

**Proteome changes in auricular lymph nodes and serum after dermal sensitization
to toluene diisocyanate in mice**

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Abbreviations

2D-DIGE	Two-dimensional difference gel electrophoresis
AOO	Acetone olive-oil
APOE	Apolipoprotein E
BAL	Bronchoalveolar lavage
COPD	Chronic obstructive pulmonary disease
COR1A	Coronin 1a
HPX	Hemopexin
IFN- γ	Interferon gamma
Ig	Immunoglobulin
LCP-1	Lymphocyte cytosolic protein-1
LSP-1	Lymphocyte specific protein-1
MDI	Methylene diphenyl diisocyanate
PCA	Principal component analysis
TDI	Toluene diisocyanate

Abstract

Some reactive chemicals, such as diisocyanates, are capable of initiating an allergic response, which can lead to occupational asthma after a latency period. Clinical symptoms such as cough, wheezing and dyspnea occur only late, making it difficult to intervene at an early stage. So far, most studies using proteomics in lung research have focused on comparisons of healthy vs. diseased subjects. Here, using two-dimensional difference gel electrophoresis (2D-DIGE), we explored proteome changes in the local draining lymph nodes and serum of mice dermally sensitized once or twice with TDI before asthma is induced.

In the lymph nodes, we found 38 and 58 differentially expressed proteins after one and two treatments, respectively, between toluene diisocyanate-treated and vehicle-treated mice. In serum, 7 and 16 differentially expressed proteins were detected after one and two treatments, respectively. We identified 80-85% of the differentially expressed proteins by mass spectrometry. Among them, lymphocyte specific protein-1, coronin 1a and hemopexin were verified by Western blotting or ELISA in an independent group of mice.

This study revealed alterations in the proteomes early during sensitization in a mouse model before the onset of chemical-induced asthma. If validated in humans, these changes could lead to earlier diagnosis of TDI-exposed workers.

Key Words

2D-DIGE; coronin1a, isocyanate-induced asthma; lymphocyte specific protein-1; sensitization;

Introduction

Occupational asthma (OA), characterized by reversible airway obstruction, non-specific airway hyperreactivity to stimuli like cold air or cigarette smoke, and bronchial inflammation, has become one of the most common work-related lung diseases [1;2].

Two forms of OA can be distinguished. First, non-immunologic OA occurs after one or multiple exposures to high concentrations of inhaled irritants and is, therefore, called irritant-induced OA [2;3]. Second, immunologically mediated OA is characterized by a complaints-free latency period of weeks, months or years during which workers become sensitized to a protein or a chemical. After sensitization, clinical symptoms such as cough, wheezing and shortness of breath occur upon inhalation of even low amounts of the offending agent [4].

Diisocyanates constitute one of the most common causes of immunologically mediated OA [5]. The exact mechanisms by which isocyanates cause OA, are not fully understood [2]. Despite numerous efforts to diagnose isocyanate-sensitized workers, no method has proven successful and no early markers are available. Specific IgE or IgG antibodies against toluene-2,4-isocyanate (TDI)-albumin conjugates are only detected in 0-50% of subjects with TDI-induced asthma [6-8], which is neither sensitive nor specific enough to serve as biomarkers.

Some researchers found, using a proteomics approach, decreased ferritin and increased transferrin levels in bronchoalveolar lavage and serum of workers with methylene diphenyl diisocyanate (MDI)-induced OA [9]. In our previous study, these findings were confirmed in a mouse model of TDI-induced asthma [10]. However, a recent study from Sastre *et al.* failed to find significant differences in ferritin and

transferrin levels in serum between workers with clinically determined TDI-induced asthma, non-sensitized exposed workers and healthy controls [11].

Recent improvements in separation techniques (2D-DIGE) and increases in the sensitivity of identification technologies (mass spectrometry) have led to an increase in the use of proteomics techniques in different research domains. Most proteomic studies in the field of (pulmonary) toxicology have concentrated on the comparison of healthy versus known (TDI-) asthmatic subjects [9;12;13]. New efforts should be focused on developing early diagnostic biomarkers to distinguish isocyanate-sensitized but clinically silent workers from equally silent non-sensitized exposed workers [7]. There are yet no good tests to detect chemical sensitization by humoral tests (unlike IgE-mediated allergy to biological proteins).

Therefore, in this study, we compared the proteome of auricular lymph nodes and serum between TDI-sensitized and vehicle-treated control mice early after one or two sensitizations, i.e. before asthma was induced [14-16]. A subset of the differentially expressed proteins was verified by Western blotting or ELISA. This study should provide insights in the early events taking place during sensitization and could lead to early biomarkers of sensitization, after validation in human TDI-exposed workers.

Materials and methods

Toluene-2,4-diisocyanate (TDI, 98%; Fluka, CAS 584-84-9) was dissolved in a vehicle consisting of a mixture of 2 volumes of acetone and 3 volumes of olive oil (AOO) to obtain a final concentration of 0.3% TDI for dermal sensitization. All experiments were performed with approval of the Local Ethical Committee for Animal Experiments of the KU Leuven.

Animal procedures

Male BALB/c mice (6 weeks old, 20 g) received dermal applications of 0.3% TDI or vehicle (AOO, control) on the dorsum of both ears (20 μ l/ear) once (day 1) with sacrifice on day 4 or twice (days 1 and 8) with sacrifice on day 11. Mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (90 mg/kg body weight). Blood was taken from the retro-orbital plexus and afterwards, mice were sacrificed by section of the abdominal vessels. Auricular lymph nodes were isolated, weighed (per mouse) and kept on liquid nitrogen.

Lymphocyte subpopulations

Flow cytometry was performed as described by De Vooght et al. [17]. Briefly, auricular lymph nodes were collected from an independent group of mice that were treated, as above, once or twice with TDI (n=3) or AOO (n=6). Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 μ m) (BD Bioscience).

Five-hundred thousand cells were stained with anti-CD3⁺ (APC Hamster Anti-Mouse CD3e), anti-CD4⁺ (APC-Cy7 Rat Anti-Mouse CD4), anti-CD8⁺ (PerCP-Cy5.5-conjugated Rat Anti-Mouse CD8a (Ly-2) Monoclonal antibody) and anti-CD25⁺ (PE Rat Anti-Mouse

CD25), or received a single staining with anti-CD19⁺ (PE Rat Anti-Mouse CD19) labeled antibodies, according to standard procedures. All antibodies were purchased from BD Biosciences (Erembodegem, Belgium)

Sample preparation for 2D-DIGE analysis

Blood was kept for 30 min at room temperature, after which it was centrifuged (13000 x g, 10 min, 4 °C); the serum was stored at -80 °C. Following removal of the three most abundant proteins (albumin, transferrin and IgG) using a multi affinity removal system (MARS, spin cartridge, Agilent technologies), samples were up-concentrated using Amicon-Ultra 4 centrifugal tubes (Millipore). Afterwards, 1 volume of lysis buffer (40 mM Tris base, pH 8.8, 7 M urea, 2 M thiourea, 4% CHAPS and 1% dithiothreitol) with protease inhibitor (40 µl of ¼ tablet Complete in 500 µl MilliQ water, per ml lysis buffer) were added to 2 volumes of serum sample.

Auricular lymph nodes were homogenized in 200 µl of lysis buffer with protease inhibitor using a cone sonicator (3x10 s with 30 s on ice in between). The homogenate was centrifuged for 10 min (13000 x g, 4 °C) to remove cell debris and supernatant was collected.

The pH of serum and auricular lymph node samples was measured and adjusted if necessary (between pH 8-9) by adding small amounts of lysis buffer (pH 8.35). Afterwards, samples were desalted by dialysis (Mini Dialysis kit 1 kDa cut-off, GE Healthcare). Protein concentrations were spectrophotometrically measured using the Bradford assay.

2D-DIGE

The proteomes of the serum and auricular lymph nodes of 12 mice that had received one TDI sensitization and 12 mice that had received two TDI sensitizations were individually compared each time to 12 AOO-treated mice (controls) that had received one or two AOO-applications (12 biological replicates in each condition and at each time point), respectively, after CyDye DIGE Fluor minimal labelling (GE Healthcare), as previously described [10]. A two-dimensional gel electrophoresis separation was carried out as described by Haenen *et al.* [10]. Afterwards, gels were scanned with the Ettan DIGE Imager according to manufacturer's instructions. The digital images were analysed with Decyder 2D Differential Analysis 7.0 software (GE Healthcare). A more advanced statistical analysis, including principal component analysis (PCA), was performed in the Extended Data Analysis module.

Differentially expressed protein spots were excised by means of an automated spot picker (GE Healthcare) from deep purple stained preparative gels containing 300 µg of protein sample, and transferred to a 96 well plate.

In gel trypsinization and sample preparation for mass spectrometry

Protein digestion with trypsin (Promega) and sample preparation for mass spectrometry were performed as described by Haenen *et al.* [10]. Briefly, after in-gel digestion and peptide extraction, desalting and concentration, 1 µl of elution was spotted together with 1 µl of a saturated solution of α -cyano-4-hydroxy-cinnamic acid (matrix) in acetone on a MALDI target plate.

Protein identification by mass spectrometry

Peptide mass fingerprinting was performed on a matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF/TOF) mass spectrometer in positive ion reflectron mode (Ultraflex II, Bruker Daltonics). More detailed information concerning the experimental conditions for the mass spectrometry is provided in the supporting information of this journal. External calibration was done for each five samples with a peptide mixture containing Angiotensin II (1046.5420 Da), Angiotensin I (1296.6853 Da), Substance P (1347.7361 Da), Bombesin (1619.8230 Da), ACTH 1–17 (2093.0868 Da), and ACTH 18–39 (2465.1990 Da) (Bruker Daltonics). FlexAnalysis 3.0 was used to smooth the spectra and to subtract baseline following manufacturer's instructions. Peaks of known contaminants, such as keratin, were removed before database searching.

Protein database (NCBI nr database 20090422 - *Mus musculus* taxonomy [142790 sequences]) searching was performed on an 'in-house' Mascot server (Matrix Science, London, UK). Trypsin was selected as enzyme, peptide tolerance was set to 0.2 Da, one missed cleavage per peptide was allowed, carbamidomethylation of cysteins, as a result of the equilibration with iodoacetamide, was set as a fixed modification and methionine oxidation was set as variable modification. The probability-based MOWSE (Molecular Weight Search) scores greater than the given cut-off value were considered as significant ($p < 0.05$).

The identified proteins from serum and auricular lymph nodes were classified according to their biological function retrieved from Gene Ontology (www.geneontology.org).

Verification of differentially expressed proteins

A subset of the identified immune-related proteins, that were included in the list of proteins contributing to the separation of the TDI-treated group and AOO-treated group in the PCA, were verified via Western blotting or ELISA in lymph node and serum samples, obtained from a new, independent set of similarly treated TDI- and AOO-treated mice (n=8-10/group). Western blotting was performed on lymph node samples for lymphocyte specific protein 1 (LSP-1) and coronin 1a (COR1A). Briefly, proteins were loaded and separated on 4-12% Bis/Tris Midi-gels (Invitrogen) and subsequently transferred to a PVDF membrane (iBlot, Gel Transfer Stack, Invitrogen). Membranes were blocked (1-2h, 5% blocking agent, GE Healthcare) and incubated overnight with primary antibody (LSP-1: 1/1000, goat Ab, Santa Cruz Biotechnology; COR1A: 1/1000, mouse Ab, Santa Cruz Biotechnology; GAPDH, internal standard, 1/200000, mouse Ab, Dako). Following secondary Ab incubation (LSP-1: 1/50000, donkey anti-goat IgG, Santa Cruz Biotechnology; COR1A and GAPDH: 1/100000, goat anti mouse IgG, Dako) protein bands were visualized using chemiluminescence detection (Supersignal West Dura, Thermo Scientific) on ECL hyperfilm (GE healthcare). The proteins bands were semiquantitatively evaluated by densitometry (ImageQuant TL v2009, GE healthcare). A hemopexin-ELISA kit was used on independent serum samples according to manufacturer's instructions (GenWay Biotech).

Pathway analysis

A subset of the identified proteins was also introduced in Pathway Studio 8 (Ariadne Genomics, Rockville, MD). With this program, common regulators of the identified proteins and the pathways in which they are involved were visualized.

Statistical analysis

Graphpad Prism 4.01 (Graphpad Software Inc) was used for statistical analysis of ELISA and Western blot data. All data were normally distributed (tested using Kolmogorov-Smirnov). An unpaired t-test was used to compare both groups (AOO vs. TDI). A level of $p < 0.05$ was considered significant.

Results

Lymph node characteristics

Figure 1 shows a significant increase in weight of auricular lymph nodes of TDI-treated mice compared to AOO-treated mice, both after one and two treatments. Figure 2 shows the distribution of T-lymphocytes (CD3⁺), T-helper lymphocytes (CD4⁺), T-cytotoxic lymphocytes (CD8⁺), activated regulatory T-lymphocytes (CD25⁺) and B-lymphocytes (CD19⁺) after one and two treatments with TDI or AOO. Lymph nodes obtained from TDI-treated mice contained significant increases in all lymphocyte subpopulations compared to AOO-treated control mice, both after one and two sensitizations.

2D-DIGE analysis and protein identifications

Comparing the proteome of auricular lymph nodes between TDI-treated and control mice revealed 38 and 58 protein spots after one and two treatments, respectively, that were differentially ($p < 0.01$) expressed. In serum, 7 and 16 protein spots were differentially expressed ($p < 0.01$) after one and two treatments, respectively.

After one sensitization, principal component analysis (PCA) was able to discriminate between the TDI and AOO-treated group based on the different proteomes from the auricular lymph nodes (except for 1 mouse in each condition), but not with serum protein samples. However, PCA clearly separated TDI and AOO samples from the two times TDI- or AOO-treated mice on the spotmap level, both in lymph nodes and serum (Figure 3).

MALDI-TOF MS analysis allowed the identification of 26 and 35 unique proteins from the auricular lymph nodes after one and two sensitizations, respectively (Table 1). In serum samples from once and twice sensitized mice, 3 and 10 unique proteins, respectively, were successfully identified (Table 1; Figure S1).

Proteins were grouped in different functional classes according to their GO terms (<http://www.geneontology.org>; Figure S2). In the auricular lymph nodes, a quarter of the differentially expressed proteins are structural proteins (27% and 20%, for one and two sensitizations, respectively), a smaller fraction is related to cell metabolism (11% and 20%) or is involved in stress response (15% and 20%) and a small group of immune related proteins (8%) was identified after two sensitizations.

Most of the identified proteins in serum, after one (67%) or two sensitizations (50%), are involved in binding/transport or take part in cell metabolism (33% and 30%, respectively).

Western blot or ELISA verification

We verified three of the differentially expressed proteins resulting from the 2D-DIGE analysis via Western blotting or ELISA in an independent group of TDI-treated and AOO-treated mice, after one and two applications. We selected these proteins for verification based on two criteria: (1) biological relevance in the process of exposure and sensitization and (2) contribution to the discrimination between TDI- and AOO-treated in the principal component analysis (PCA).

Western blotting confirmed that the expression of LSP-1 was significantly downregulated ($p=0.0008$) in auricular lymph nodes, whereas COR1A was significantly

upregulated ($p=0.003$) after two sensitizations with TDI (Figure 4). After one sensitization, the expression of COR1A was also significantly ($p<0.0001$) upregulated in TDI-treated mice. LSP-1 expression was not significantly different after one sensitization, in agreement with our 2D-DIGE analysis.

Hemopexin, evaluated via ELISA (Figure 4), was significantly upregulated in serum of TDI-treated mice after one sensitization ($p=0.05$) and downregulated after two sensitizations ($p=0.05$), also in agreement with the 2D-DIGE results.

Pathway analysis

Figure 5 depicts common regulators, such as cytokines, of three differentially expressed proteins identified in the auricular lymph nodes (LSP-1, Cor1A and LCP-1) and two differentially expressed proteins from serum (HPX and APOE), built by the use of Pathway Studio 8 (Ariadne Genomics, Rockville, MD).

Discussion

The introduction of proteomics has led to the discovery of proteins that had previously not been associated with a particular disease. Now, with recent technical advances, the potential to work in a high-throughput manner has increased the potential of proteomics even further [12]. Nevertheless, the use of proteomics in the field of pulmonary diseases is still rather limited. So far, proteomic studies conducted in pulmonology have investigated changes in the proteome of healthy subjects versus diseased individuals (asthma, COPD, ...) using samples from either animal models or patients.

Allergic diseases induced by exposures at the workplace are only recognized and reported after the occurrence of clinical complaints, i.e. when the disease is already established. However, during sensitization against a chemical (latency period), before clinical symptoms of asthma or allergy appear, workers are asymptomatic. In this proteomics study, we shifted the focus from studying changes in the proteome at the stage of established disease¹⁰ to changes in the proteome during the sensitization phase, i.e. before the occurrence of disease. We applied the initial dermal sensitization regimen of a well-established mouse model of chemical-induced asthma[14-16], but before challenging the airways we collected the auricular lymph nodes (draining site of sensitization) and serum (systemic outcome) to investigate the changes in the proteome. We only exposed the mice once or twice and collected samples three days after the final sensitization. Admittedly, two sensitizations may seem a limited number of contact events compared to the human scenario where industrial workers could be exposed daily to a sensitizing agent. Nevertheless, two dermal sensitizations lead to

significantly increased levels of T and B lymphocytes (Figure 2) indicating a profound local immune response, resembling human sensitization against TDI [2;18].

Our current data show that already after two sensitizations, substantial differences occur in the proteome, which we presume relate to the onset of an immune response leading to asthma. In this discussion we shall concentrate on lymphocyte specific protein-1 (LSP-1), coronin 1a and hemopexin. Among the identified proteins in the auricular lymph nodes after two sensitizations, 8% are immune-related proteins. In accordance with our previous study [10], despite a 5 day difference in sampling and the lack of an airway challenge, LSP-1 was significantly downregulated in TDI-treated mice. LSP-1, a regulator of neutrophil chemotaxis, is negatively controlled by IL-4 [19]. This means that a decrease in LSP-1 allows a higher neutrophil influx. Previous studies by our group already showed that IL-4 is upregulated in the auricular lymph nodes of TDI-treated mice and that TDI-sensitization followed by a TDI-challenge leads to a pronounced neutrophilic airway inflammation [20]. The downregulation of LSP-1 after two sensitizations was also confirmed in an independent group of TDI-treated mice via Western blotting. After one sensitization, no downregulation of LSP-1 was observed, however, since we analyzed the samples three days after sensitization we cannot exclude that LSP-1 could be differentially expressed at a later time point after the first sensitization.

Coronin 1a (COR1A) expression was already significantly upregulated in TDI-treated mice after one sensitization and further increased after two sensitizations. COR1A, a member of the well conserved WD repeat family of coronin proteins, mediates Ca^{2+} release from intracellular stores, which is a key event in T lymphocyte activation upon

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contact with an antigen. In mice lacking COR1A, resulting in a profound defect in Ca^{2+} mobilization, peripheral T lymphocytes are impaired in survival, migration and activation [21]. The upregulation of COR1A after one and two sensitizations was also confirmed in an independent group of TDI- and AOO-treated mice via Western blotting. The changes in the proteome of the auricular lymph nodes indicate that as soon as three days after a single contact with TDI, local immunological changes occur, as shown by the upregulation of COR1A, involved in T lymphocyte activation and proliferation. In our model, we find a T lymphocyte proliferation and activation (Figure 2) after only one sensitization, compatible with the importance of COR1A. This proliferation was even higher after two sensitizations and was accompanied by a significant increase in the number of B lymphocytes, which was less pronounced after one sensitization.

Serum is an easily accessible body fluid which makes it attractive to search for biomarkers, yet it is also a technically complicated fluid to handle, because the potentially interesting proteins or peptides (cytokines, chemokines, ...) are only present in low concentrations (picomolar range) and, therefore, could be masked by very abundant proteins such as albumin, transferrin, IgA, IgM which together constitute more than 90% of the serum proteome. In this study, we depleted the three most abundant proteins from the serum samples with a multi-affinity removal system. This way, a number of lower abundance proteins could be visualized. One sensitization only resulted in a low number of differentially expressed proteins, with small fold changes, in the serum of TDI-treated mice, accompanied by a poor discrimination between the TDI- and AOO-treated groups in the principal component analysis (PCA). On the other hand,

this discrimination was complete after two sensitizations, although fold changes were relatively small. This could indicate that changes caused by one application on the ears are not yet reflected in the serum proteome three days later. Still, we were able to detect and identify three differentially expressed proteins already three days after one treatment.

Hemopexin (HPX) was significantly upregulated after one treatment, and then downregulated after two applications in TDI-treated mice. HPX belongs to the type II acute phase proteins which are involved in the innate body defense when encountering danger signals (e.g. infection, tissue damage...) and is known to be increased with an inflammatory event. In our previous study, we found a more than two-fold up regulation of HPX in bronchoalveolar lavage (BAL) and serum of TDI-sensitized and challenged mice when there was also a large influx of neutrophils in the BAL fluid [10]. In a 2D-DIGE analysis by Verrills *et al.*, comparing serum from patients with asthma or COPD and healthy controls, HPX was negatively associated with asthma and COPD [12]. In contrast, Lee *et al.*, also found an upregulation of HPX in BAL fluid of asthmatic subjects compared to healthy controls [22]. At this moment, we cannot explain these apparent discrepancies. They can rest on various differences, from time of sampling to cause of asthma. Nevertheless, it is clear from our 2D-DIGE results and our validation results in an independent group of mice, that this protein is associated with exposure to a chemical sensitizer, such as TDI, as soon as three days after one application.

Apolipoprotein E (APOE), known for its key role in lipid metabolism, exerts also important immune modulatory functions. APOE was also found associated with airway hyperreactivity in a mouse model of house dust mite induced asthma [23]. Moreover,

APOE-deficient mice show higher levels of IL-6, IL-12, TNF- α and IFN- γ both at the mRNA and protein level after LPS stimulation[24], while in another study apoE^{-/-} mice showed decreased levels of IL-4, IL-10 and granulocyte-macrophage colony-stimulating factor [25], indicating that APOE may suppress T_H1 immune response [26]. In this study, we show that APOE is differentially upregulated in serum of TDI-treated mice that were sensitized twice. According to the findings by Zhang *et al.*, this APOE upregulation could be related to the more pronounced T_H2 profile found in our mice. Although validation of APOE via Western blot or ELISA has not been successful so far, our 2D-DIGE result combined with data from literature justifies further interest in this protein.

Pathway analysis, using Pathway studio 8, was focused on a subset of differentially expressed proteins in the auricular lymph nodes (LSP-1, COR1A and LCP-1) and serum (HPX and APOE) (Figure 5). Several cytokines such as IL-4, IL-1 β and IL-6, previously associated with asthma, but also other proteins such as mitogen-activated protein kinase 14 (MAPK14) and formyl peptide receptor 1 (FPR1), are involved in the regulation of the identified proteins and could therefore be directly linked to processes activated during sensitization [20;27;28].

In conclusion, we found several differentially expressed proteins when comparing the proteomes of the auricular lymph nodes and serum of TDI-sensitized and AOO-treated mice after one and two dermal applications. Some of these proteins show only limited differences in levels between treated and non-treated mice. We did not apply a cut-off for the fold changes (e.g. > 1.5) since biological processes are regulated in such way that even small changes in homeostasis may have important biological implications.

Moreover, there is no gold standard concerning the use of a cut-off and multiple other studies also do not use a minimum fold change [29-31]. Verification of several of the proteins in an independent group of mice confirmed our 2D-DIGE results. This opens the possibility to evaluate these proteins in workers exposed to TDI. Therefore, we will evaluate these proteins in TDI-symptomatic, TDI-asymptomatic workers, as well as non-TDI-exposed workers, to evaluate specificity and sensitivity.

Supplementary data

Supporting information is added to the online repository of this journal.

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Conflicts of interest

None

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Table 1: 2D-DIGE results showing differentially expressed proteins ($p < 0.01$)**Auricular lymph nodes: 1 sensitization**

Gene Ontology Spot no.	Name	No. of Spots	Accession ID	Score	Seq Cov (%)	Peptides matched/unmatched	Fold Change	pI ^a	Mw (Da) ^a
Structural									
870	beta-actin	2	gi 49868	66	32	10/52	1.75	5.78	39446
551	actin-related protein 3 homolog	1	gi 23956222	92	33	19/63	1.41	5.61	47783
721	gamma-actin	1	gi 123298587	133	69	22/71	1.36	5.15	32941
304	lamin A	1	gi 161760667	106	38	23/47	1.18	6.37	65464
127	gelsolin	3	gi 90508	85	25	16/51	-1.36	5.53	81113
280	lamin B1	1	gi 188219589	159	50	38/62	-1.87	5.11	66973
1202	growth factor receptor bound protein 2	1	gi 123228035	70	31	9/22	-2.47	6.31	23687
Metabolism									
776	fructose-bisphosphate aldolase A	1	gi 6671539	136	62	19/76	1.68	8.31	39787
217	ubiquinol-cytochrome c reductase core protein 1	1	gi 14548301	81	33	17/74	1.42	5.75	53420
1117	phosphoglycerate mutase 1	1	gi 114326546	91	50	11/35	1.24	6.67	28928
Stress response									
901	aldo-keto reductase family 1, member B1	1	gi 160707894	135	50	15/38	1.48	6.71	36052
244	heat shock protein 5	1	gi 31981722	235	41	27/15	-2.02	5.01	72492
1193	glutathione S-transferase Mu 2	1	gi 121718	53	40	10/44	-2.16	6.90	25871
1184	glutathione S-transferase Mu 1	1	gi 28386202	55	34	10/38	-3.44	7.71	26067
Immune response									
420	coronin 1A ^b	1	gi 6753492	150	38	16/25	1.33	6.05	51641
Binding/Transport									
241	albumin	4	gi 163310765	192	49	30/65	-2.02	5.75	70700
Protein folding									
367	mKIAA0002 protein / cct8	1	gi 50510319	136	30	21/32	1.79	5.44	60817
886	GRB2-related adaptor protein 2	1	gi 148672651	64	40	10/65	1.49	8.70	28532
496	chaperonin subunit 2	1	gi 126521835	141	39	21/28	1.36	5.97	57783
Other									
422	PRP19/PSO4 pre-mRNA processing factor 19 homolog	1	gi 148709447	79	29	13/40	1.57	6.06	52581
1246	proteasome subunit beta type 2	1	gi 14198410	56	42	8/63	1.4	6.52	23063
557	glutathione synthetase	1	gi 74198134	73	16	9/19	1.32	5.41	52457
1129	proteasome subunit, beta type 10	1	gi 7305417	68	50	11/59	-1.57	6.40	29372
809	serine (or cysteine) proteinase inhibitor, clade B	1	gi 114158675	142	47	22/49	-1.76	5.85	42719
1130	endoplasmic reticulum protein ERp29 precursor	1	gi 19526463	64	26	8/19	-1.79	5.90	28862
1018	annexin A5	1	gi 6753060	170	59	20/39	-2.46	4.83	35787

Auricular lymph nodes: 2 sensitizations

Gene Ontology Spot no.	Name	No. of spots	Accession ID	Score	Seq Cov (%)	Peptides matched/ unmatched	Fold Change	pI ^a	Mw (Da) ^a
Structural	www.proteomics-journal.com		Page 30			Proteomics			
1669	beta-actin	2	gi 49868	66	32	10/52	1.81	5.78	39446
1639	gamma-actin	2	gi 809561	97	55	20/80	1.60	5.56	41335
996	vimentin	2	gi 55408	228	62	38/62	1.54	5.06	53746
1710	capping protein alpha 1 subunit	1	gi 595917	123	49	11/20	1.46	5.34	32902
1184	actin-related protein 3 homolog	1	gi 23956222	92	33	19/69	1.37	5.61	47783
105	collagen, type VI, alpha 1	1	gi 6753484	66	15	14/35	-1.66	5.20	109562
2569	transgelin 2	1	gi 30519911	89	65	20/80	-1.80	8.39	22552
Metabolism									
391	eukaryotic translation elongation factor 2	3	gi 33859482	80	22	25/49	1.61	6.41	96222
1218	serine hydroxymethyltransferase 2	1	gi 21312298	120	33	21/51	1.51	8.73	56237
1473	citrate synthase	1	gi 13385942	81	29	15/55	1.23	8.72	51988
315	programmed cell death 6 interacting protein	1	gi 148677002	68	15	13/29	1.21	6.14	96923
1459	phosphoglycerate kinase 1 ^c	1	gi 70778976	124	51	19/43	1.20	8.02	44921
2240	phosphoglycerate mutase 1	1	gi 114326546	91	50	11/35	1.18	6.67	28928
2442	U8 snoRNA-decapping enzyme ^c	1	gi 12844714	66	50	10/70	-1.39	8.89	23806
Stress response									
2330	biliverdin reductase B ^b	1	gi 21450325	81	59	12/56	1.20	6.49	22297
319	tumor rejection antigen gp96	1	gi 6755863	129	35	34/66	-1.20	4.74	92703
2442	glutathione peroxidase 1 ^c	1	gi 121666	61	36	7/36	-1.39	6.74	22553
2336	peroxiredoxin 6	1	gi 6671549	67	35	7/24	-1.40	5.98	24925
615	hspa8 protein	1	gi 74143862	75	23	13/33	-1.57	5.28	71041
2347	glutathione S-transferase Mu 1	1	gi 28386202	55	34	10/38	-1.70	7.71	26067
2365	glutathione S-transferase Mu 2	1	gi 121718	53	40	10/44	-1.72	6.90	25871
Immune response									
2912	lymphocyte cytosolic protein 1	3	gi 31543113	216	52	38/53	1.25	5.20	70732
1023	coronin 1A	1	gi 6753492	115	40	19/80	1.24	6.05	51641
1011	lymphocyte specific protein 1	1	gi 209862951	141	55	16/49	-1.53	4.77	36806
Binding/ Transport									
677	albumin	4	gi 163310765	213	51	30/49	-1.42	5.75	70700
Protein folding									
1042	t-complex protein 1	1	gi 148666718	140	53	23/55	1.40	6.65	44331
925	mKIAA0002 protein / cct8	1	gi 50510319	136	30	21/32	1.38	5.44	60817
1122	chaperonin subunit 2	1	gi 126521835	141	39	21/28	1.16	5.97	57783
Other									
1585	serine (or cysteine) proteinase inhibitor, clade B	2	gi 114158675	142	47	22/49	2.05	5.85	42719
2272	rho GDP-dissociation inhibitor 2	1	gi 33563236	55	40	9/50	1.33	4.97	22894
1895	annexin A5	2	gi 6753060	170	59	20/39	-1.29	4.83	35787
582	transglutaminase 2, C polypeptide	1	gi 6678329	141	34	24/49	-1.30	4.98	78153
836	ulip2 protein	1	gi 1915913	70	23	12/36	-1.35	5.95	62531
1094	G protein-regulated inducer of neurite outgrowth 1	1	gi 5901688	64	12	10/20	-1.44	8.63	85042
2243	proteasome subunit, beta type 10	1	gi 7305417	68	50	11/59	-1.55	6.40	29372

Serum: 1 sensitization

Gene Ontology Spot no.	Name	No. of Spots	Accession ID	Score	Seq Cov (%)	Peptides matched/ unmatched	Fold Change	pI ^a	Mw (Da) ^a
Metabolism 382	inter alpha-trypsin inhibitor, heavy chain 4	1	gi 151357765	62	14	13/35	1.16	6.12	100446
Binding/ Transport 927 803	beta-2 glycoprotein I hemopexin ^b	3 2	gi 1938223 gi 160358829	215 120	53 31	26/48 16/42	1.70 1.18	8.54 7.92	37551 52049

Serum: 2 sensitizations

Gene Ontology Spot no.	Name	No. of Spots	Accession ID	Score	Seq Cov (%)	Peptides matched/ unmatched	Fold Change	pI ^a	Mw (Da) ^a
Metabolism 843 803 695	apolipoprotein A-I apolipoprotein E protein-arginine deiminase type-6	1 1 1	gi 61402210 gi 74146419 gi 119372294	140 85 57	59 37 15	17/31 17/35 13/54	1.54 1.49 -1.19	7.00 5.56 5.36	23008 35917 77755
Stress response 865	glutathione peroxidase 3	2	gi 15011841	81	40	13/50	1.53	8.33	25580
Binding/ Transport 864 707 180 181 467	retinol-binding protein 4 protein SDA1 homolog beta-2 glycoprotein I murinoglobulin 1 hemopexin	1 1 2 1 2	gi 74142057 gi 74141265 gi 1938223 gi 31982171 gi 160358829	87 70 205 76 232	38 22 51 14 47	9/38 17/48 25/49 17/28 25/37	1.50 -1.14 -1.17 -1.19 -1.26	5.67 8.22 8.54 6.00 7.92	20473 73940 37551 166618 52049
Protein folding 809	clusterin	1	gi 149266012	70	20	10/35	-1.31	5.92	52140

Proteins are ranked from most up- to most down-regulated

^a pI and Mw are depicted as theoretical values

^b Proteins significantly differentially expressed at p<0.05

^c Two proteins were identified in the same protein spot

Figure legends

Figure 1: Auricular lymph nodes weight

Auricular lymph nodes weights were collected three days after one application (day 4, left panel) and three days after the second application (day 11, right panel). Figure shows individual values and group mean. n=12 per group, *** p<0.0001

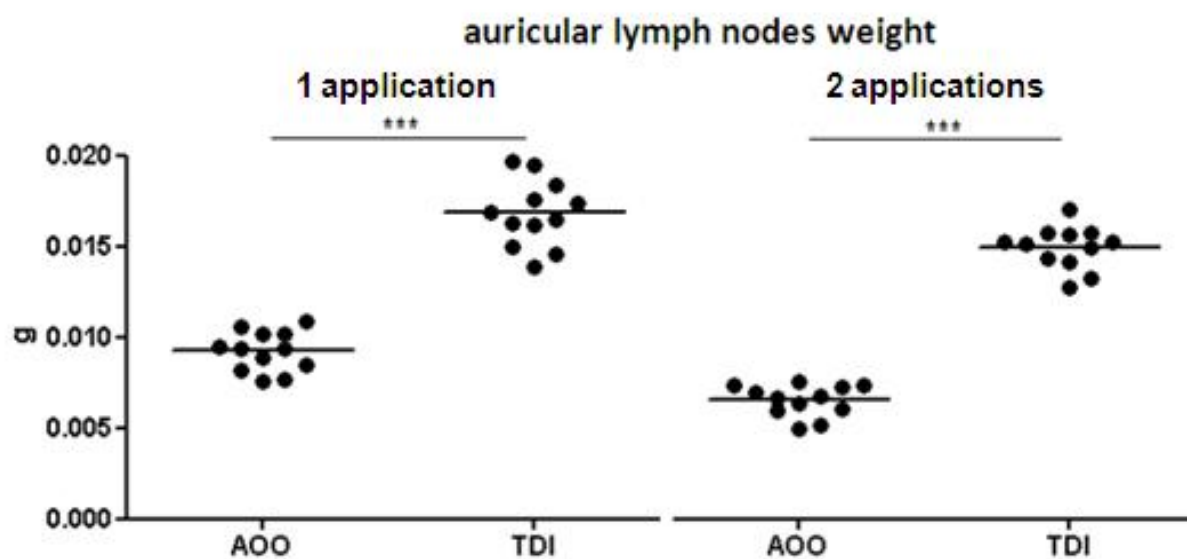


Figure 2: Auricular lymphocyte subpopulations

Freshly isolated lymphocytes from the auricular lymph nodes three days after one application (day 4, left panel) and three days after the second application (day 11, right panel) were analyzed by flow cytometry using the following markers: anti-CD3 (T lymphocytes), anti-CD4 (T-helper lymphocytes), anti-CD8 (cytotoxic T lymphocytes), anti-CD25 (activated T lymphocytes) and anti-CD19 (B lymphocytes).

Data are presented as mean + SD per lymph node, n= 3-6 per group, * p<0.01, ** p<0.001 compared to the AOO group.

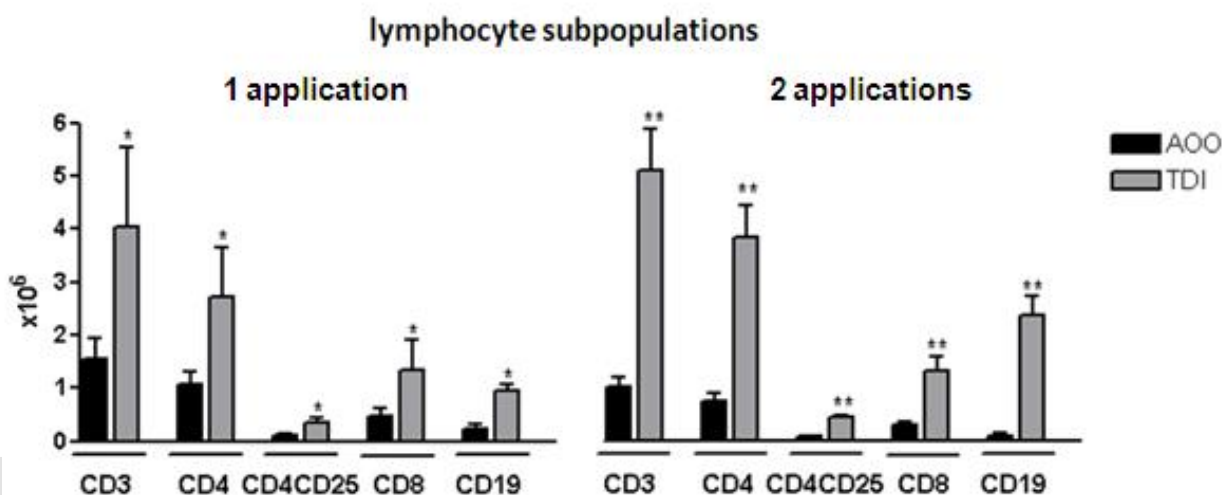


Figure 3: Principal component analysis (PCA).

PCA of the proteome maps of auricular lymph nodes and serum samples after one and two sensitizations. Green symbols represent AOO-treated mice. Red symbols represent TDI-treated mice. n=10-12 per group.

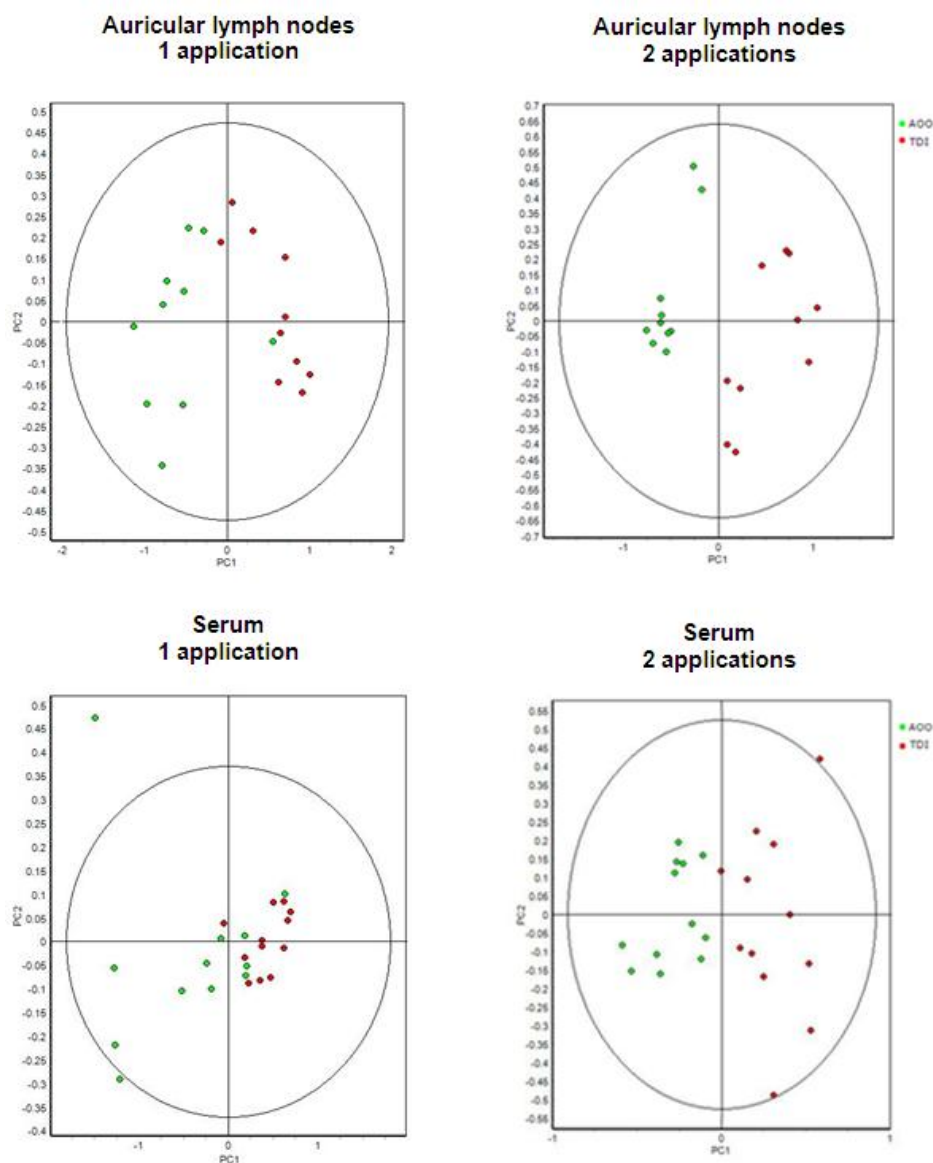


Figure 4: Validation of differentially expressed proteins using Western blotting or ELISA. Validation experiments were performed in a separate set of mice.

(A) Lymphocyte specific protein-1 (LSP-1) and (B) Coronin 1a (COR1A) validation in auricular lymph nodes using Western blotting. In (A) and (B), two lanes were excluded due to aberrant detection. GAPDH was used as an internal standard. (C) Hemopexin (HPX) validation in serum using ELISA. Figure shows individual mice and group means. n=8-10; *p<0.05, **p<0.01, ***p<0.001.

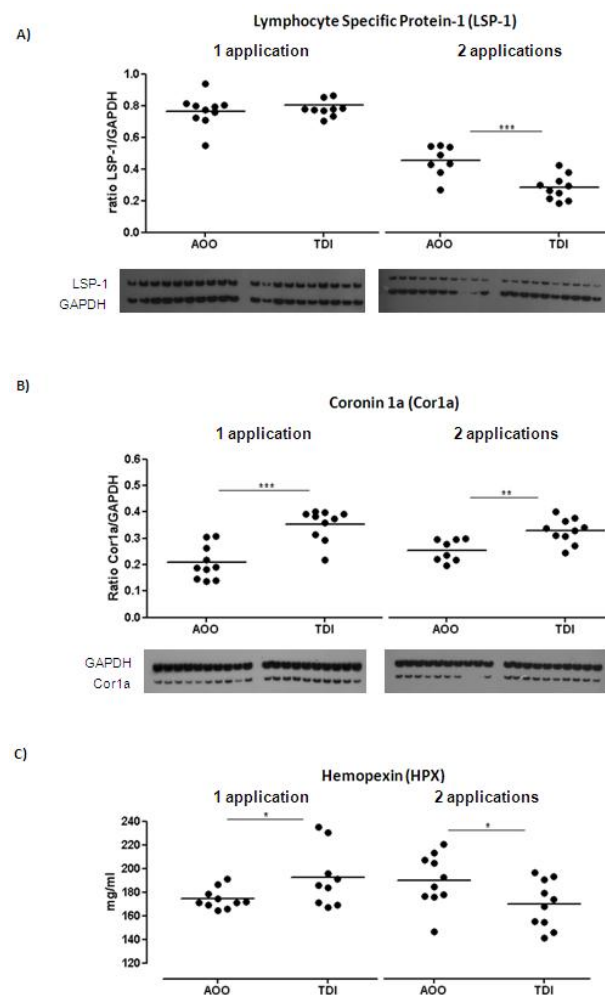
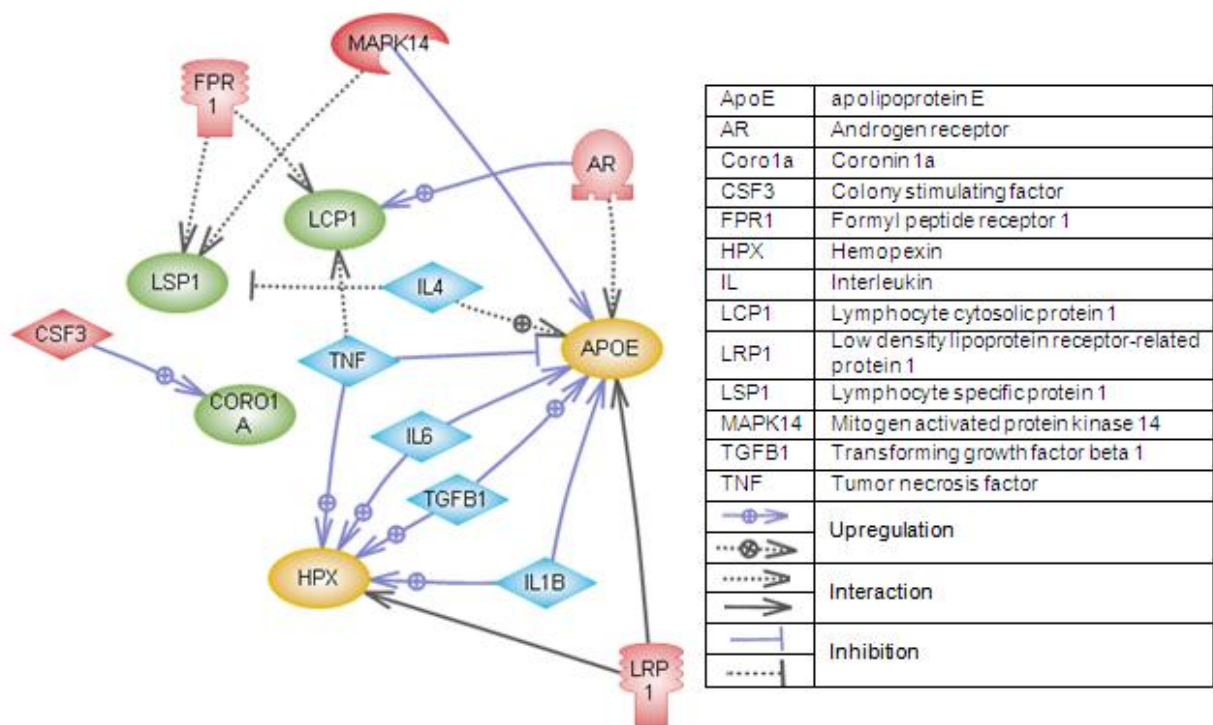


Figure 5: Common regulators of a subset of differentially expressed proteins

Pathway analysis, using Pathway Studio 8, was performed on three differentially expressed proteins (shown in green) and two differentially expressed proteins of serum (shown in orange). Common known regulators of these proteins are depicted.



Supporting Information

Mass spectrometry and post acquisition settings for peptide mass finger printing.

Peptide mass fingerprinting was performed on a matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF/TOF) mass spectrometer in positive ion reflectron mode (Ultraflex II, Bruker Daltonics), which is equipped with a Smartbeam 1 (100Hz) laser and an Acquiris DP 240 (version 1.9) digitizer. Voltage settings for Ion source 1, ion source 2, the lens, reflector 1 and reflector 2 were 25kV, 21.65kV, 9.3kV, 26.3kV and 13.71kV, respectively. Ions smaller than 700 kDa were suppressed and ions were recorded between 700 Da and 5000 Da.

FlexAnalysis 3.0 was used to smooth the spectra and to subtract baseline following manufacturer's instructions. Briefly, the SNAP peak detection algorithm was used after baseline subtraction (TopHat) and smoothing (SavitzkyGolay) the mass spectra. Afterwards, peak lists were generated.

Protein database (NCBI nr database 20090422 - *Mus musculus* taxonomy [142790 sequences]) searching was performed on an 'in-house' Mascot server (Matrix Science, London, UK). Trypsin was selected as enzyme, peptide tolerance was set to 0.2 Da, one missed cleavage per peptide was allowed, carbamidomethylation of cysteins, as a result of the equilibration with iodoacetamide, was set as a fixed modification and methionine oxidation was set as variable modification. The probability-based MOWSE (Molecular Weight Search) scores greater than the given cut-off value were considered as significant ($p < 0.05$).

Table 1: 2D-DIGE results showing differentially expressed proteins (p<0.01)

Auricular lymph nodes: 1 sensitization

Gene Ontology Spot no.	Name	Accession ID	Score	E value	Seq Cov (%)	Peptides matched/ unmatched	Fold Change	pI *	Mw (Da) *
Structural									
870	beta-actin	gi 49868	66	0.039	32	10/52	1.75	5.78	39446
551	actin-related protein 3 homolog	gi 23956222	92	8.8e-05	33	19/63	1.41	5.61	47783
721	gamma-actin	gi 123298587	133	7.2e-09	69	22/71	1.36	5.15	32941
304	lamin A	gi 161760667	106	3.6e-06	38	23/47	1.18	6.37	65464
127	gelsolin	gi 90508	85	0.00044	25	16/51	-1.36	5.53	81113
280	lamin B1	gi 188219589	159	1.8e-11	50	38/62	-1.87	5.11	66973
1202	growth factor receptor bound protein 2	gi 123228035	70	0.014	31	9/22	-2.47	6.31	23687
Metabolism									
776	fructose-bisphosphate aldolase A	gi 6671539	136	3.6e-09	62	19/76	1.68	8.31	39787
217	ubiquinol-cytochrome c reductase core protein 1	gi 14548301	81	0.001	33	17/74	1.42	5.75	53420
1117	phosphoglycerate mutase 1	gi 114326546	91	0.00011	50	11/35	1.24	6.67	28928
Stress response									
901	aldo-keto reductase family 1, member B1	gi 160707894	135	4.5e-09	50	15/38	1.48	6.71	36052
244	heat shock protein 5	gi 31981722	235	4.5e-19	41	27/15	-2.02	5.01	72492
1193	glutathione S-transferase Mu 2	gi 121718	53	0.05	40	10/44	-2.16	6.90	25871
1184	glutathione S-transferase Mu 1	gi 28386202	55	0.043	34	10/38	-3.44	7.71	26067
Immune response									
420	coronin 1A **	gi 6753492	150	1.4e-10	38	16/25	1.33	6.05	51641
Binding/ Transport									
241	albumin	gi 163310765	192	9e-15	49	30/65	-2.02	5.75	70700
Protein folding									
367	mKIAA0002 protein / cct 8	gi 50510319	136	3.6e-09	30	21/32	1.79	5.44	60817
886	GRB2-related adaptor protein 2	gi 148672651	64	0.05	40	10/65	1.49	8.70	28532
496	chaperonin subunit 2	gi 126521835	141	1.1e-09	39	21/28	1.36	5.97	57783
Other									
422	PRP19/PSO4 pre-mRNA processing factor 19 homolog	gi 148709447	79	0.0017	29	13/40	1.57	6.06	52581

1246	proteasome subunit beta type 2	gi 14198410	56	0.033	42	8/63	1.4	6.52	23063
557	glutathione synthetase	gi 74198134	73	0.0077	16	9/19	1.32	5.41	52457
1129	proteasome subunit, beta type 10	gi 7305417	68	0.022	50	11/59	-1.57	6.40	29372
809	serine (or cysteine) proteinase inhibitor, clade B	gi 114158675	142	9e-10	47	22/49	-1.76	5.85	42719
1130	endoplasmic reticulum protein ERp29 precursor	gi 19526463	64	0.05	26	8/19	-1.79	5.90	28862
1018	annexin A5	gi 6753060	170	1.4e-12	59	20/39	-2.46	4.83	35787

Auricular lymph nodes: 2 sensitizations

<i>Gene Ontology Spot no.</i>	<i>Name</i>	<i>Accession ID</i>	<i>Score</i>	<i>E value</i>	<i>Seq Cov (%)</i>	<i>Peptides matched/ unmatched</i>	<i>Fold Change</i>	<i>pI[*]</i>	<i>Mw (Da)[*]</i>
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1639	gamma-actin	gi 809561	97	3e-05	55	20/80	1.60	5.56	41335
996	vimentin	gi 55408	228	2.3e-18	62	38/62	1.54	5.06	53746
1710	capping protein alpha 1 subunit	gi 595917	123	7.2e-08	49	11/20	1.46	5.34	32902
1184	actin-related protein 3 homolog	gi 23956222	92	8.8e-05	33	19/69	1.37	5.61	47783
105	collagen, type VI, alpha 1	gi 6753484	66	0.032	15	14/35	-1.66	5.20	109562
2569	transgelin 2	gi 30519911	89	0.00018	65	20/80	-1.80	8.39	22552
Metabolism									
391	eukaryotic translation elongation factor 2	gi 33859482	80	0.0015	22	25/49	1.61	6.41	96222
1218	serine hydroxymethyltransferase 2	gi 21312298	120	1.4e-07	33	21/51	1.51	8.73	56237
1473	citrate synthase	gi 13385942	81	0.0012	29	15/55	1.23	8.72	51988
315	programmed cell death 6 interacting protein	gi 148677002	68	0.025	15	13/29	1.21	6.14	96923
1459	phosphoglycerate kinase 1	gi 70778976	124	5.7e-08	51	19/43	1.20	8.02	44921
2240	phosphoglycerate mutase 1	gi 114326546	91	0.00011	50	11/35	1.18	6.67	28928
2442	U8 snoRNA-decapping enzyme [†]	gi 12844714	66	0.038	50	10/70	-1.39	8.89	23806
Stress response									
2330	biliverdin reductase B ^{**}	gi 21450325	81	0.0012	59	12/56	1.20	6.49	22297
319	tumor rejection antigen gp96	gi 6755863	129	1.8e-08	35	34/66	-1.20	4.74	92703
2442	glutathione peroxidase 1 [†]	gi 121666	61	0.0099	36	7/36	-1.39	6.74	22553
2336	peroxiredoxin 6	gi 6671549	67	0.029	35	7/24	-1.40	5.98	24925
615	hspa8 protein	gi 74143862	75	0.0044	23	13/33	-1.57	5.28	71041
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1011	lymphocyte specific protein 1	gi 209862951	141	1.1e-09	55	16/49	-1.53	4.77	36806
Binding/Transport									
677	albumin	gi 163310765	213	7.2e-17	51	30/49	-1.42	5.75	70700
Protein folding									
1042	t-complex protein 1	gi 148666718	140	1.4e-09	53	23/55	1.40	6.65	44331
925	mKIAA0002 protein / cct8	gi 50510319	136	3.6e-09	30	21/32	1.38	5.44	60817
1122	chaperonin subunit 2	gi 126521835	141	1.1e-09	39	21/28	1.16	5.97	57783
Other									
1585	serine (or cysteine) proteinase inhibitor, clade B	gi 114158675	142	9e-10	47	22/49	2.05	5.85	42719
2272	rho GDP-dissociation inhibitor 2	gi 33563236	55	0.041	40	9/50	1.33	4.97	22894
1895	annexin A5	gi 6753060	170	1.4e-12	59	20/39	-1.29	4.83	35787
582	transglutaminase 2, C polypeptide	gi 6678329	141	1.1e-09	34	24/49	-1.30	4.98	78153
836	ulip2 protein	gi 1915913	70	0.015	23	12/36	-1.35	5.95	62531
1094	G protein-regulated inducer of neurite outgrowth 1	gi 5901688	64	0.06	12	10/20	-1.44	8.63	85042
2243	proteasome subunit, beta type 10	gi 7305417	68	0.022	50	11/59	-1.55	6.40	29372

Serum: 1 sensitization

Gene Ontology Spot no.	Name	Accession ID	Score	E value	Seq Cov (%)	Peptides matched/ unmatched	Fold Change	pI *	Mw (Da) *
Metabolism 382	inter alpha-trypsin inhibitor, heavy chain 4	gi 151357765	62	0.08	14	13/35	1.16	6.12	100446
Binding/ Transport 927 803	beta-2 glycoprotein I hemopexin **	gi 1938223 gi 160358829	215 120	4.5e-17 1.4e-07	53 31	26/48 16/42	1.70 1.18	8.54 7.92	37551 52049

Serum: 2 sensitizations

Gene Ontology Spot no.	Name	Accession ID	Score	E value	Seq Cov (%)	Peptides matched/ unmatched	Fold Change	pI *	Mw (Da) *
Metabolism 843 803 695	apolipoprotein A-I apolipoprotein E protein-arginine deiminase type-6	gi 61402210 gi 74146419 gi 119372294	140 85 57	1.4e-09 0.00043 0.024	59 37 15	17/31 17/35 13/54	1.54 1.49 -1.19	7.00 5.56 5.36	23008 35917 77755
Stress response 865	glutathione peroxidase 3	gi 15011841	81	0.0012	40	13/50	1.53	8.33	25580
Binding/ Transport 864 707 180 181 467	retinol-binding protein 4 protein SDA1 homolog beta-2 glycoprotein I murinoglobulin 1 hemopexin	gi 74142057 gi 74141265 gi 1938223 gi 31982171 gi 160358829	87 70 205 76 232	0.00031 0.016 4.5e-16 0.004 9e-19	38 22 51 14 47	9/38 17/48 25/49 17/28 25/37	1.50 -1.14 -1.17 -1.19 -1.26	5.67 8.22 8.54 6.00 7.92	20473 73940 37551 166618 52049
Protein folding 809	clusterin	gi 149266012	70	0.016	20	10/35	-1.31	5.92	52140

Proteins are ranked from most up- to most down-regulated

* pI and Mw are depicted as theoretical values

** Proteins significantly differentially expressed at $p < 0.05$

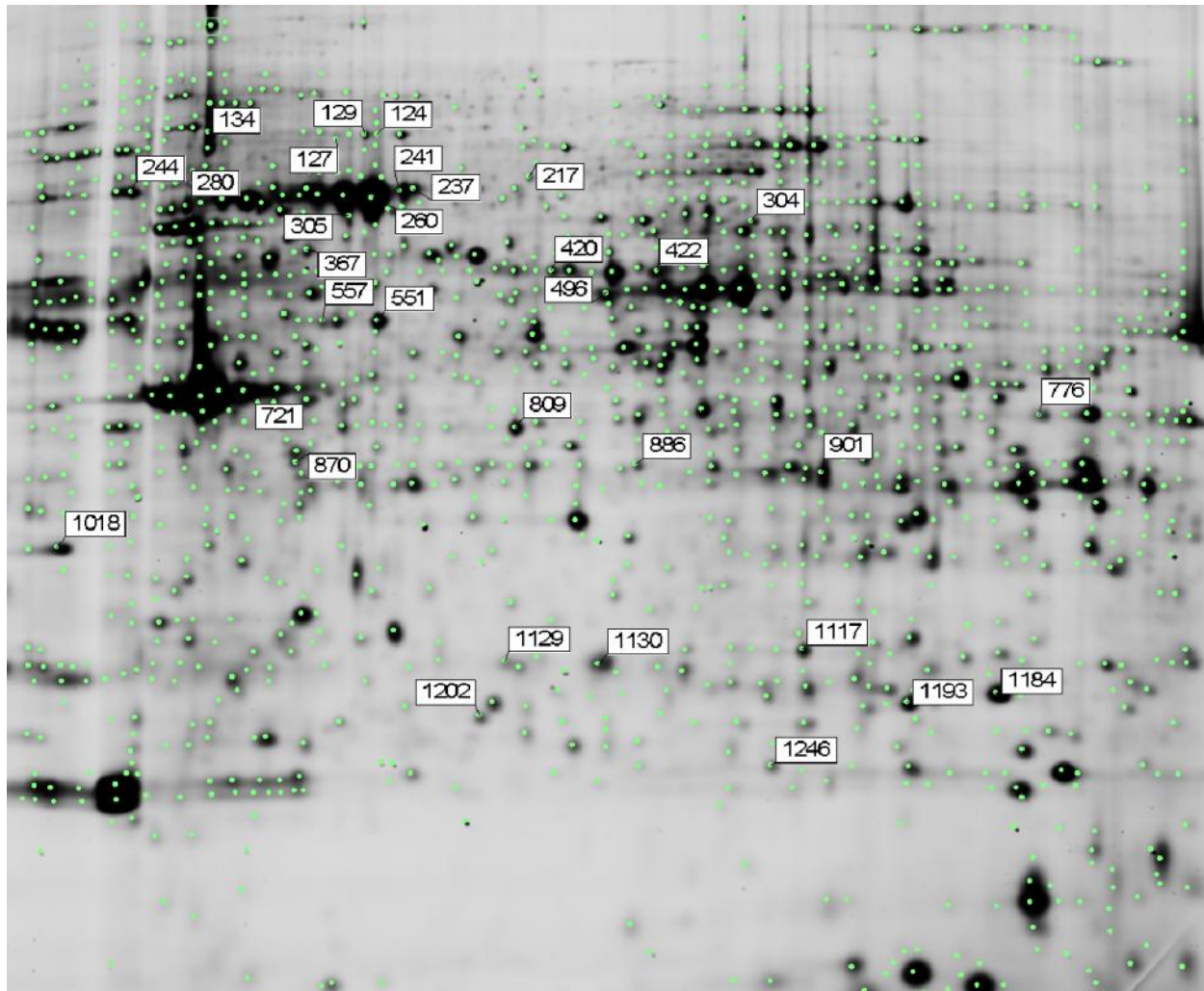


Fig S1a: Raw 2D-DIGE gel image of the auricular lymph nodes after 1 sensitization

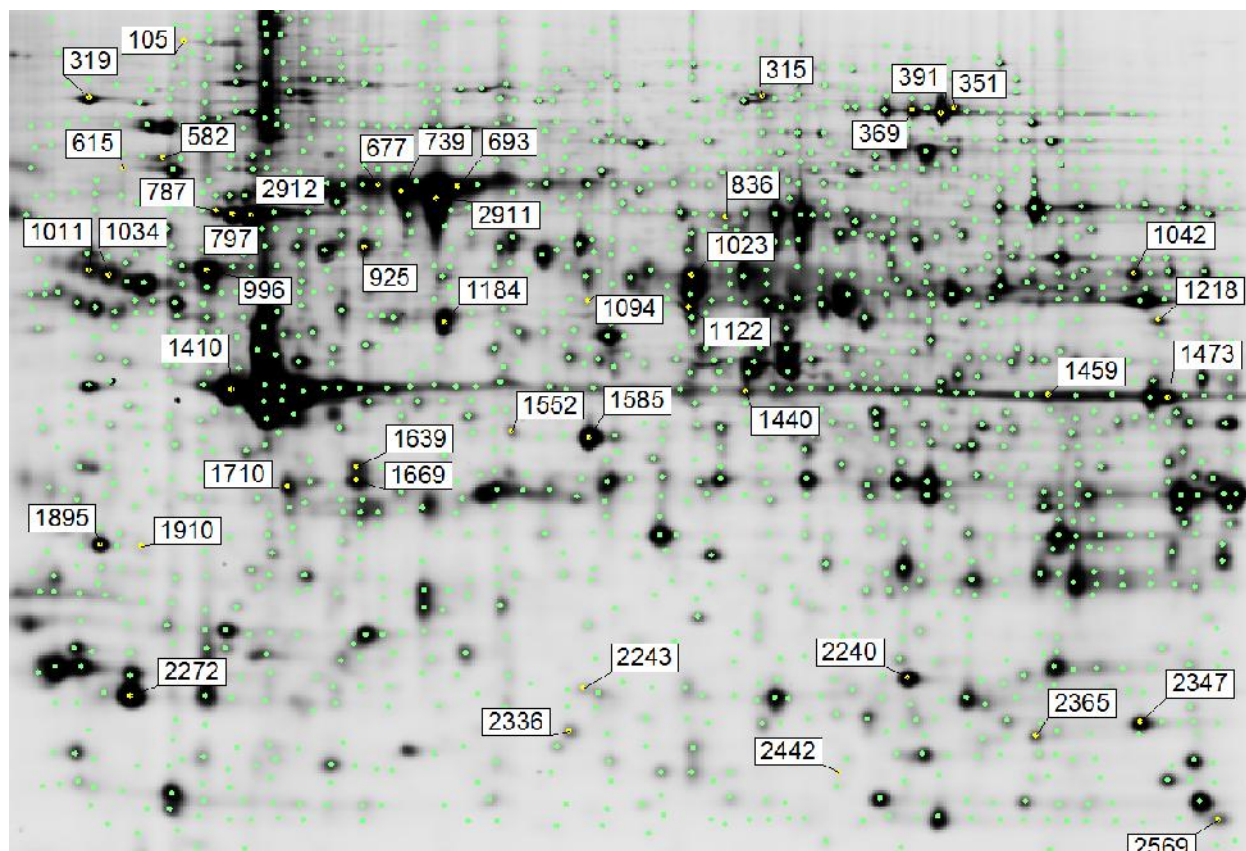


Fig S1b: Raw 2D-DIGE gel image of the auricular lymph nodes after 2 sensitizations

Differences between Fig. S1a and Fig. S1Bb are due to contrast settings in Decyder 7.0

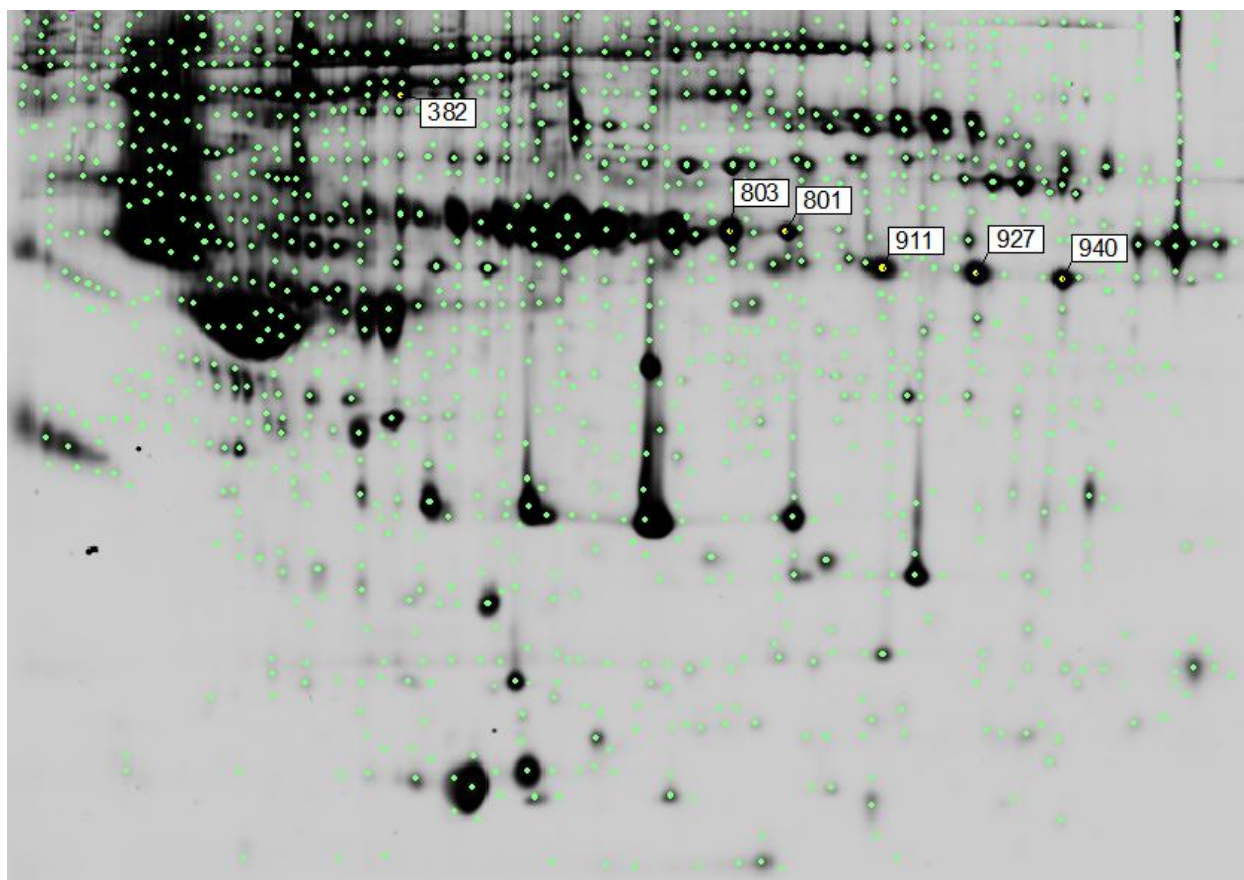


Fig S1c: Raw 2D-DIGE gel image of serum after 1 sensitization

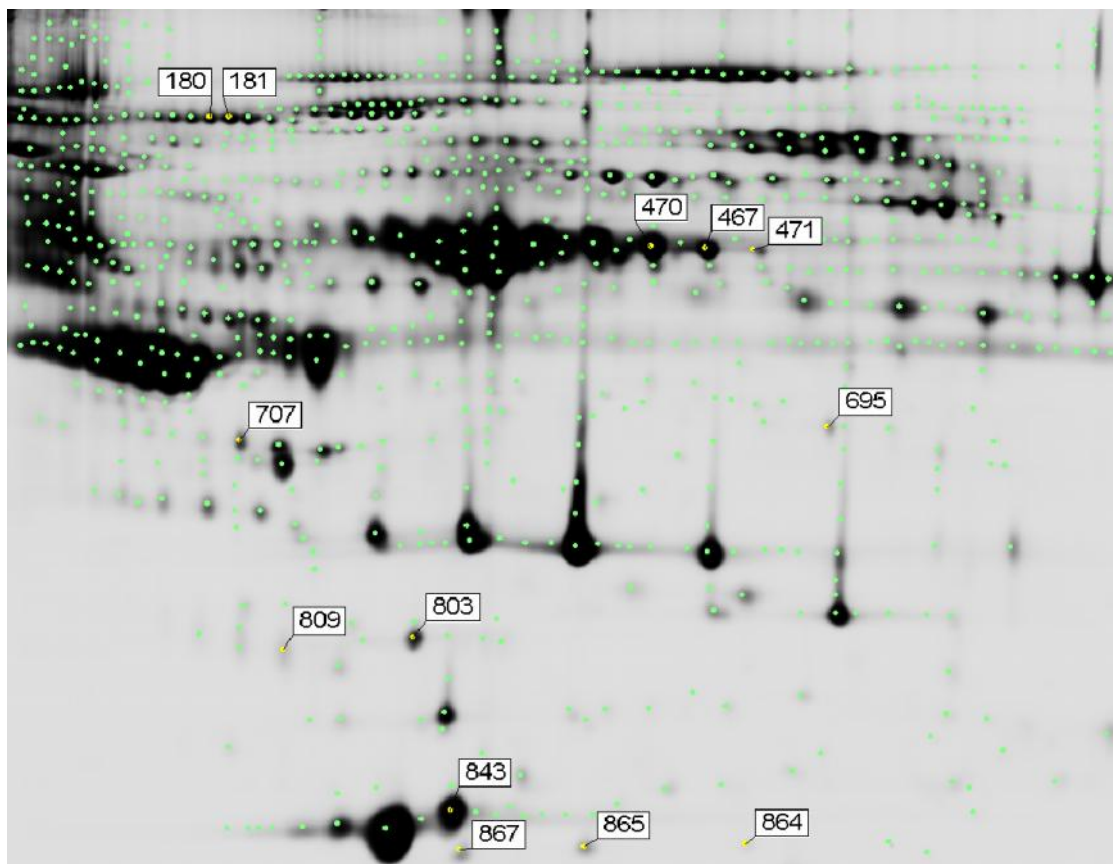


Fig S1d: Raw 2D-DIGE gel image of serum after 2 sensitizations

Differences between Fig. S1c and Fig. S1d are due to contrast settings in Decyder 7.0

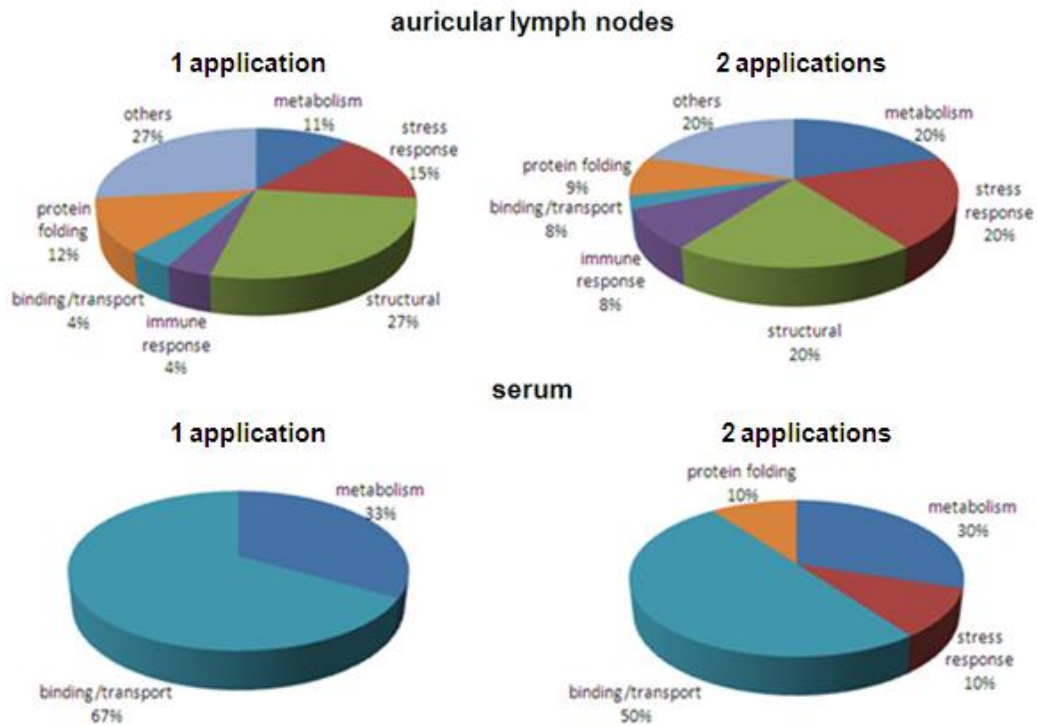


Figure S2: Classification of the identified differentially expressed proteins

The differentially expressed proteins in auricular lymph node and serum samples after one and two sensitizations were classified according to their biological function (retrieved from Gene Ontology).