 1 on the expression of DC surface markers

Monocyte-derived immature DCs were exposed to LPS for 24 h in the presence of either proteolytically active or inactive

Der p 1. To ascertain the phenotype of the DCs that have been matured in this way, the cells were stained for CD11c, CD80, CD83, CD86 and HLA-DR. This has shown initiation of the final maturation of the DCs, as evidenced by the appearance of CD83 and the up-regulation of CD11c, CD80, CD86 and HLA-DR. The level of expression of these markers was the same regardless of whether the DCs were matured in the presence of proteolytically active or inactive Der p 1 (Fig. 2).

The proteolytic activity of Der p 1 conditions DCs to produce significantly less IL-12

We have generated immature DCs from peripheral blood monocytes, matured them with LPS in the presence of either proteolytically active or inactive Der p 1, washed them and then stimulated them with anti-CD40 antibody and rhIFN- γ . Here we show that DCs that have been matured in the presence of proteolytically active Der p 1 produce significantly less IL-12 ($P < 0.001$) compared to DCs that have been matured in the presence of proteolytically inactive Der p 1. The reduction in the number of IL-12-producing cells was also accompanied by a 1.5-fold decrease in the fluorescence intensity, indicating that less IL-12 was produced per cell (Fig. 3).

The suppression of IL-12 production by DCs that have been matured in the presence of proteolytically active Der p 1 is due to cleavage of CD40

We then asked the question of whether the suppression of IL-12 production by DCs was due to proteolytic cleavage of CD40 or the IFN- γ receptor by Der p 1, thereby making the cells less responsive to stimulation by anti-CD40 and rhIFN- γ , respectively. We found that DCs that have been matured in the

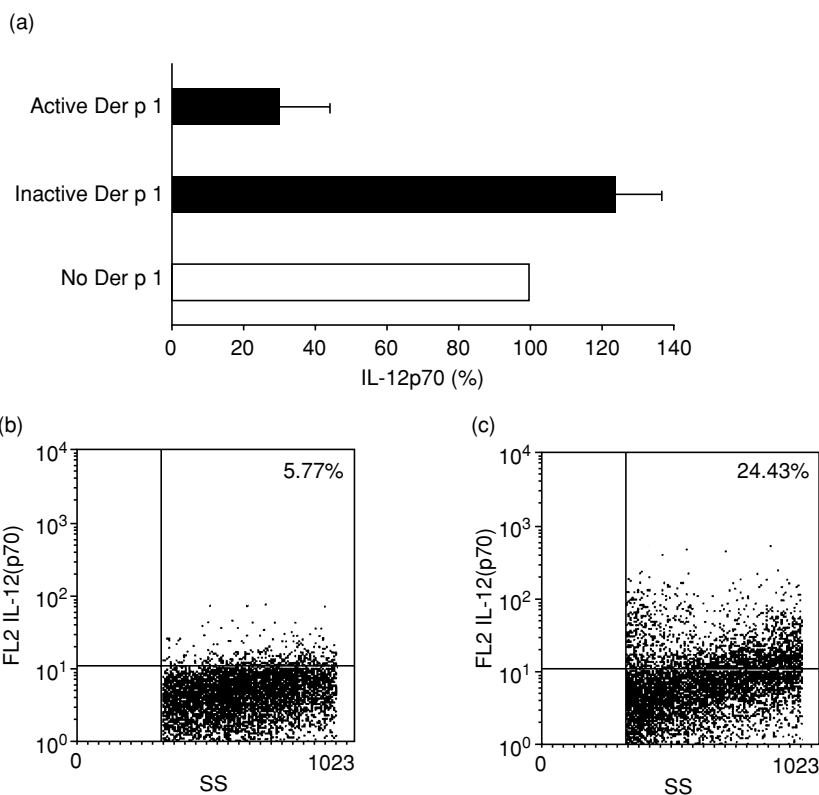


Fig. 3. Intracellular IL-12 expression by DCs that have been matured with LPS in the presence of an optimal concentration (5 μ g/mL) of either proteolytically active or inactive Der p 1 and re-stimulated with immobilized anti-CD40 and IFN- γ . (a) Given the variation in the absolute amount of IL-12 production by different donors, the level of IL-12 produced by DCs which have been matured with LPS in the presence of Der p 1 is expressed as percentage of IL-12 produced by DCs from the same donor which have been matured with LPS plus cysteine (blank bar represents 100%). Data shown represent mean $\pm$ SD values obtained with DCs from six individuals, and correspond to percentage of cells expressing IL-12 (range 1.3–28.7%; $P < 0.001$). (b and c) are representative dot plots corresponding to IL-12 expression in DCs that have been matured in the presence of proteolytically active (b) or inactive (c) Der p 1. SS, side scatter.

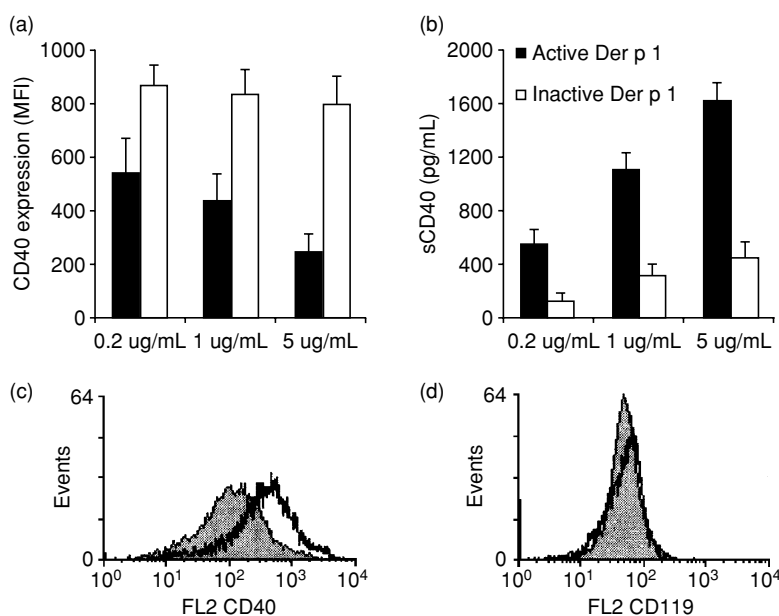


Fig. 4. Expression of CD40 on DCs that have been matured with LPS in the presence of either proteolytically active or inactive Der p 1. (a) Dose-dependent decrease ($P < 0.001$) in the expression of CD40 on DCs expressed as mean fluorescence intensity (MFI). (b) Dose-dependent release ($P < 0.001$) of sCD40 detected in the supernatant of DC cultures. Data shown represent the mean $\pm$ SD values obtained with DCs from five individuals. (c and d) are representative histograms corresponding to CD40 and CD119 expression on DCs that have been matured with proteolytically active (solid profile) or inactive (blank profile) Der p 1, respectively.

presence of proteolytically active Der p 1 express significantly less CD40 ($P < 0.001$) compared to DCs that have been matured in the presence of proteolytically inactive Der p 1. This was due to cleavage of the CD40 molecule by the proteolytic activity of Der p 1, as evidenced by the release of sCD40 in the culture supernatant. However, no such decrease in expression was demonstrable for the IFN- γ receptor (Fig. 4).

The proteolytic activity of Der p 1 does not affect the innate ability of DCs to produce IL-12

In order to confirm that the observed suppression of IL-12 production by DCs is due to cleavage of CD40, we monitored IL-12 production after stimulation with STAg, a microbial agent known to stimulate IL-12 production independently of CD40 ligand (CD40L) [18]. This has shown that DCs that have been matured in the presence of proteolytically active Der p 1 are just as capable of producing IL-12 upon stimulation with STAg as those matured in the presence of proteolytically inactive Der p 1 (Fig. 5). This means that DCs that have been in contact with the proteolytic activity of Der p 1 do not lose their innate ability to produce IL-12. This confirms the notion that the suppression of IL-12 production demonstrated here is due to the cleavage of CD40 by the proteolytic activity of Der p 1, hence rendering the DCs less responsive to stimulation with anti-CD40.

DCs that have been matured in the presence of proteolytically active Der p 1 bias the differentiation of naïve CD4 T cells towards Th2

Having demonstrated that the proteolytic activity of Der p 1 conditions DCs to produce significantly less IL-12, we tested their ability to influence the cytokine profile of naïve T cells. DCs that have been matured in the presence of either proteolytically active or inactive Der p 1 were cultured with naïve T cells in the presence of the superantigen SEB and later rhIL-2, the cells were harvested, washed and then stimulated with anti-CD3 and anti-CD28 antibodies. This has demonstrated that DCs

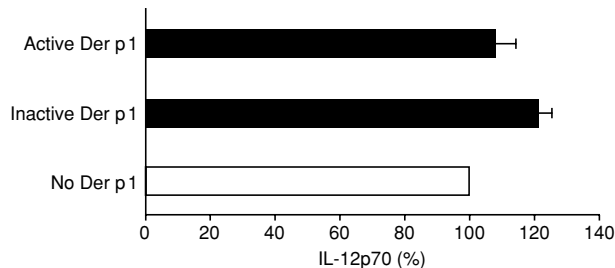


Fig. 5. Intracellular IL-12 expression by DCs that have been matured with LPS in the presence of an optimal concentration (5 µg/mL) of either proteolytically active or inactive Der p 1 and re-stimulated with STAg. Results are expressed as explained in caption to Fig. 3. Data shown represent mean $\pm$ SD values obtained with DCs from three individuals, and correspond to percentage of cells expressing IL-12 (range 4.5–15.1%).

that have been matured in the presence of proteolytically active Der p 1 induce the production of significantly less IFN- γ ($P < 0.001$) in both CD4 and CD8 (CD3⁺CD4⁺) naïve T cells, and significantly more IL-4 ($P < 0.002$) in naïve CD4 T cells, compared to DCs that have been matured in the presence of proteolytically inactive Der p 1 (Fig. 6).

Discussion

IgE-mediated hypersensitivity is the underlying mechanism of atopic conditions, such as asthma, that afflict about 20% of the population [19]. 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, may bias Th cell subset development in favour of Th2 [9, 10]. Apart from its direct effect on T cells, it is conceivable that the proteolytic activity of Der p 1 may induce the generation of DCs

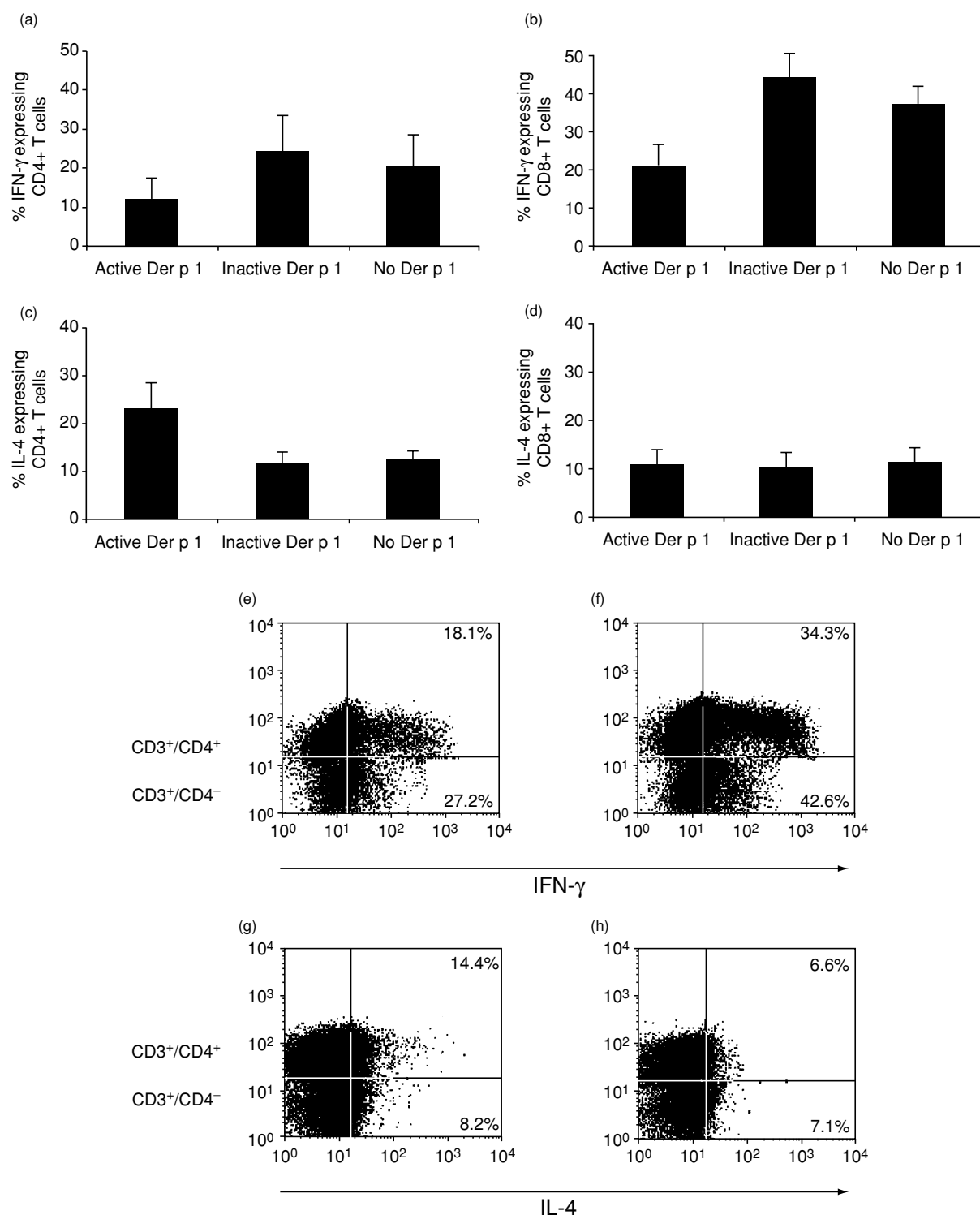


Fig. 6. Effect of DCs that have been matured with LPS in the presence of an optimal concentration (5 μ g/mL) of either proteolytically active or inactive Der p 1 on IFN- γ ($P < 0.001$) and IL-4 ($P < 0.002$) expression by naïve CD4 and CD8 (CD3⁺CD4⁺) T cells following re-stimulation with anti-CD3 and anti-CD28 antibodies. The data in a–d show the mean $\pm$ SD values obtained with DCs from three individuals. The figure also shows representative dot plots demonstrating that DCs that have been matured in the presence of proteolytically active Der p 1 suppress IFN- γ and enhance IL-4 production by T cells (e and g), compared to those which have been matured in the presence of proteolytically inactive Der p 1 (f and h).

that favour a Th2 response and consequent IgE production. In other words, the proteolytic activity of Der p 1 may condition DCs, which then become empowered to stimulate naïve T cells to become Th2 cells.

Here we demonstrate that the proteolytic activity of Der p 1 cleaves CD40 from the DC surface, thereby leading to suppression of IL-12 production in response to stimulation by anti-CD40 antibody. Clearly, the cleavage of CD40 by the

proteolytic activity of Der p 1 will deprive DCs of the ability to receive CD40L-mediated signals from T cells, which is an important pathway for stimulating IL-12 production [20–23]. Thus, in light of recent observations showing that signalling through CD40 is essential for the up-regulation of IL-12 (p35) [22], we believe that the down-regulation of IL-12 (p70), observed in DCs that have been matured in the presence of proteolytically active Der p 1, is due to low levels of IL-12 (p35) production.

This decrease in IL-12 production by DCs may enable them to direct the differentiation of naïve T cells towards a Th2 cytokine profile. The significance of the observed changes in IL-12 production by DCs is underlined by previous work which showed that the concentration of IL-12 [7, 24] in the microenvironment plays a pivotal role in the differential priming of naïve Th cells by DCs. We have indeed been able to demonstrate that DCs that have been matured in the presence of proteolytically active Der p 1 induce the production of less IFN- γ by CD4 and CD8 T cells and more IL-4 by CD4 T cells.

Clearly, any extrapolation of these effects to the *in vivo* situation would partly depend on the expected concentration of Der p 1 in the target organ, namely the lungs. Der p 1 accounts for about 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 very high concentrations in the lungs. Passage of Der p 1 across the mucosal surface would bring this allergen in direct contact with DCs, an encounter which would trigger DC migration into the proximal lymphoid organs, where they mature into a developmental state that allows them to select and activate specific T cells. Clearly, prior cleavage of CD40 in peripheral tissues by Der p 1 would deprive the DCs of the ability to receive costimulatory signals from T cells in the lymphoid organs, thereby leading to a significant reduction in IL-12 production.

Collectively, our data provide compelling evidence for the role of the proteolytic activity of Der p 1 in the decision making process of the DCs. Thus, we believe that DCs that have been conditioned by the proteolytic activity of Der p 1 will provide the initial driving force for Th2 cell development, whilst Der p 1-induced cleavage of CD25 from the T cell surface [9, 10] will serve to further amplify this process. Our results provide new insights into the mechanism by which the proteolytic activity of an important allergen, namely Der p 1, leads to powerful IgE antibody responses and consequent allergic disease. The novelty of this work is the demonstration that the intrinsic (enzymatic) property of an allergen determines the outcome of the immune response.

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