

Differential Protein Expression by Dendritic Cells from Atopic and Non-Atopic Individuals after Stimulation by the Major House Dust Mite Allergen Der p 1

C. Horlock^a F. Shakib^a N.S. Jones^b H.F. Sewell^a A.M. Ghaemmamghami^a

^aInstitute of Infection, Immunity and Inflammation, School of Molecular Medical Sciences and Biomedical Research Unit, and ^bDivision of Otorhinolaryngology, School of Clinical Sciences, University of Nottingham, Nottingham, UK

Key Words

Allergy · Dendritic cells · Der p 1 · Dust mite · Proteomics

Abstract

Background: Dendritic cells (DCs) are sentinels of the immune system and are known to play a key role in allergic responses. However, it is not clear how DCs that have been exposed to an allergen support Th2 type immune responses. It is possible that DCs from atopic individuals are inherently programmed to support allergic disease, or it is the exposure of dendritic cells to allergens that is key to the development of allergic sensitisation. **Methods:** We used 2D gel electrophoresis and MALDI mass spectrometry to compare the proteome of DCs from atopic and non-atopic individuals in both the resting state and after stimulation with the major house dust mite allergen Der p 1. **Results:** Our data show that unstimulated DCs from atopic and non-atopic individuals are very similar at the whole cell proteome level, showing few differentially expressed proteins. However, upon stimulation with Der p 1, a number of additional proteins are differentially expressed, and of these several were of potential relevance to Th2 cell differentiation and the allergic response, including GTP-binding regulatory protein Gi alpha-2, frabin and cathepsin D. **Conclusion:** Whilst there are inherent differences between DCs from atopic and non-

atopic individuals, it seems that exposure to allergen plays a key role in differential expression of proteins by these key immune cells. Further studies should now focus on establishing the biological relevance of these proteins as biomarkers in house dust mite allergy and their role in allergen induced Th2 cell differentiation.

Copyright © 2009 S. Karger AG, Basel

Introduction

Allergic diseases are thought to be the result of an interaction between genetic and environmental factors [1]. A number of studies have been carried out to investigate this interaction, but no definitive etiology has yet been identified. Allergens are proteins that are endowed with the ability to elicit powerful T helper type 2 (Th2) responses, culminating in excessive IgE antibody production and the development of allergic diseases, such as asthma and rhinitis. The house dust mite *Dermatophagoides pteronyssinus* group 1 inhaled allergen (Der p 1) has long been recognised as the most immunodominant allergen involved in the development of IgE-mediated hypersensitivity [2]. This potent IgE-eliciting property of Der p 1 is likely to be due to its cysteine protease activity, which we have previously shown to digest cell surface

molecules involved in the homeostatic control of IgE synthesis, including CD23 on B cells [3, 4], CD25 on T cells [5, 6] and CD40 and DC-SIGN on dendritic cells (DCs) [7, 8].

Of the 3 cell types targeted by Der p 1, DCs are particularly vulnerable to proteolytic attack, given their close proximity to the external environment, and this is important since DCs play a crucial role in orchestrating T helper cell responses. The central role of DCs in the induction of Th2-mediated allergic diseases has been clearly established in the last few years [9–11], but the underlying mechanism of allergen-induced modulation of DC behaviour remains elusive. For instance, we are still largely unaware of how DCs acquire an activated phenotype after encountering allergens and why allergen-activated DCs induce Th2, rather than Th1, responses.

Given the central role of DCs in the initiation of Th1 and Th2 responses, it is highly likely that the expression of genes and proteins by DCs, after exposure to allergens, would provide important clues as to why allergen-primed DCs support Th2 responses. Several recent studies have suggested that the protein profile (i.e. proteome) of DCs changes dramatically when challenged with antigen and that this varies according to their maturation state [12]. Therefore, in an attempt to obtain a global picture of DC protein changes following exposure to allergen, we have undertaken a proteomic analysis of monocyte-derived DCs, from atopic and non-atopic individuals, stimulated with either proteolytically active or inactive Der p 1. It is hoped that this may help uncover signalling pathways and molecules of key importance to the allergic response, leading to biomarker discovery and enabling development of new intervention strategies in allergic disease.

Materials and Methods

Patient Population

Whole blood was taken from healthy donors and volunteers with positive skin prick tests for house dust mite. Serum was then measured for total and house dust mite specific IgE levels. Samples defined as atopic ($n = 8$) for proteomic analysis had total IgE levels of above 130 kU/l, measured using the AlaStat (Allergy Immunoassay System; DPC, Oxford, UK), and house dust mite specific IgE at levels greater than 3.5 kUa/l (grade III or above) as measured by the ImmunoCap system (Phadia AB, Uppsala, Sweden). None of the atopic patients were undergoing treatment with steroids. Individuals with total serum IgE levels below 114 kU/l were considered non-atopic ($n = 8$). Further details can be found in table 1. Non-atopic and atopic sample pools, with equal numbers of cells from each donor, were created for running 2-dimensional gels and proteomic analysis. All samples were obtained with prior consent and ethics committee approval.

Table 1. Atopic and non-atopic donor information

	Atopics	Non-Atopic
Age, years	36 ± 12.7	33 ± 10.5
Gender (M/F), n	3/5	5/3
Total IgE, kU/l	413 ± 312	29 ± 19
Positive skin prick test to house dust mite (wheal >3 mm), n	8	0

Cell Cultures and Stimulation

Heparinized whole blood from healthy volunteers and atopic individuals was used for separation of peripheral blood mononuclear cells on a Histopaque density gradient (Histopaque-1077; Sigma, Poole, UK). CD14⁺ monocytes were isolated by positive selection from the mononuclear cells, and cell purity was verified by flow cytometry. Immature CD1a⁺CD83⁻ DCs were generated as previously described [8, 13]. Briefly, this involved culturing CD14⁺ monocytes (purity >95% as measured by flow cytometry) in the presence of interleukin 4 (IL-4, 250 IU/ml; R&D Systems, Oxford, UK) and granulocyte-macrophage colony stimulating factor (GM-CSF, 50 ng/ml; R&D Systems) for 6 days. Cells were cultured at 1×10^6 /ml in RPMI 1640 medium (Sigma), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (Gibco Life Technologies, Paisley, UK) and 10% v/v foetal calf serum (FCS; Harlan Sera-Lab, Loughborough, UK), at 37°C in 5% CO₂. On day 3, cultured cells were fed with fresh medium containing relevant cytokines. On day 6, cells were washed 3 times in serum-free AIM V medium, in which Der p 1 remains proteolytically active [6], then replated and stimulated. Der p 1 purification was performed as described previously [14].

The Der p 1 enzyme active site becomes oxidised during purification, therefore activation of purified Der p 1 with a reducing agent such as cysteine was carried out to produce proteolytically active Der p 1. DCs were stimulated with 5 µg/ml Der p 1, either in the presence of 5 mM cysteine (proteolytically active, AC) or in the presence of 5 mM cysteine and 40 µM of the protease inhibitor anti-pain (proteolytically inactive, IN). After stimulation for 48 h, cell supernatant was collected and frozen at -80°C for future cytokine analysis, and the cells were then washed 3 times with PBS (Sigma) followed by resuspension in electrophoresis lysis buffer [13]. Samples were vortexed for up to 10 minutes and then frozen at -80°C until processing.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis (2DE) was performed as previously described [13]. Briefly, differentially treated DCs from atopics and non-atopics were harvested and resuspended in lysis buffer containing 7 M urea (Fisher Scientific, Loughborough, UK), 2 M thiourea (Sigma), 4% CHAPS (Fisher Scientific), 50 mM dithiothreitol (DTT; Fisher Scientific), 5 mM TBP (Bio-Rad, Hercules, Calif., USA), 0.5% carrier ampholytes (Invitrogen, Paisley, UK), 1X protease inhibitor (Amersham, Little Chalfont, UK), 150 U/ml benzonase (Novagen; Merck Biosciences, Nottingham, UK) and a trace of bromophenol blue (Sigma). Samples were frozen at -80°C until processing. Cell lysates from 8 individuals were combined and a protein concentration assay (2D Quant Kit; Amer-

sham) was carried out. Samples of 30 µg were made up to 320 µl with lysis buffer, vortexed for 5 min at room temperature and centrifuged at 14,000 rpm for 30 min.

Immobilised pH gradient strips (Bio-Rad) were passively re-hydrated by the protein samples at 20°C for approximately 17 h. A low voltage run at 50 V was then performed for 6 h. Isoelectric focusing was run with the following conditions: rapid ramping 250 V for 15 min, 10,000 V for 3 h followed by total 60,000 V/h and a subsequent 500 V hold. Immobilised pH gradient strips were equilibrated for 30 min in equilibration buffer, containing 6 M urea, 2% SDS, 0.05 M Tris and 20% Glycerol (Fisher Scientific), for 15 min with 2% DTT and 15 min with 2.5% iodoacetamide (Bio-Rad). The second dimension separation was carried out on precast vertical 10–20% SDS-polyacrylamide gels (BioRad). Gels were typically run at 20 mA per gel for 18 h.

Protein Visualisation and Image Analysis

Gels were stained using the Dodeca Silver Stain Kit (Bio-Rad) which was shown, by us [13] and others [15, 16], to be linear, reproducible and mass spectrometry compatible. While, in comparison with silver staining, Coomassie staining is reported to have better linearity and compatibility with mass spectrometry, with the low protein quantities used in this study we found the Coomassie staining not sensitive enough. An alternative fluorescent stain, Sypro Ruby (Molecular Probes, Eugene, Oreg., USA), although reported to have better linearity and similar sensitivity to silver staining, was found to be less sensitive to silver staining in our hands and had a high non-specific background, making quantification problematic.

Gels were scanned on a GS-800 calibrated imaging densitometer (Bio-Rad). Image analysis was performed as described before [13]. Briefly, gel images were analysed using PDQuest gel analysis software version 7.1 (Bio-Rad). Spots were automatically detected, and then manually checked for undetected or incorrectly detected spots. Scanned images were uploaded to PDQuest and then cropped to produce the same gel area across all samples. Spots were detected using the 'spot detection wizard' tool, which is a spot detection filter allowing the detection parameters to be selected. For comparison of the gels, the same parameters were used to ensure identical staining and scanning methods for all samples.

PDQuest uses Gaussian modelling to create 'ideal' spots that can be easily identified and quantified. Using Gaussian modelling, it is possible to accurately quantitate overlapping spots, spots in gel streaks, and multiple spots in dense clusters; all challenges for software-based gel analysis. Following matching of all gels in the comparison, all spots were checked using the spot review tool, which allowed for the matches to be confirmed by eye, enabling any missed spots or inaccurately matched spots to be identified and changed accordingly. As well as comparing gels from different samples, it was also confirmed that multiple gels from identical samples were reproducible. This was confirmed by comparing samples within a matchset to confirm gel-to-gel reproducibility. All images were normalized according to total quantity in valid spots in each gel before any comparisons were made, to ensure the sample load did not create any bias. To carry out further analysis, data from PDQuest was exported and analysed using Excel (Microsoft, Redmond, Wash., USA). For each group, spot quantity was exported. Spot quantity is the total density of a defined spot in a gel image, representing the actual amount of protein in a gel.

To calculate the difference in expression of spots from sample gels, the spot quantity in each sample set was divided to create a 'factor of difference'. Spots with differential expression between groups were chosen and then protein identification was attempted using MALDI-TOF mass spectrometry.

In-Gel Digestion

Gel pieces were excised and placed in a 96 well plate, then loaded onto a MassPrep robotic liquid handling system (Waters Corp., Elstree, UK). This was used to destain gel pieces, reduce and alkylate cysteine residues using DTT and iodoacetamide, carry out an in-gel tryptic digest and extract the resulting peptide mixture into a 96 well PCR plate. The extracted peptide mixture was manually desalted using C18 loaded zip-tips (Millipore, Livingston, UK). Two microlitres were routinely spotted onto sample wells of a stainless steel MALDI target plate previously spotted with 1 µl matrix solution, comprising 1 mg/ml α -cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile, 50% ethanol and an internal standard, adeno corticotrophic hormone (Sigma) at a final concentration of approximately 100 fmol/µl in 0.1% formic acid (Romil, Cambridge, UK). Samples were left to air dry and the plate placed in the MALDI mass spectrometer.

MALDI-TOF Mass Spectrometry Analysis of Protein Spots

Peptide mixtures eluted from each gel spot were analysed using a matrix assisted laser desorption/ionisation time of flight mass spectrometer (MALDI TOF MS; Waters Corp.) operating at a resolution of greater than 10,000 full width at half maximum in reflectron mode. Spectra were acquired at 5 Hz using a nitrogen laser (337 nm wavelength). Typically, 10 data collection events were combined to generate each spectrum. Data acquisition was achieved by randomly sampling from the target well. Peak lists (peptide mass fingerprints, PMF) were entered into a Mascot PMF database search engine (Matrix Science, London, UK; www.matrixscience.com) or Aldente (www.expasy.org). Peptide masses corresponding to keratin tryptic digests, tryptic autolysis peptides and peptides detected in the negative control (a sample of a spot-free area of gel) were excluded from the analysis. Search parameters used included a peptide mass tolerance of ± 0.2 Da, which allows for modifications such as alkylation of cysteine during the tryptic digest procedure and the formation of methionine sulfoxide. Mass values were monoisotopic, protein mass was unrestricted, peptide charge state was 1+ and the maximum number of mixed cleavages was 1.

Mascot assigns each database search item with a protein score. This score is characterised as $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Using this calculation protein scores of 64 or above correspond to a statistically significant match ($p < 0.05$). In addition to this, Mascot also generates an expectation value which is the number of times we would expect to obtain an equal or higher score, purely by chance. These values enabled assessment of the validity of the protein matches using the PMF generated by our gel spots of interest.

Statistical Analysis

The paired Student's t test was used to compare datasets of differentially stimulated DCs from the same population, and the unpaired Student's t test was used to compare atopic with non-atopic samples. $p < 0.05$ was considered significant.

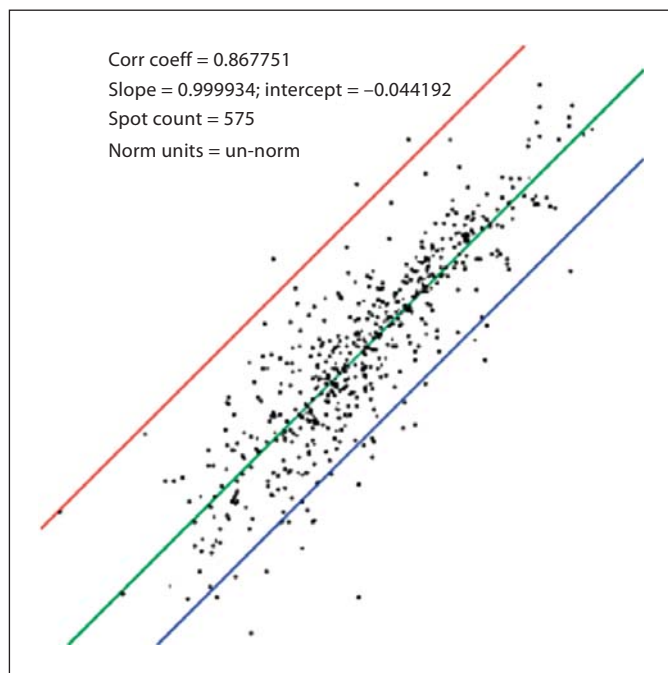


Fig. 1. Correlation between spots from gels of unstimulated DCs isolated from atopic (x-axis) and non-atopic (y-axis) individuals. The centre line is the regression line, and the area outside the 2 outer lines represent spots greater than 2× differentially expressed in the 2 populations.

Results and Discussion

Comparative Studies of Immature DCs from Atopics and Non-Atopics

A comparison of unstimulated, immature DCs from non-atopic and atopic donors was carried out to see if DCs of atopic individuals were inherently programmed to initiate allergic disease compared to those from healthy non-atopic donors. Multiple gels from atopic and non-atopic sample pools (samples from 8 donors in each pool with the same number of cells from each donor) were compared using PDQuest. This analysis revealed that samples from the same population (i.e. atopic or non-atopic) were highly reproducible, as evidenced by correlation coefficients for atopic and non-atopic samples of 0.90, when comparing samples run on the same percentage gels.

Comparing the proteomes of immature DCs from the 2 populations showed a high degree of similarity (correlation coefficient of 0.87; fig. 1), but there were a number of quantitative differences with higher expression of 6 proteins in DCs isolated from non-atopic individuals and 11

proteins had higher expression in atopic donors. Figure 2 shows representative gels from unstimulated DCs isolated from atopic or non-atopic donors; labelled spots on the gel were successfully identified using mass spectrometry (table 2). Of these, 2 proteins, namely glutathione-S-transferase (GST) and heat shock protein 27 (HSP27), were found to be of possible importance to the allergic response. GST was more highly expressed ($p < 0.05$) in unstimulated DCs isolated from atopic donors compared with those isolated from non-atopic donors, suggesting that internal glutathione levels may be lower in atopy. This correlates with published data showing that low levels of intracellular glutathione supports a Th2 type response [17–19].

GSTs are enzymes that catalyse the conjugation of reduced glutathione with electrophilic groups of a wide variety of compounds [20]. The GST superfamily is made up of at least 5 main classes: alpha (A), mu (M), pi (P), theta (T) and zeta (Z) [21, 22]. Members of M, T and P GSTs are known to play a critical role in protection of cells from the toxic products of reactive oxygen species, which has led several researchers to propose a link between polymorphisms in GST and allergy and asthma [22–25].

The gene for GST is found on chromosome 11q13, known to be a ‘hot spot’ for asthma-related genes [26], and it has been shown that cytosolic GSTs (mu and pi) demonstrate activity towards both leukotrienes and prostaglandins, and may influence the synthesis of eicosanoids, critical mediators of the asthmatic response [23, 27]. Several researchers have linked polymorphisms in GSTP1 with atopy and asthma [22–25].

Furthermore, HSP27 was found to be more highly expressed ($p < 0.05$) in DCs from the atopic population compared with the non-atopic population. HSP27 is a small stress protein that belongs to the superfamily of heat shock proteins. It is an oligomeric phosphoprotein that negatively regulates cell death [28–30], and has also been implicated in modulation of actin polymerisation and cytoskeletal architecture [31, 32]. It appears that the latter function may be unique to monomeric non-phosphorylated HSP27 [30, 33]. There are several studies that suggest an association between HSP27 expression and allergy or asthma [28, 34]. Hastie et al. [28] showed elevated levels of HSP27 in allergic individuals (in mild inflammation) compared with non-allergic subjects; this would appear to be in agreement with our proteomic data. The atopic patients we studied were not suffering from severe allergic disease, which would suggest that HSP27 may indeed play a role in mild allergic inflammation found in

Fig. 2. Two dimensional gels of unstimulated dendritic cells generated from atopic (**a**) and non-atopic (**b**) donors. These are representative of triplicate 10% polyacrylamide gels. Spots labelled were identified by mass spectrometry.

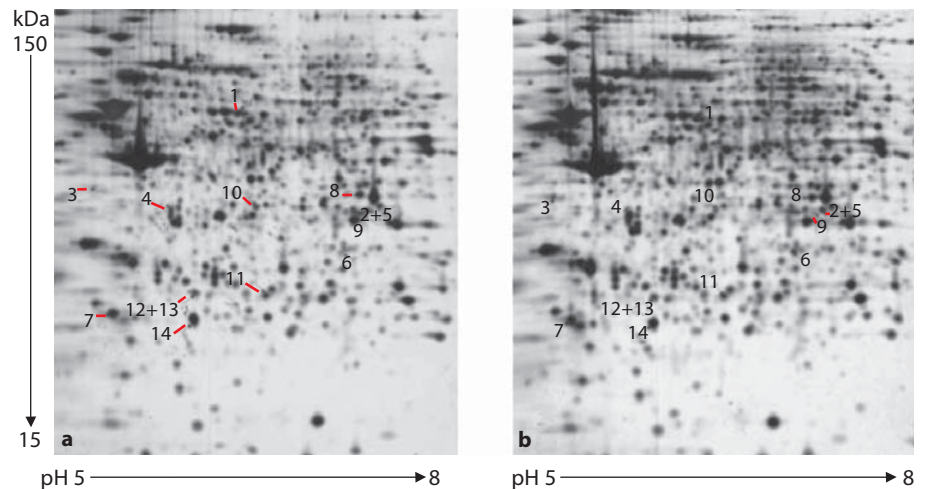
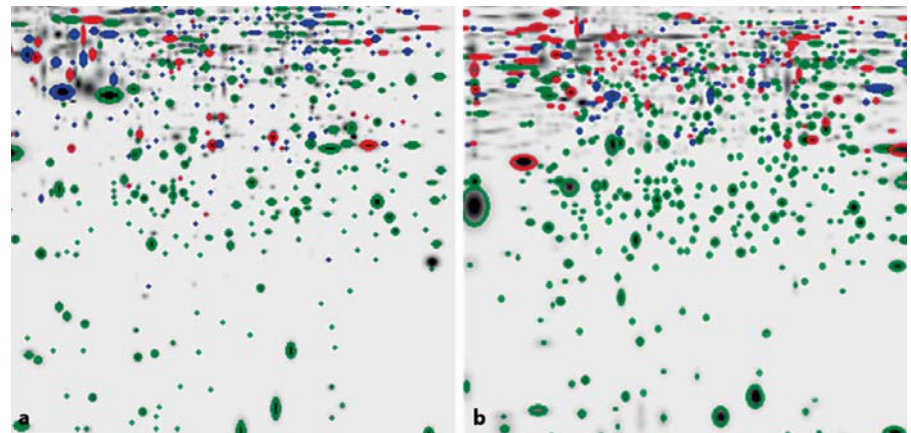


Fig. 3. Gel overlay images comparing gels of DCs stimulated with proteolytically active (AC; blue) or inactive (IN; red) Der p 1. Spots in both gels are in green, those unique to either condition are in blue or red. **a** DCs isolated from non-atopic donors. **b** DCs isolated from atopic donors. Der p 1 stimulated gels were run on 14% acrylamide gels.



constitutively atopic individuals, as reported by Hastie et al. [28].

Proteomic Profile of DCs after Allergen Stimulation

Whole cell proteomes of DCs were compared after stimulation with either proteolytically active or inactive Der p 1. For this we created whole cell lysate pools of DCs stimulated under each condition, with each pool containing cells from 8 donors who were either atopic or non-atopic as determined by measuring total and specific serum IgE levels. From each pool at least triplicate gels of each condition were run by 2DE, and a comparison carried out. Gels from identical pools were found to be highly reproducible with a very similar correlation coefficient (correlation coefficient >0.9), when compared using PDQuest, and a comparison of the protein expression from samples stimulated under different conditions also

showed fairly similar correlation in spots (correlation coefficient >0.80).

Comparing the relative abundance of gel spots of stimulated DCs revealed numerous differentially expressed proteins. Figure 3 shows an overlay image of gels of DC lysate from non-atopic (fig. 3a) and atopic individuals (fig. 3b). Images of gels from DCs stimulated with proteolytically active (AC) and proteolytically inactive (IN) Der p 1 have been overlaid to reveal spots unique to either condition. Most spots were matched to all gels using the PDQuest spot detection wizard and are shown in green (fig. 3); however, there did appear to be some unique spots when comparing differentially stimulated DCs (red and blue spots, fig. 3).

All spots that appeared to be unique to a gel set, or were differentially expressed [measured by the difference in spot quantity (optical density)] when comparing DC

Table 2. Differentially expressed proteins in unstimulated DCs from atopic and non-atopic samples using MALDI TOF mass spectrometry

Spot No.	Protein identification	Accession No. (Matrix)	Factor of difference (atopy/non-atopy) ^a	Expectation value ^b	Expected pI/Mr (ExPASy)	Mascot score	Biological process
1	Tankyrase-related protein	gi 10953952	-1.29*	1.9	6.76/126917.94	49	May regulate vesicle trafficking, and is involved in regulation of telomere length.
2	LIM and SH3 protein (LASP-1), or, zinc finger protein 227	gi 5453710/ gi 2135552 gi 32698786	-1.58	0.0029	6.61/29717.16	81	Plays an important role in the regulation of dynamic actin-based, cytoskeletal activities. Transcriptional regulation
				0.0029	9.10/92032.87	77	
3	Immunoglobulin heavy chain variable region	gi 112698912	1.10	0.046	8.67/10445.67	65	Immune response
4	Cathepsin X precursor	gi 3650498	-2.12*	0.02	5.48/27149.11	69	Exhibits carboxy-monopeptidase as well as carboxy-dipeptidase activity.
5	DNA replication complex GINS protein PSF1, or LIM and SH3 protein 1 (Lasp-1)	gi 63999832	-1.58	0.028	6.96/22988.41	67	The GINS complex plays an essential role in the initiation of DNA replication. Plays an important role in the regulation of dynamic actin-based, cytoskeletal activities.
		gi 5453710/ gi 2135552		0.16	6.61/29717.16	60	
6	Peroxisomal enoyl-coenzyme A, hydratase-like protein	gi 70995211	-1.40	0.098		62	Metabolism
7	Rho GDP dissociation inhibitor (GDI) beta	gi 56676393	1.05	0.00084		83	Role in cell motility, development, immune response, negative regulation of cell adhesion, and signal transduction.
8	Aldehyde reductase, or aldo-keto reductase family 1, member A1	gi 225939 gi 5174391	1.06	0.0022	5.84/16516.87	104	Involved in glucose metabolism
				6.1e-06		104	
9	Heterogeneous nuclear ribonuclear H3 isoform b	gi 14141159	1.02	0.27		58	RNA splicing
10	LIM and SH3 protein (LASP-1)	gi 5453710/ gi 2135552	2.23	0.025	6.61/29717.16	68	Plays an important role in the regulation of dynamic actin-based, cytoskeletal activities.
11	Hypothetical protein, or NADH dehydrogenase	gi 6624942 gi 4758788/ gi 62898071	1.08*	1.9e-05 0.00012	9.04/14187.52	99 91	Involved in mitochondrial electron transport.
12/13	Heat shock protein 27	gi 662841	1.08	0.88	5.98/22782.52	52	Involved in stress resistance and actin organization.
14	Glutathione S transferase P1	gi 2204207	1.11*	0.072	8.84/25719.98	64	Metabolism
15	Chain 1, succinyl coA 3 ketoacid-coenzyme A	P55809 (Swiss-Prot)	1.71	n/a	6.0/52089.89	n/a	Key enzyme for ketone body catabolism.
16	Protein similar to alpha fetoprotein	P02768 (Swiss-Prot)	1.12	n/a	5.67/66472.21	n/a	Involved in transport, regulation of cell death, maintenance of mitochondrion localization, and cellular response to starvation
17	Splice isoform 2 of zinc finger protein 278 (ZNF278) (MAZR)	Q9HBE1 (Swiss-Prot)	1.44	n/a	8.71/74059.97	n/a	DNA-dependent regulation of transcription.

The Mascot protein score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Protein scores >64 are significant ($p < 0.05$). * $p < 0.05$, atopic vs. non-atopic samples.

^a Corresponds to the factor of difference between spot volume (as measured by calibrated densitometer) in DCs from atopic individuals compared with DCs from non-atopic individuals.

^b The expectation value is the number of times we would expect to obtain an equal or higher score purely by chance.

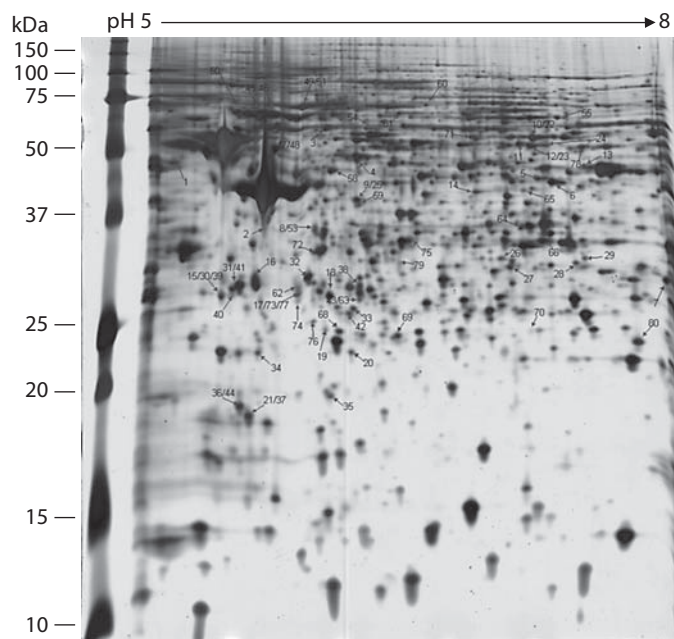


Fig. 4. Master gel showing spots identified by mass spectrometry. Numbers correspond to the numbering in table 3.

stimulations, were identified using the PDQuest software program. Gel data was exported from PDQuest, and a comparison of spot quantity in each gel set was carried out in Excel. These spots of interest were then removed from the gel, and their identification attempted using MALDI-TOF mass spectrometry. All spots identified are shown on the master gel in figure 4. In total, 71 spots were identified from gels of DCs generated from non-atopic donors, and of these 61 were more highly expressed in gels of DCs stimulated with active Der p 1 (AC), while 10 were more highly expressed in gels of DCs stimulated with inactive Der p 1 (IN) (table 3). In DCs generated from atopic donors, 56 proteins were identified, and of these 44 were expressed higher in gels of DCs stimulated with active Der p 1 (AC), while 8 were more highly expressed in gels of DCs stimulated with inactive Der p 1 (IN). Of the spots identified, most were involved in general cell processes, such as metabolism, transcription, and transport, with 8 proteins involved in the immune response. Interestingly all 8 proteins involved in the immune response were more highly expressed in DCs stimulated with proteolytically active Der p 1 (AC). A summary of various protein spots biological activities and their possible relevance to allergy is provided in tables 2 and 3.

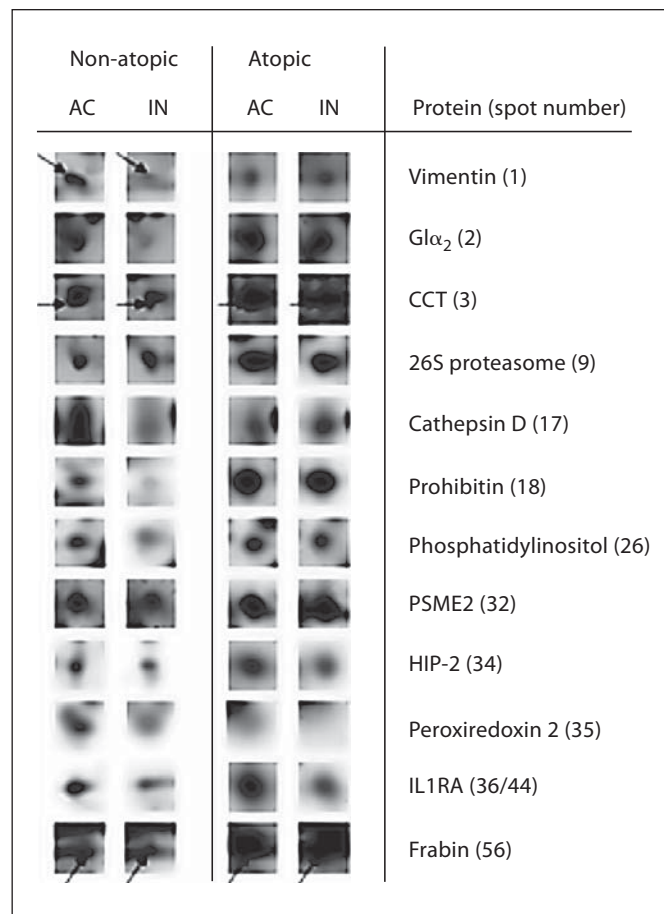


Fig. 5. Protein spots differentially expressed in DCs stimulated with proteolytically active (AC) Der p 1 compared with those stimulated with proteolytically inactive (IN) Der p 1. Shown here are spots from DCs isolated from both non-atopic (left column) and atopic (right column) donors. Numbers in brackets refer to the spot number as used in figure 3 and table 3. Spots shown are representative of $n > 3$.

Proteolytically Active Der p 1 Induces a Unique Protein Expression Profile in DCs

Proteins differentially expressed by DCs after stimulation by proteolytically active Der p 1 (AC) had functions covering a number of biological processes with possible relevance to the allergic response (table 3). These included the Gla₂ protein, CCT, 26S proteasome, c src tyrosine kinase, prohibitin, fascin, proteasome activator subunit 2, thioredoxin peroxidase, IL-1RA, HSP70, and frabin (fig. 5; table 3).

Our data indicate that DCs, isolated from non-atopic and atopic donors, have on the whole responded to proteolytically active Der p 1 in a similar manner, with most

Table 3. Spots of interest from 2D gels of differentially stimulated DCs from atopic and non-atopic individuals were excised and their identification attempted using MALDI-TOF mass spectrometry

Spot No.	Protein ID	Accession No.	Factor of difference ^a AC/IN non-atopic	Factor of difference ^a AC/IN atopic	Expectation value ^b	Mascot score	Biological function	Possible role in the allergic response
1	Vimentin	gi 340219	5.17	-1.12*	1.9e ⁻¹⁶	209	Protein binding/cell motility	Role in airway remodelling
2	GTP-binding regulatory protein Gi alpha-2 chain (G α 2)	gi 4218034	2.76*	1.83*	4.7e ⁻⁰⁷	115	G-protein coupled receptor protein signalling pathway/signal transduction	Th1/Th2 bias G α 2 knockout leads to Th1
3	Chaperonin containing TCP1 subunit 8 (CCT)	gi 47940653	1.94*	2.35*	1.9e ⁻⁰⁵	99	ATP binding/unfolded protein binding/protein folding	Role in cell morphology and immune synapse
4	Actin related protein 3 (ARP3)	gi 27806335	1.33	1.34	1.5e ⁻¹⁰	132	Protein binding	
5	Aldehyde dehydrogenase	gi 21361176	1.73	1.27	0.001	82	Metabolism	
6	Glucose 6 phosphate dehydrogenase	gi 12653141	1.48	1.45	5.9e ⁻²⁵	207	Carbohydrate metabolism/glucose metabolism	
7	Vacuolar ATPase	gi 4502317, gi 313014	Unique to AC (3,154.6)	n/a	2.9e ⁻⁰⁶	107	Transport/ATP synthesis	
8	Cathepsin X	gi 7546546	-3.18*	1.83	2.9e ⁻⁰⁸	127	Proteolysis and peptidolysis	Inflammation
9	26S proteasome	gi 3618343, gi 12654533, gi 62897349	1.41	1.49*	7.4e ⁻¹¹	153	Antigen processing	Antigen processing; Regulation of NF κ B activation
10	Glucose 6 phosphate dehydrogenase	gi 12653141	1.22	2.27	3.7e ⁻²⁰	246	Carbohydrate metabolism/glucose metabolism	
11	Aldehyde dehydrogenase	gi 21361176	1.73	1.27	0.0011	81	Metabolism (vitamin A)	
12	Cytosol aminopeptidase	gi 12643394	1.48	1.45	1.2e ⁻¹⁶	211	Proteolysis and peptidolysis	
13	c-src tyrosine kinase	gi 62898984	-1.50	3.59*	0.25	58	Intracellular signalling cascade/protein amino acid phosphorylation	Innate immune response and Th1/Th2 associations
14	Unknown protein	gi 62822169	3.15	1.01	0.0004	86		
15	EF hand domain family, member D2	gi 20149675	1.42	n/a	0.0029	77	Calcium ion binding	
16	Cathepsin B	gi 24158607	-1.17	-1.85*	2.9e ⁻⁰⁶	107	Proteolysis and peptidolysis/regulation of enzyme activity	Th1/Th2 bias
17	Cathepsin D	gi 5822091	2.19*	-2.46*	0.38	56	Regulation of enzyme activity	Th1/Th2 bias
18	Prohibitin	gi 46360168	1.21*	1.31*	7.3e ⁻⁰⁷	113	Transcription regulation/cell cycle regulation/signal transduction	Role in inflammation and glutathione levels
19	NADH dehydrogenase	gi 4758788	1.53	1.21	0.00068	83	Electron transport	
20	Unnamed protein product	gi 28193110	1.01	n/a	0.51	55		
21	ATP synthase	gi 51479152	1.15	1.88	0.13	60	Proton transport	

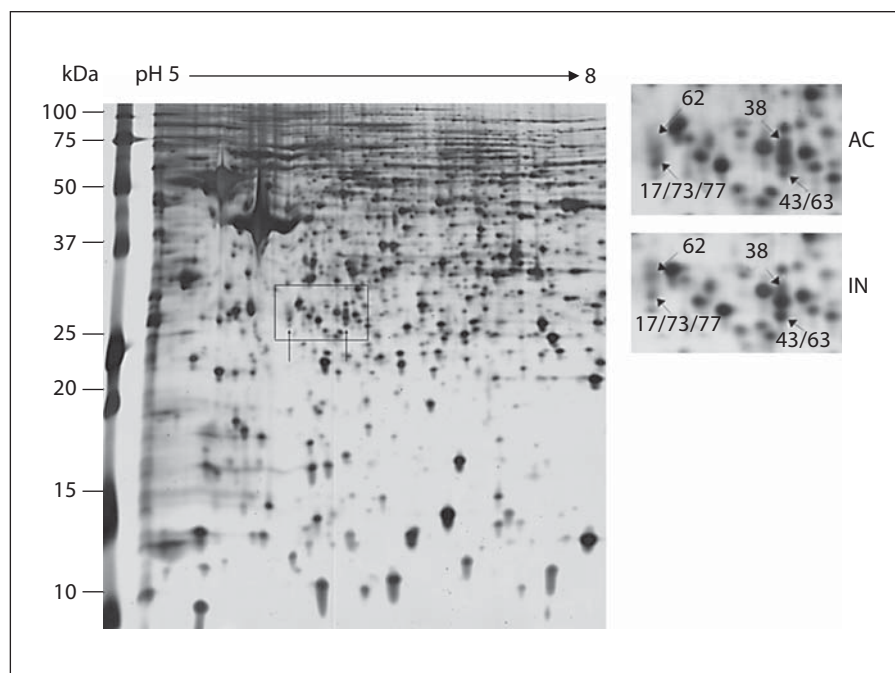
22	Glucose 6 phosphate dehydrogenase	gi 12653141	1.22	2.27	$9.2e^{-21}$	252	Carbohydrate metabolism/glucose metabolism
23	Cytosol aminopeptidase	gi 12643394	1.48	1.45	$2.3e^{-12}$	168	Proteolysis and peptidolysis
24	Fascin 1	gi 13623415	1.17	2.10	$1.5e^{-07}$	120	Actin cytoskeleton organisation and biogenesis/cell proliferation
25	Proteasome 26S subunit	gi 5106785,	1.41	1.49	$1.1e^{-06}$	89	Antigen processing
26	Phosphatidylinositol	gi 47169319	2.23	1.74	0.00033	86	Lipid metabolism
27	Peroxisomal enoyl-coenzyme A hydratase-like protein	gi 15080016	1.35	1.67	0.0024	78	Metabolism
28	glucosamine 6 phosphate deaminase	gi 13027378 (P46926)	5.63	n/a	0.00013	90	Metabolism
29	phosphoribosyl pyrophosphate synthetase 1	gi 57209589	4.16*	-1.12	0.36	56	Nucleoside metabolism and biosynthesis
30	EF hand domain family, member D2	gi 20149675	1.42*	n/a	$2.8e^{-07}$	117	Calcium ion binding
31	Chloride intracellular channel 1	gi 55961619 (O00299)	1.62	2.16	$7e^{-11}$	153	Signal transduction and transport
32	Proteasome activator subunit 2 (PSME2)	gi 13325110, gi 49456275	1.26*	1.28*	$7e^{-09}$	133	Antigen processing
33	Thioredoxin peroxidase (PRDX4)	gi 5453549, gi 49456297	1.05	2.12*	0.034	66	Redox regulation, regulation of apoptosis, response to stress
34	Huntington interacting protein 2 (HIP-2)	gi 60594412, gi 55622402	1.88 (moved)	1.34	0.24	58	Apoptosis regulation
35	Peroxiredoxin 2 isoform b	gi 33188452	1.89	1.49	0.049	65/63	Apoptosis regulation, response to stress
36	Interleukin 1 receptor antagonist isoform 4	gi 27894321	1.98	1.33*	0.0035	76	Immune response
37	ATP synthase	gi 51479152	2.23* (moved)	1.39	0.00034	86	Proton transport/ATP synthesis
38	Cathepsin D	gi 5822091	3.47*	5.91*	$7e^{-07}$	113	Regulation of enzyme activity
39	EF hand domain family, member D2	gi 20149675	1.42*	n/a	$1.8e^{-07}$	119	Calcium ion binding
40	SOX-12 protein	gi 3024656	1.24* (moved)	2.16	0.13	60	Transcription regulation
41	Chloride intracellular channel 1	gi 55961619	2.15*	2.16	$1.1e^{-12}$	171	Signal transduction and transport
42	Actin, alpha 1	gi 56204817	1.51	2.12	0.47	55	Cell motility, structure
43	Cathepsin D (chain H)	gi 5822091	-2.78*	-3.05	0.0061	74	Regulation of enzyme activity
							Th1/Th2 bias

Spot No.	Protein ID	Accession No.	Factor of difference ^a AC/IN non-atopic	Factor of difference ^a AC/IN atopic	Expectation value ^b	Mascot score	Biological function	Possible role in the allergic response
44	Interleukin 1 receptor antagonist inform 4	gi 27894321	1.99	1.33	0.13	60	Immune response	Inflammation
45	L-plastin (lymphocyte cytosolic protein 1)	P13796 (Swiss-Prot)	-1.4	n/a	n/a	0.87 (peptident)	Proteolysis and peptidolysis (peptident)	
46	L-plastin (lymphocyte cytosolic protein 1)	P13796 (Swiss-Prot)	1.24	n/a	n/a	0.91 (peptident)	Proteolysis and peptidolysis (peptident)	
47	Actin (chain 1)	P60709 (Swiss-Prot)	1.17 (modified)	n/a	n/a	0.67 (peptident)	Cell motility, structure (peptident)	Cell morphology and immune synapse
48	Actin (chain 1)	P60709 (Swiss-Prot)	1.19	n/a	n/a	0.83 (peptident)	Cell motility, structure (peptident)	Cell morphology and immune synapse
49	HSP70	P38646 (Swiss-Prot)	1.08	n/a	n/a	0.86 (peptident)	Involved in stress resistance (peptident)	Innate immune response and inflammation
50	L-plastin	P13796 (Swiss-Prot)	-2.2	n/a	n/a	0.86 (peptident)	Proteolysis and peptidolysis (peptident)	
51	HSP70	P38646 (Swiss-Prot)	3.4	n/a	n/a	0.82 (peptident)	Involved in stress resistance (peptident)	Innate immune response and inflammation
52	Vimentin	P08670 (Swiss-Prot)	3.8	n/a	n/a	8.86 (peptident)	Protein binding/cell motility (peptident)	Airway remodelling
53	Cathepsin Z	Q9UBR2 (Swiss-Prot)	-1.46*	1.83*	n/a	1.09 (peptident)	Proteolysis and peptidolysis (peptident)	Local inflammation
54	Protein disulphide isomerase A3	P30101 (Swiss-Prot)	1.11	1.30	n/a	5.9 (peptident)	Electron transport	
55	MOP-5 (SAM domain and HD domain containing protein 1).	Q9Y3Z3 (Swiss-Prot)	1.26	1.57	n/a	1.41 (peptident)	Immune response	
56	Frabin (FYVE, RhoGEF and PH domain containing protein 4) (FGD1-related F-actin binding protein)	Q96M96 (Swiss-Prot)	2.4	2.10	n/a	0.62 (peptident)	Cell motility, structure (peptident)	Cell morphology and immune synapse
57	Cathepsin D	gi 4503143	1.41	1.49	0.052	65	Regulation of enzyme activity	Th1/Th2 bias
58	Ubiquinol cytochrome c reductase core protein I.	gi 41152104, gi 46593007	1.26	1.68	1.5, 5.9	50, 44	Metabolism	
59	Guanylate binding protein 4	gi 37999758	2.08*	n/a	4.6	45	Cell communication, signal transduction	
60	Gamma actin, or, ACTG1 protein, or, actin beta	gi 178045, gi 40226101, gi 14250401	-1.33	n/a	0.036, 0.086, 0.086	70, 66, 62	Cell motility, structure	Cell morphology and immune synapse
61	Chain A, Structure Of Leukotriene A4 Hydrolase	gi 29726225	1.30	n/a	0.00023	88	Immune response	

62	Cathepsin B	gi 16307393	1.45	-2.46*	1.5	50	Proteolysis and peptidolysis/regulation of enzyme activity	Th1/Th2 bias
63	Cathepsin D	gi 5822091	-2.97*	-3.05*	0.038	66	Regulation of enzyme activity	Th1/Th2 bias
64	Vacuolar H ⁺ ATPase	gi 522193	1.45 (moved)	n/a	0.00051	85	Proton transport/ATP synthesis	
65	Lasp-1 protein (LIM and SH3 protein 1)	gi 2135552	1.09		1.28 ⁻⁰⁸	131	Cytoskeleton, ion transport	
66	Cathepsin X	gi 7546546	1.40	-1.56*	1.2e ⁻⁰⁵	101	Proteolysis and peptidolysis	Local inflammation
67	Adipophilin, or, adipose differentiation-related protein	gi 1806040, gi 34577059	1.92	2.07	0.0071, 0.0071	73, 73	Fatty acid synthesis	
68	Thioredoxin peroxidase, PRXD4	gi 49456297	1.05	2.12*	0.0091	72	Redox regulation, regulation of apoptosis, response to stress	Recruitment and activation of macrophages. Secretion of IL-10 and PGE ₂ (Th2 bias).
69	Prosome beta-subunit; HSBpros26, or, Proteasome (prosome, macropain) subunit, beta type, 4, or, PSMB4	gi 551547	1.33	2.41	0.026	68	Antigen processing	
70	UBL3, or, axonemal dynein, heavy chain	gi 49065342, gi 4165265	1.13	2.00	2.4, 5.1	48, 45	Motility	
71	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1, or, serine/threonine phosphatase	gi 45768728, gi 1177478	-1.03*	3.54	2.6, 16	48, 40	Metabolism	

The Mascot protein score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Protein scores >64 are significant ($p < 0.05$). * $p < 0.05$, atopic vs. non-atopic samples. ^a Factor of difference corresponds to the factor of difference between spot volume (as measured by calibrated densitometer) in DCs from atopic individuals compared with DCs from non-atopic individuals. ^b The expectation value is the number of times we would expect to obtain an equal or higher score, purely by chance.

Fig. 6. Multiple isoforms of cathepsin B and D located in the 2D gel. A representative 14% polyacrylamide gel is shown. The area containing multiple cathepsin isoforms is boxed, and the respective enlarged gel detail shown to the right of the gel. Data shown are for DCs isolated from non-atopic donors.



proteins following the same expression trend (table 3). It has to be noted, however, that our approach widely excludes low abundance proteins (e.g. those involved in signalling) which are difficult to identify by 2DE on whole cell lysates. Despite this limitation there were a few exceptions, with spots 1, 8, 13, 17, 53, 62 and 66 showing an opposing expression pattern in atopics versus non-atopics when stimulated with proteolytically active or inactive Der p 1. These included multiple isoforms of cathepsins B, D and X (spots 8, 17, 53, 62 and 66).

Cathepsins B and D are both involved in antigen processing and may additionally be able to degrade MHC class II li chain [35]. Cathepsin B was identified from 3 spots in the DC proteome. These showed a trend of lower expression in active Der p 1 treated DCs compared with inactive Der p 1 treated DCs (spots 16, 62 and 77). Recently, Zhang et al. [36] found that inhibition of cathepsin B caused a shift from Th2 to Th1 in OVA-sensitised BALB/c mice. This would suggest a role for cathepsin B in processing antigens for specific activation of Th2 cells. Cathepsin D was identified from numerous spots from the gel (spots 17, 38, 43, 57, 63, 73 and 75). Some of these spots were expressed more in proteolytically active Der p 1 treated DCs, whilst others were expressed higher in proteolytically inactive Der p 1 treated DCs (fig. 6). It is unclear whether the different cathepsins have clearly distinct roles in creating antigenic epitopes or not [36] but

our data would suggest that different isoforms may have differing functions in relation to the allergic response.

Furthermore, a number of proteins appeared to have been modified upon differential stimulation, as evidenced by slight changes in their molecular weight or electric charge. Whilst we were not able to fully investigate these changes, observed differences in spot position are likely to be due to post-translational modifications, such as phosphorylation and glycosylation, and these often have important implications for protein function [37]. Interestingly, some of these spots were proven to be a number of isoforms of cathepsins B and D.

Role of Der p 1 Induced Proteins in Th2 Cell Differentiation

Of the proteins differentially expressed when DCs were stimulated with either proteolytically active (AC) or inactive (IN) Der p 1, several had a clear role in allergy as evidenced by previously published data. As well as these, there were additional proteins with tentative links to the inflammatory response or other pathophysiological characteristics associated with the allergic response. Among these $G\alpha_2$, Farbin and IL-1RA are of particular interest.

The GTP binding regulatory protein $G\alpha_2$ was expressed 2.76 times higher in DCs stimulated with proteolytically active Der p 1 compared with those stimulated with inactive Der p 1 ($p < 0.05$). Interestingly, it has pre-

viously been shown that genetic knockout of $\text{Gi}\alpha_2$ proteins in mice leads to increased expression of Th1-type cytokines and a disease state similar to ulcerative colitis [38]. In addition, Fan et al. [39] showed that $\text{Gi}\alpha_2$ deficient mice produced significantly increased amounts of IFN- γ and IL-12. Indeed, our cytokine expression data appeared to show increased IL-12 production in the presence of proteolytically inactive Der p 1, corresponding to decreased $\text{Gi}\alpha_2$ levels (data not shown). This data would appear to link the expression of $\text{Gi}\alpha_2$ protein to the allergic response.

Frabin is a novel actin filament (F-actin) binding protein [40], which was found to be more expressed in active Der p 1 treated DCs by a factor of 2.4 ($p < 0.05$). Frabin plays a key role in cell morphology, and may be important in regulation of the immunological synapse, which clearly plays an important role in the differentiation of CD4^+ T lymphocytes. Frabin is capable of inducing not only the direct activation of cdc42 by its GEP activity, which induces filopodium-like structure formation, but also the cdc42-independent indirect activation of Rac, which induces the formation of lamellipodium-like structures or ruffles in H1H3T3 and L-fibroblasts [41]. In addition to frabin, chaperonin containing TCP1 (CCT) was expressed 1.94 times higher in active Der p 1 stimulated DCs compared with those stimulated with inactive Der p 1. CCT is a group II chaperonin, and is different to most chaperonins because it only appears to be involved in the assistance of the folding of only a small set of proteins. Two major folding substrates are the cytoskeletal proteins actin and tubulin, which both require the interaction (in vivo and in vitro) with CCT to fold to their native states [42]. CCT may therefore also play a key role in the morphology of DCs, which is important for the integrity of the immune synapse.

Furthermore, our data showed that IL-1RA was expressed 2 times higher in DCs treated with active Der p 1 compared with DCs stimulated with inactive Der p 1 ($p < 0.05$). IL-1RA is an important anti-inflammatory cytokine that counter-regulates the proinflammatory effects of IL-1 by binding IL-1R and preventing IL-1 binding. It has been found in a variety of inflammatory disorders including asthma [43] and it has been shown that IL-1RA plays an important role in allergen-specific Th2 cell activation and the development of airway hypersensitivity [44]. The IL-1RA gene is found on mouse chromosome 2, which is linked to allergen-induced BHR [45], and IL-1RA mRNA and protein was induced in both asthma susceptible and asthma resistant mice upon OVA exposure, but to a greater extent in asthma susceptible

mice at the earlier timepoints (6 + 24 h) [46]. We identified a number of other differentially expressed proteins with possible link to allergic sensitisations (table 3).

Concluding Remarks

Apart from some quantitative differences our data indicate that there is a high degree of similarity at the whole proteome level of unstimulated DCs between atopic and non-atopic individuals. However, upon stimulation of DCs with proteolytically active Der p 1 the number of unique and differentially expressed proteins increased, revealing novel proteins that might have a role in allergic sensitisation. Clearly the observed similarity between unstimulated DCs from atopic and non-atopic individuals at the whole proteome level does not rule out other more subtle differences particularly at the level of low abundant signalling proteins and difficult to dissolve membrane proteins. Therefore, to maximise the ability of 2DE to identify proteins with low abundance, including those directly involved in immune responses, the pre-fractionation of cell lysates may be necessary [47]. Furthermore, future work should investigate the role of post-translational modifications of DC proteins in the allergic responses in more detail. The application of techniques such as tandem mass spectrometry which provides the full sequence of identified proteins, or 2DE coupled with ProQ Diamond staining, which detects phosphoproteins will give detailed information on post-translational modifications processes including phosphorylation.

In summary, this study clearly highlights the value of the proteomic approach as a tool for studying allergic sensitisation but future efforts should focus on establishing the biological relevance of the differentially expressed proteins in relation to the role of DCs in this process.

Acknowledgments

The authors would like to thank John Keyte, Kevin Bailey and Matt Carlile (School of Biomedical Sciences, University of Nottingham) for their technical assistance with regard to mass spectrometry. This work was supported by the BRC (University of Nottingham) and in part by the Nasal Research Fund (Nottingham; grant Z50712).

References

- Vercelli D: Discovering susceptibility genes for asthma and allergy. *Nat Rev Immunol* 2008;8:169–182.
- Tovey ER, Chapman MD, Platts-Mills TA: Mite faeces are a major source of house dust allergens. *Nature* 1981;289:592–593.
- Schulz O, Laing P, Sewell HF, Shakib F: Der p 1, a major allergen of the house dust mite, proteolytically cleaves the low-affinity receptor for human IgE (CD23). *Eur J Immunol* 1995;25:3191–3194.
- Schulz O, Sutton BJ, Beavil RL, Shi J, Sewell HF, Gould HJ, Laing P, Shakib F: Cleavage of the low-affinity receptor for human IgE (CD23) by a mite cysteine protease: nature of the cleaved fragment in relation to the structure and function of CD23. *Eur J Immunol* 1997;27:584–588.
- Ghaemmaghami AM, Robins A, Gough L, Sewell HF, Shakib F: Human T cell subset commitment determined by the intrinsic property of antigen: the proteolytic activity of the major mite allergen Der p 1 conditions T cells to produce more IL-4 and less IFN-gamma. *Eur J Immunol* 2001;31:1211–1216.
- Schulz O, Sewell HF, Shakib F: Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J Exp Med* 1998;187:271–275.
- Furmonaviciene R, Ghaemmaghami AM, Boyd SE, Jones NS, Bailey K, Willis AC, Sewell HF, Mitchell DA, Shakib F: The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses. *Clin Exp Allergy* 2007;37:231–242.
- Ghaemmaghami AM, Gough L, Sewell HF, Shakib F: 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. *Clin Exp Allergy* 2002;32:1468–1475.
- Duez C, Gosset P, Tonnel AB: Dendritic cells and toll-like receptors in allergy and asthma. *Eur J Dermatol* 2006;16:12–16.
- Eisenbarth SC, Piggott DA, Bottomly K: The master regulators of allergic inflammation: dendritic cells in Th2 sensitization. *Curr Opin Immunol* 2003;15:620–626.
- Lambrecht BN: Dendritic cells and the regulation of the allergic immune response. *Allergy* 2005;60:271–282.
- Pereira SR, Faca VM, Gomes GG, Chammas R, Fontes AM, Covas DT, Greene LJ: Changes in the proteomic profile during differentiation and maturation of human monocyte-derived dendritic cells stimulated with granulocyte macrophage colony stimulating factor/interleukin-4 and lipopolysaccharide. *Proteomics* 2005;5:1186–1198.
- Horlock C, Shakib F, Mahdavi J, Jones NS, Sewell HF, Ghaemmaghami AM: Analysis of proteomic profiles and functional properties of human peripheral blood myeloid dendritic cells, monocyte-derived dendritic cells and the dendritic cell-like KG-1 cells reveals distinct characteristics. *Genome Biol* 2007;8:R30.
- Gough L, Schulz O, Sewell HF, Shakib F: The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999;190:1897–1902.
- Zhan X, Desiderio DM: Differences in the spatial and quantitative reproducibility between two second-dimensional gel electrophoresis systems. *Electrophoresis* 2003;24:1834–1846.
- Zhan X, Desiderio DM: Spot volume vs. amount of protein loaded onto a gel: a detailed, statistical comparison of two gel electrophoresis systems. *Electrophoresis* 2003;24:1818–1833.
- Agrawal A, Kaushal P, Agrawal S, Gollapudi S, Gupta S: Thimerosal induces Th2 responses via influencing cytokine secretion by human dendritic cells. *J Leukoc Biol* 2007;81:474–482.
- Dobashi K, Aihara M, Araki T, Shimizu Y, Utsugi M, Iizuka K, Murata Y, Hamuro J, Nakazawa T, Mori M: Regulation of LPS induced IL-12 production by IFN-gamma and IL-4 through intracellular glutathione status in human alveolar macrophages. *Clin Exp Immunol* 2001;124:290–296.
- Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C: Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci USA* 1998;95:3071–3076.
- Watson MA, Stewart RK, Smith GB, Massey TE, Bell DA: Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. *Carcinogenesis* 1998;19:275–280.
- Hayes JD, Pulford DJ: The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* 1995;30:445–600.
- Tamer L, Ercan B, Camsari A, Yildirim H, Cicek D, Sucu N, Ates NA, Atik U: Glutathione S-transferase gene polymorphism as a susceptibility factor in smoking-related coronary artery disease. *Basic Res Cardiol* 2004;99:223–229.
- Spiteri MA, Bianco A, Strange RC, Fryer AA: Polymorphisms at the glutathione S-transferase, GSTP1 locus: a novel mechanism for susceptibility and development of atopic airway inflammation. *Allergy* 2000;55(suppl 61):15–20.
- Aynacioglu AS, Nacak M, Filiz A, Ekinci E, Roots I: Protective role of glutathione S-transferase P1 (GSTP1) val105val genotype in patients with bronchial asthma. *Br J Clin Pharmacol* 2004;57:213–217.
- Fryer AA, Bianco A, Hepple M, Jones PW, Strange RC, Spiteri MA: Polymorphism at the glutathione S-transferase GSTP1 locus: a new marker for bronchial hyperresponsiveness and asthma. *Am J Respir Crit Care Med* 2000;161:1437–1442.
- Doull IJ, Lawrence S, Watson M, Begishvili T, Beasley RW, Lampe F, Holgate T, Morton NE: Allelic association of gene markers on chromosomes 5q and 11q with atopy and bronchial hyperresponsiveness. *Am J Respir Crit Care Med* 1996;153:1280–1284.
- Hayes JD, Strange RC: Potential contribution of the glutathione S-transferase supergene family to resistance to oxidative stress. *Free Radic Res* 1995;22:193–207.
- Hastie AT, Everts KB, Zangrilli J, Shaver JR, Pollice MB, Fish JE, Peters SP: Hsp27 elevated in mild allergic inflammation protects airway epithelium from H₂O₂ effects. *Am J Physiol* 1997;273:L401–L409.
- Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E, Garrido C: Hsp27 negatively regulates cell death by interacting with cytochrome C. *Nat Cell Biol* 2000;2:645–652.
- Paul C, Manero F, Gonin S, Kretz-Remy C, Viot S, Arrigo AP: Hsp27 as a negative regulator of cytochrome C release. *Mol Cell Biol* 2002;22:816–834.
- Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, Landry J: Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. *J Cell Sci* 1997;110:357–368.
- Mounier N, Arrigo AP: Actin cytoskeleton and small heat shock proteins: how do they interact? *Cell Stress Chaperones* 2002;7:167–176.
- Benndorf R, Hayess K, Ryazantsev S, Wieske M, Behlke J, Lutsch G: Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. *J Biol Chem* 1994;269:20780–20784.
- Merendino AM, Paul C, Vignola AM, Costa MA, Melis M, Chiappara G, Izzo V, Bousquet J, Arrigo AP: Heat shock protein-27 protects human bronchial epithelial cells against oxidative stress-mediated apoptosis: possible implication in asthma. *Cell Stress Chaperones* 2002;7:269–280.
- Villadangos JA, Bryant RA, Deussing J, Driessen C, Lennon-Dumenil AM, Riese RJ, Roth W, Saftig P, Shi GP, Chapman HA, Peters C, Ploegh HL: Proteases involved in MHC class II antigen presentation. *Immunol Rev* 1999;172:109–120.

- 36 Zhang T, Maekawa Y, Hanba J, Dainichi T, Nashed BF, Hisaeda H, Sakai T, Asao T, Himeno K, Good RA, Katunuma N: Lysosomal cathepsin B plays an important role in antigen processing, while cathepsin D is involved in degradation of the invariant chain in ovalbumin-immunized mice. *Immunology* 2000;100:13–20.
- 37 Colvis C, Garland D: Posttranslational modification of human alphaA-crystallin: correlation with electrophoretic migration. *Arch Biochem Biophys* 2002;397:319–323.
- 38 Niessner M, Volk BA: Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). *Clin Exp Immunol* 1995;101:428–435.
- 39 Fan H, Zingarelli B, Peck OM, Teti G, Tempel GE, Halushka PV, Spicher K, Boulay G, Birnbaumer L, Cook JA: Lipopolysaccharide- and gram-positive bacteria-induced cellular inflammatory responses: Role of heterotrimeric Galpha(I) proteins. *Am J Physiol Cell Physiol* 2005;289:C293–C301.
- 40 Obaishi H, Nakanishi H, Mandai K, Satoh K, Satoh A, Takahashi K, Miyahara M, Nishiooka H, Takaishi K, Takai Y: Frabin, a novel FGD1-related actin filament-binding protein capable of changing cell shape and activating C-jun N-terminal kinase. *J Biol Chem* 1998;273:18697–18700.
- 41 Ono Y, Nakanishi H, Nishimura M, Kakizaki M, Takahashi K, Miyahara M, Satoh-Horikawa K, Mandai K, Takai Y: Two actions of frabin: direct activation of CDC42 and indirect activation of RAC. *Oncogene* 2000;19:3050–3058.
- 42 Sternlicht H, Farr GW, Sternlicht ML, Driscoll JK, Willison K, Yaffe MB: The T-complex polypeptide 1 complex is a chaperonin for tubulin and actin in vivo. *Proc Natl Acad Sci USA* 1993;90:9422–9426.
- 43 Arend WP, Malyak M, Guthridge CJ, Gabay C: Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol* 1998;16:27–55.
- 44 Nakae S, Komiyama Y, Yokoyama H, Nambu A, Umeda M, Iwase M, Homma I, Sudo K, Horai R, Asano M, Iwakura Y: IL-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response. *Int Immunol* 2003;15:483–490.
- 45 Ewart SL, Kuperman D, Schadt E, Tankersley C, Grupe A, Shubitowski DM, Peltz G, Wills-Karp M: Quantitative trait loci controlling allergen-induced airway hyperresponsiveness in inbred mice. *Am J Respir Cell Mol Biol* 2000;23:537–545.
- 46 Ramadas RA, Li X, Shubitowski DM, Samineni S, Wills-Karp M, Ewart SL: IL-1 receptor antagonist as a positional candidate gene in a murine model of allergic asthma. *Immunogenetics* 2006;58:851–855.
- 47 Zuo X, Speicher DW: A method for global analysis of complex proteomes using sample prefractionation by solution isoelectrofocusing prior to two-dimensional electrophoresis. *Anal Biochem* 2000;284:266–278.