

Proteome analysis of multiple compartments in a mouse model of chemical-induced asthma

Steven Haenen, Jeroen AJ Vanoirbeek, Vanessa De Vooght, Evelynne
Maes, Liliane Schoofs, Benoit Nemery, Peter H Hoet, and Elke Clynen

J. Proteome Res., **Just Accepted Manuscript** • DOI: 10.1021/pr100638m • Publication Date (Web): 22 September 2010

Downloaded from <http://pubs.acs.org> on September 24, 2010

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.



ACS Publications
High quality. High impact.

Proteome analysis of multiple compartments in a mouse model of chemical-induced asthma

‡Steven Haenen, MSc^{1,2}, ‡Jeroen A.J. Vanoirbeek, PhD¹, Vanessa De Vooght, MSc¹, Evelyne Maes, MSc², Liliane Schoofs, PhD², Benoit Nemery, MD, PhD¹, *Peter H.M. Hoet, PhD¹ and Elke Clynen, PhD³ (‡ First two authors equally contributed to this manuscript.)

¹ Katholieke Universiteit Leuven, Research Unit Lung Toxicology, 3000 Leuven, Belgium
² Katholieke Universiteit Leuven, Research Group Functional Genomics and Proteomics, 3000 Leuven, Belgium
³ Hasselt University, BIOMED Research Institute, Agoralaan Building C, 3590 Diepenbeek, Belgium

Corresponding author:
Peter Hoet
Katholieke Universiteit Leuven
Research Unit Lung Toxicology
Herestraat 49 mailbox 706
B-3000 Leuven, Belgium
Tel.: +32 16 33 01 97
Fax.: +32 16 34 71 24
E-mail: peter.hoet@med.kuleuven.be

Short title: Proteomics in a mouse model of occupational asthma

Abstract

Occupational asthma is the principal cause of work-related respiratory disease in the industrial world. Toluene-2,4-diisocyanate (TDI) is one of the most common respiratory sensitizers leading to occupational asthma. Using a mouse model of chemical-induced asthma we explored proteome changes in multiple compartments of mice sensitized and challenged with TDI or acetone-olive oil (AOO; vehicle).

Airway reactivity to methacholine and a bronchoalveolar lavage (BAL) cell count was assessed in treated and control mice, 1 day after challenge. Subsequently, two-dimensional differential gel electrophoresis (2D-DIGE) was performed on auricular lymph nodes, bronchoalveolar lavage (BAL) and serum comparing TDI-treated and control mice. The differentially expressed proteins were identified by mass spectrometry and pathway analysis was performed.

TDI-treated mice exhibit increased airway reactivity (2.6 fold increase) and a neutrophilic inflammation in the BAL fluid, compared to control mice. 2D-DIGE showed 53, 210 and 40 differentially expressed proteins in the auricular lymph nodes, BAL and serum of TDI-treated versus vehicle-treated mice, respectively. Several of the identified proteins could be linked with inflammation, neutrophil chemotaxis and/or oxidative stress.

Physiologic and immunologic readouts of the asthmatic phenotype, such as inflammation, were confirmed in three compartments by several of the differentially expressed proteins via 2D-DIGE and computerized pathway analysis.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

42 **Key words**
43 2D-DIGE; auricular lymph nodes; BALB/c mouse; bronchoalveolar lavage; serum; toluene-2,4-
44 diisocyanate

Introduction

Genomics and proteomics can help to unravel (new) genes or proteins related to the pathophysiology or onset of asthma and allergy, including occupational asthma (OA)¹. He *et al.* (2001) analyzed differences in gene expression using oligonucleotide arrays for the auricular lymph nodes of mice treated with sensitizers (toluene diisocyanate and oxazolone) and an irritant (nonanoic acid)². Ku *et al.* (2009) described gene expression differences between skin sensitization and skin inflammation³.

While genomics is already incorporated in both immuno-toxicological and respiratory research, the use of proteomics is rather limited, although proteomics has gained a lot of interest in other research domains^{4,5}. Calvo *et al.* (2009) used a SELDI-TOF MS approach on lung samples to identify biomarkers in ovalbumin-induced airway inflammation and remodeling⁶.

Diisocyanates are the most common cause of chemical-induced asthma. Due to the highly reactive isocyanate groups ($-N=C=O$), it is generally accepted that they react easily with functional groups of proteins⁷. Several studies reported a prevalence of occupational asthma between 2-15% among isocyanate-exposed workers^{8,9}.

The clinical presentation of diisocyanate asthma is variable and hence this type of asthma is hard to diagnose. Moreover, diisocyanate asthma is characterized by a latency period during which workers become sensitized without having complaints, which makes it difficult to intervene at an early stage. Only weeks, months or even years after initial exposure, symptoms of cough, wheezing and shortness of breath, occur¹⁰. So far, no early diagnostic or screening tests are available. Furthermore, the exact mechanisms by which isocyanate-induced OA develops are unclear. Therefore, (new) markers of sensitization and/or the onset of chemical-induced asthma are required.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

68 In this study we implemented a systematic proteome analysis in our validated mouse model of
69 chemical-induced asthma to see if physiological and immunological changes (sensitization,
70 inflammation and airway hyperresponsiveness) are reflected by changes in the proteome^{11,12,13}.
71 This was achieved using two-dimensional difference gel electrophoresis (2D-DIGE) and a
72 computerized pathway analysis. Proteins present in the draining auricular lymph nodes,
73 bronchoalveolar lavage and serum of mice sensitized and challenged with toluene-2,4-
74 diisocyanate (TDI) were compared with those of vehicle-treated control mice. To our knowledge
75 this is the first systematic study to investigate changes in the proteome of multiple compartments
76 in the same animals with experimentally-induced asthma.

Experimental section

Toluene-2,4-diisocyanate (TDI) was dissolved in a vehicle consisting of a mixture of 2 volumes of acetone and 3 volumes of olive oil (AOO) for dermal sensitization, and 1 volume of acetone and 4 volumes of olive oil for oropharyngeal aspiration. Concentrations of TDI are given as percent (v/v) in AOO. All experiments were performed with approval of the Local Ethical Committee of the K.U.Leuven.

Mouse model

A more detailed description of the mouse model can be found in the Supporting Information of this journal. On days 1 and 8, 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 µl/ear). One week later, on day 15, a single challenge (20 µl) with 0.01% TDI or AOO was given via oropharyngeal aspiration under light isoflurane anesthesia¹¹.

Methacholine airway hyperreactivity (AHR) responses were measured 24h after the oropharyngeal challenge, using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada) as described before^{11,14}.

Based on the intensity of the AHR, 12 out of 20 TDI-treated mice (responders) and 12 out of 18 vehicle-treated mice (non-responders) were selected. The selected mice were pooled in three groups of four, yielding three biological replicates for each condition.

Sample collection

After lung function measurements, the mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (90 mg/kg body weight). Blood was taken from the retro-orbital plexus

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

100 and then mice were sacrificed by section of the abdominal vessels. Auricular lymph nodes were
101 isolated, weighed (per mouse) and kept on liquid nitrogen.
102 Lungs were washed three times with 0.7 ml phosphate buffered saline (PBS) to obtain
103 bronchoalveolar lavage (BAL) fluid and the recovered fluids were pooled. Total cells were
104 counted using a Bürker hemocytometer. For differential cell counts, cells were spun onto
105 microscope slides, air-dried and stained (Diff-Quik[®], Medical Diagnostics, Dürdingen, Germany).
106 For each mouse, 200 cells were counted for the number of macrophages, eosinophils, neutrophils
107 and lymphocytes.
108
Sample preparation for 2D-DIGE analysis
110 The blood was kept for 30 min at room temperature. Following centrifugation (14.000 x g, 10
111 min, 4°C), serum was collected and stored at -80°C. The three most abundant proteins (albumin,
112 transferrin and IgG) were removed using the multi affinity removal system (MARS, spin
113 cartridge, Agilent technologies, Diegem, Belgium) and were up-concentrated using Amicon-
114 Ultra 4 centrifugal tubes (Millipore, Brussels, Belgium). Afterwards, 1 volume of lysis buffer (40
115 mM Tris base, pH 8.8, 7 M urea, 2 M thiourea, 4% CHAPS and 1% dithiothreitol) with protease
116 inhibitor (40 µl of ¼ tablet Complete in 500 µl MilliQ water, per ml lysis buffer) were added to 2
117 volumes of serum sample.
118 Auricular lymph nodes were pooled and homogenized in 500µl of lysis buffer with protease
119 inhibitor using a cone sonicator (3x10s with 30s on ice in between). The homogenate was
120 centrifuged for 10 min (13.000 x g, 4°C) to remove cell debris and supernatant was collected.

121 Protease inhibitor (160 μ l) was added to bronchoalveolar lavage samples. Samples were up-
122 concentrated using Amicon Ultra-4 centrifugal tubes and lysis buffer was added in a 1:2 ratio
123 (lysis buffer : BAL sample).

124 Prior to 2D-DIGE analysis, pH of all samples was measured and adjusted if necessary (between
125 pH 8-9) by adding small amounts of lysis buffer (pH 8.35). Afterwards samples were desalted by
126 dialysis.

127

128 **Two-Dimensional Difference Gel Electrophoresis (2D-DIGE)**

129 A schematic overview of the 2D-DIGE setup is available in the Supporting Information of this
130 journal (Table E1). Proteins of AOO-treated mice (controls) and TDI-treated mice, sampled from
131 serum, auricular lymph nodes and bronchoalveolar lavage fluid were compared after CyDye
132 DIGE Fluor minimal labelling (GE Healthcare, Freiburg, Germany). Cy3 and Cy5 were used to
133 differentially label the proteins of the distinct treatment groups (TDI vs AOO in 3 biological
134 replicates); technical repeats were introduced by a dye swap to exclude preferential binding of a
135 label to a set of proteins. An internal standard, consisting of equal amounts of proteins from each
136 sample, was labelled with Cy2. Labelled proteins were separated in a first dimension on IPG
137 strips (pH 3-10NL, 24cm, GE Healthcare) that were rehydrated with Destreak reagent (GE
138 Healthcare). Iso-electric focussing was performed using the IPGphor I (GE Healthcare) with
139 following parameters: 3h at 150V step-n-hold, 3h at 300V step-n-hold, 6h at 1000V gradient, 3h
140 at 8000V gradient continued by 8000V step-n-hold until a total of 50000Vhrs. Afterwards, the
141 strips were equilibrated in 6 M urea, 30% glycerol, 50 mM TrisHCl (pH 8.8), 2% SDS and 1%
142 DTT (w/v) for 15 min at room temperature followed by 15min equilibration in the same solution
143 with DTT replaced by 4% iodoacetamide (w/v) and bromophenol blue was added as a tracking

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

dye. Strips were subsequently loaded on a gradient SDS-polyacrylamide gel (11.5%) to separate the proteins according to their molecular weight. Electrophoresis was carried out in an ETTANdaltsix unit (GE Healthcare) for one hour at 48 mA followed by a constant current of 72 mA overnight. 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, was performed in the Extended Data Analysis module.

In gel trypsinization

Preparative gels containing 300 µg of protein sample were run and stained with silver nitrate (VWR, Leuven, Belgium). Spots of interest were picked manually in a laminar flow hood with a sterile scalpel. Silver was removed by adding 25 µl of 30 mM potassium ferricyanide and 25 µl of 100 mM sodium thiosulfate to the picked gel pieces. After rinsing with MilliQ water, they were dehydrated three times with 50 µl of acetonitrile. Next, rehydration was achieved with 50 µl of 100 mM ammonium bicarbonate and gel pieces were dehydrated again with acetonitrile for 10min. After repeating the last two steps, the gel pieces were dried in a vacuum centrifuge. The enzymatic digestion was started by adding 25 µl of digestion buffer (50 mM ammonium bicarbonate and 5 mM CaCl₂ containing 6 ng/µl trypsin (Promega, WI 53711, USA)). After 45 min on ice, enzymatic digestion was continued overnight at 37°C. The resulting peptides were extracted once with 80 µl 50 mM ammonium bicarbonate (30 min) and two times with 80 µl of 50% acetonitrile and 5% formic acid (30 min). Supernatans of the three extraction steps were pooled together and dried in a vacuum centrifuge.

167 **Sample preparation for mass spectrometry**

168 Tryptic digests were redissolved in 50 μ l of 2% acetonitrile/0.1% aqueous trifluoroacetic acid
169 and concentrated and desalted using Millipore Ziptips C18 (15 μ m), according to manufacturer's
170 instructions. Elution was done with 4.5 μ l of 70% acetonitrile/0.1% formic acid. 1 μ l was then
171 spotted together with 1 μ l of a saturated solution of α -cyano-4-hydroxy-cinnamic acid (matrix) in
172 acetone on a MALDI target plate.

174 **Protein identification by mass spectrometry**

175 Peptide mass fingerprinting was performed on a matrix-assisted laser desorption ionisation time-
176 of-flight (MALDI-TOF) mass spectrometer in positive ion reflectron mode (Reflex IV, Bruker
177 Daltonics, Bremen, Germany). Settings of the mass spectrometer were adopted from Vierstraete
178 *et al.* (2003) with minor adjustments¹⁵. External calibration was done with a peptide mixture
179 containing Angiotensin II (1046.5420 Da), Angiotensin I (1296.6853 Da), Substance P
180 (1347.7361 Da), Bombesin (1619.8230 Da), ACTH 1–17 (2093.0868 Da), and ACTH 18–39
181 (2465.1990 Da) (Bruker Daltonics).

182 Laser power was optimized for best resolution and signal to noise ratio. For each sample 400
183 laser shots were summed.

184 Protein database (NCBI database - *Mus musculus* taxonomy) searching was performed on an
185 'in-house' Mascot server (Matrix Science, London, UK). Trypsin was selected as enzyme,
186 peptide tolerance was set to 0.2 Da, one missed cleavage per peptide was allowed,
187 carbamidomethylation of cysteins, as a result of the equilibration with iodoacetamide, was set as
188 a fixed modification and methionine oxidation was set as variable modification. The probability-

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

189 based MOWSE (Molecular Weight Search) scores greater than the given cutoff value were
190 considered as significant ($p < 0.05$).

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

193 **Pathway Analysis**

194 The identified proteins were also introduced in Pathway Studio 7 (Ariadne Genomics, Rockville,
195 MD). With this program, additional information could be retrieved, such as the direct
196 interactions between the proteins and their regulators and the pathways in which they are
197 involved.

Results

Twenty four hours after the oropharyngeal challenge, the selected TDI-treated mice showed a 2.6-fold higher responsiveness to methacholine, measured by the FlexiVent, compared to the selected control mice (Fig. 1A-B). Figure 1C shows the difference in weight of auricular lymph nodes of TDI-treated mice compared to control mice. In the selected TDI-mice higher numbers of neutrophils (50%) and eosinophils (10%) were found compared to selected control mice, whereas macrophages did not differ and no lymphocytes were found (Fig. 1D).

2D-DIGE analysis revealed 53, 210 and 40 protein spots that were differentially ($p < 0.01$) expressed in the auricular lymph nodes, BAL and serum respectively between TDI- and AOO-treated mice (Fig. 2). Principal component analysis clearly separated TDI and AOO samples on the spotmap level (Fig. E1 Supporting Information). MALDI-TOF MS analysis allowed the identification of 27 spots from the auricular lymph nodes, corresponding to 25 different proteins, 72 spots from the BAL fluid, corresponding to 41 different proteins and 18 spots from serum, corresponding to 9 different proteins (Table 1). Proteins were grouped in different functional classes according to their GO terms (<http://www.geneontology.org>; Fig. 3). Most of the proteins identified in the auricular lymph nodes belong to structural classes (32%) or are related with cell metabolism (16%). Other proteins identified are involved with stress response (12%), binding/transport (20%) or with the immune response (4%). The majority of proteins identified in BAL fluid and serum are involved in binding/transport (32% and 50% respectively). In BAL fluid, most of the remaining proteins were classified in inflammation (18%), metabolism (14%) and complement system (12%) whereas in serum they were classified in structural (13%), immune response (13%), metabolism (12%) and stress response (12%).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

220 Figure 4 shows an inflammation pathway built by the use of Pathway Studio (Ariadne Genomics,
221 Rockville, MD) of the proteins from auricular lymph nodes, BAL and serum. These proteins
222 were linked with cytokines and chemokines, known to be involved with asthma and already
223 measured in previous studies via ELISA-assays¹¹. The lines between the different proteins
224 indicate a stimulatory or a inhibitory interaction as shown by literature.

Discussion

Experimental mouse models of OA have mainly investigated traditional endpoints such as cytokine production of draining lymph nodes and BAL or serum IgE levels after the elicitation of asthma^{16,17}. In humans, only few studies have applied a proteomics approach to map changes in the proteome of BAL fluid or sputum in respiratory diseases^{5,18}.

In an attempt to investigate proteome changes of multiple compartments influenced by the induction of chemical-induced asthma we used a robust and well-validated mouse model^{11,12,13}. After dermal sensitization and an airway challenge with TDI or AOO mice showed, as repeatedly found in previous experiments, phenotypical characteristics of asthma, including airway hyperreactivity, BAL airway neutrophilia, lung epithelial shedding and necrosis, increased levels of total serum IgE and T and B cell proliferation in the local draining lymph nodes. Another control group where mice are dermally treated with AOO and challenged with TDI was not included, because in previous studies we did not find any differences in physiological, inflammatory nor immunological outcomes compared to the complete control group (AOO-treated) and the non-sensitized, but TDI-challenged group^{11,12,19}.

Auricular lymph nodes

Previously, we showed that in TDI-treated mice, the auricular lymph nodes have a significantly larger number of B and T cells and these cells produce increased levels of cytokines such as IL-4, IL-13 and IFN- γ after Concanavalin A stimulation^{11,17}. Here, we describe the proteome changes in the auricular lymph nodes. A significant increase in lymph node weight was found in TDI-treated mice compared to control mice (Fig. 1b). Accordingly, a predominant differential expression of structural proteins (e.g. actin, tubulin, ...) was observed in the auricular lymph

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

nodes. Moreover, proteins involved in neutrophil chemotaxis (e.g. lymphocyte specific protein-1 and calreticulin), oxidative stress (e.g. peroxiredoxin 2 and tumor rejection antigen gp96), and inflammation (lymphocyte specific protein-1) were also significantly changed in the lymph nodes of TDI-treated mice. Lymphocyte specific protein-1 (LSP1), 5-fold downregulated in TDI-treated mice, is a negative regulator of neutrophil chemotaxis. Downregulation of this protein therefore results in a higher mobility of neutrophils, which is compatible with the BAL neutrophilia shown in fig. 1. This has also been described by Howard *et al.* (1998) and Jongstra-Bilen *et al.* (2000)^{20,21}. Figure 4 (left top) shows LSP1 is negatively controlled by IL-4, which is upregulated in TDI-treated auricular lymphocytes^{11,13,22}. Peroxiredoxin 2 (PrxII), 1.7 fold downregulated, is mostly expressed at the site of inflammation²³ but is also involved in the activation of T lymphocytes and the maturation of B cells^{24,25}. The downregulation is in agreement with the findings of Moon *et al.* (2006) who showed that a deletion of the *PrxII* gene led to a proliferation of the spleen and an activation of T lymphocytes and dendritic cells²⁵. Since isocyanate skin exposure can induce systemic sensitization and eventually, after inhalation exposure, lead to asthma-like symptoms in mice^{26,27}, serum and bronchoalveolar lavage from the same groups of mice were also analyzed.

Serum

The presence of high-abundant proteins in serum could mask the detection of low-abundant, interesting proteins. Serum, depleted of the three most abundant proteins (albumin, IgG and transferrin) still displayed some high-abundant proteins resulting in a relatively low number of identifications. Nevertheless, proteins related to neutrophil chemotaxis (e.g. Vitamin D binding

271 protein and serine (or cysteine) proteinase inhibitor, clade C), oxidative stress (e.g. glutathione
272 peroxidase and hemopexin) and inflammation (e.g. serine (or cysteine) proteinase inhibitor, clade
273 C and gelsolin) were identified.

274 Serine (or cysteine) proteinase inhibitor, clade C (antithrombin III (AT III)) is known to exert
275 anti-inflammatory effects. While treatment with high doses of AT III is effective in septic
276 animals and could improve acute lung injury in rats²⁸, we found a downregulation in serum of
277 TDI-treated mice suggesting these mice are less able to cope with the neutrophilic inflammation.
278 In our study, serum of TDI-treated mice contained 2.28 fold more hemopexin (HPX) which
279 according to Fagoonee *et al.* (2008) findings, may lead to a stronger activation of CD4⁺ T
280 cells²⁹. CD4⁺ T cells are a typical T cell subset related to asthma, increased in auricular lymph
281 nodes of TDI-treated mice³⁰.

283 *Bronchoalveolar lavage*

284 HPX was also found upregulated in the BAL fluid of mice that were sensitized and challenged
285 with TDI. BAL fluid is the most common manner to represent the protein composition of the
286 pulmonary airways. However, BAL fluid has a high concentration of salts due to the sampling
287 method and a low protein concentration. Nevertheless, after ultramembrane centrifugation, we
288 were able to obtain a clear proteome map of BAL, resulting in 72 identifications of differentially
289 expressed proteins, related to neutrophil chemotaxis (e.g. vitamin D binding protein and serine
290 (or cysteine) proteinase inhibitor, clade C), oxidative stress (e.g. peroxiredoxin 2 and hemopexin)
291 and inflammation (e.g. complement factor B and fetuin A).
292 Haptoglobin (Hp) was strongly upregulated in BAL fluid of TDI-treated mice. Hp is involved in
293 immune response modulation³¹, synthesized during granulocyte differentiation and released after

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

neutrophil activation to dampen potential damage to the surrounding tissue³². The development of chemical-induced asthma goes along with a persistent lung inflammation which can explain the strong upregulation of Hp.

Vitamin D binding protein (DBP), also known as Gc-globulin (GC), has been found in BAL fluid of patients with chronic obstructive pulmonary disease (COPD) and adult respiratory distress syndrome (ARDS)³³. A significant increase of DBP has also been observed in BAL fluid of methylene diphenyl diisocyanate (MDI)-exposed workers³⁴. DBP interacts with neutrophils to enhance the chemotactic activity of complement derived C5 peptides³⁵. Neutrophils are significantly increased in the BAL fluid of TDI-treated mice and also the complement system is known to be associated with the pathogenesis of asthma³⁶⁻³⁸.

In our experiments, complement factor B was almost 2-fold upregulated. Taube *et al.* (2006) found that complement factor B is indispensable in eliciting airway hyperresponsiveness and airway inflammation in mice sensitized and challenged with ovalbumin³⁹. Our data suggest it contributes to AHR and inflammation in TDI-treated mice.

Ferritin and transferrin are both involved in iron metabolism. Ferritin is the primary intracellular iron-storage protein and prevents toxic effects of free iron while transferrin is an antioxidant capable of binding iron in plasma. Levels of ferritin and transferrin are decreased and increased, respectively, in patients with asthma^{40,41}. In our study, ferritin and transferrin were found to be down- and upregulated in mouse BAL fluid, respectively. These results were also found by Hur *et al.* (2008), who compared the BAL fluid and serum from subjects with MDI-induced occupational asthma with asymptomatic MDI-exposed controls⁴².

1
2
3 316 An influx of neutrophils in the BAL fluid, a persistent inflammation and oxidative stress are
4
5
6 317 some of the key features characterizing chemical-induced asthma. Pathway analysis, using
7
8 318 Pathway Studio 7, was mainly focused on these characteristics to find proteins involved in these
9
10 319 pathways. We were able to highlight some important proteins associated with inflammation (Fig.
11
12 320 4). Proteins such as LSP1, GC, and plasminogen have been previously associated with
13
14 321 (neutrophilic) inflammation, but never in chemical-induced asthma. Other common
15
16 322 inflammation mediators, such as matrix metalloproteinases, were not picked up in our model.
17
18 323 When focusing on oxidative stress, proteins like haptoglobin, apolipoprotein E, hemopexin,
19
20 324 peroxiredoxin 2 and 6 were involved (figure not shown). These proteins contribute to the
21
22 325 protection against oxidative damage.
23
24
25
26
27 326

28
29 327 In this paper we discovered substantial changes in the proteome of three different compartments
30
31 328 (auricular draining lymph nodes, BAL and serum, each sampled from the same mice) in a well
32
33 329 characterized model of chemical-induced asthma. Key features of chemical-induced asthma such
34
35 330 as neutrophilic inflammation and oxidative stress, are represented by the identified proteins.
36
37 331 Further studies on earlier time points in our mouse model of chemical-induced asthma have to
38
39 332 point out which of these (or new) differentially expressed proteins can be used as early markers
40
41 333 of the onset of the disease. Hur *et al.* (2008) used a group of symptomatic MDI-exposed workers
42
43 334 to investigate which proteins are differentially expressed compared to asymptomatic MDI-
44
45 335 exposed workers^{34,42}. In this approach ferritin and transferrin were suggested as possible
46
47 336 markers. Although we were able to detect similar differences in ferritin and transferrin
48
49 337 regulations in our mouse model of TDI-induced asthma, this was not confirmed in a recent study
50
51 338 of Sastre *et al.* (2010), who studied ferritin and transferrin as serologic markers of TDI-
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

asthmatics⁴³. Further studies will be necessary to confirm whether these serologic markers are useful for the diagnosis of different types of diisocyanate-induced occupational asthma. Therefore, a first screening of possible biomarkers in mouse models can certainly be useful. In conclusion, we are confident that our approach in combination with earlier time point studies will highlight other (and perhaps more specific) markers of isocyanates sensitization and onset of occupational asthma before adverse effects arise. Such markers could be used to screen workers pre- and post isocyanate exposure.

Supporting Information description

A more detailed description of the materials and methods and two additional figures are represented in the Supporting Information of this manuscript.

Funding: The project was supported by a grant of the Interuniversity Attraction Pole Program, Belgian State, Belgian Science Policy [P6/35] and from the ‘Fund for Scientific Research Flanders’ (FWO), [FWO G.0547.08]. J.V. is a post-doctoral fellow of the FWO.

Acknowledgments

Special thanks to Koen Vandingenen for the technical assistance with the 2D-DIGE analysis.

Reference List

- (1) Cramer R., The potential of proteomics and peptidomics for allergy and asthma research. *Allergy* **2005**, 60(10), 1227-1237.
- (2) He B.; Munson AE.; Meade BJ., Analysis of gene expression induced by irritant and sensitizing chemicals using oligonucleotide arrays. *Int Immunopharmacol* **2001**, 1(5), 867-879.
- (3) Ku HO.; Jeong SH.; Kang HG.; Pyo HM.; Cho JH.; Son SW.; Yun SM.; Ryu DY., Gene expression profiles and pathways in skin inflammation induced by three different sensitizers and an irritant. *Toxicol Lett* **2009**, 190(2), 231-237.
- (4) Byrne JC.; Downes MR.; O'Donoghue N.; O'Keane C.; O'Neill A.; Fan Y.; Fitzpatrick JM.; Dunn M.; Watson RW., 2D-DIGE as a strategy to identify serum markers for the progression of prostate cancer. *J Proteome Res* **2009**, 8(2), 942-957.
- (5) Gray RD.; MacGregor G.; Noble D.; Imrie M.; Dewar M.; Boyd AC.; Innes JA.; Porteous DJ.; Greening AP., Sputum proteomics in inflammatory and suppurative respiratory diseases. *Am J Respir Crit Care Med* **2008**, 178(5), 444-452.
- (6) Calvo FQ.; Fillet M.; de Seny D.; Meuwis MA.; Maree R.; Crahay C.; Paulissen G.; Rocks N.; Gueders M.; Wehenkel L.; Merville MP.; Louis R.; Foidart JM.; Noel A.; Cataldo D., Biomarker discovery in asthma-related inflammation and remodeling. *Proteomics* **2009**, 9(8), 2163-2170.
- (7) Wisnewski AV.; Srivastava R.; Herick C.; Xu L.; Lemus R.; Cain H.; Magoski NM.; Karol MH.; Bottomly K.; Redlich CA., Identification of human lung and skin proteins

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

378 conjugated with hexamethylene diisocyanate in vitro and in vivo. Am J Respir Crit Care
379 Med **2000**, 162(6), 2330-2336.

(8) Bernstein DI., Occupational asthma caused by exposure to low-molecular-weight
chemicals. Immunol Allergy Clin North Am **2003**, 23(2), 221-234.

(9) Butcher BT.; Jones RN.; O'Neil CE.; Glindmeyer HW.; Diem JE.; Dharmarajan V.; Weill
H.; Salvaggio JE., Longitudinal study of workers employed in the manufacture of
toluene-diisocyanate. Am Rev Respir Dis **1977**, 116(3), 411-421.

(10) Redlich CA.; Cain H.; Wisnewski AV., The immunology and prevention of isocyanate
asthma: A model for low molecular weight asthma. Sem Resp Crit Care Med **1999**,
20(6), 591-599.

(11) De Vooght V.; Vanoirbeek JA.; Haenen S.; Verbeken E.; Nemery B.; Hoet PH.,
Oropharyngeal aspiration: an alternative route for challenging in a mouse model of
chemical-induced asthma. Toxicology **2009**, 259(1-2), 84-89.

(12) Vanoirbeek JAJ.; Tarkowski M.; Ceuppens JL.; Verbeken EK.; Nemery B.; Hoet PHM.,
Respiratory response to toluene diisocyanate depends on prior frequency and
concentration of dermal sensitization in mice. Toxicol Sci **2004**, 80(2), 310-321.

(13) Vanoirbeek JAJ.; De Vooght V.; Vanhooren HM.; Nawrot TS.; Nemery B.; Hoet PHM.,
How long do the systemic and ventilatory responses to toluene diisocyanate persist in
dermally sensitized mice? J Allergy Clin Immunol **2008**, 121(2), 456-463.

- 397 (14) Vanoirbeek JA.; Rinaldi M.; De Vooght V.; Haenen S.; Bobic S.; Gayan-Ramirez G.;
398 Hoet PH.; Verbeken E.; Decramer.; Nemery B.; Janssens W., Noninvasive and Invasive
399 Pulmonary Function in Mouse Models of Obstructive and Restrictive Respiratory
400 Diseases. *Am J Respir Cell Mol Biol* **2009**, 42(1), 96-104.
- 401 (15) Vierstraete E.; Cerstiaens A.; Baggerman G.; Van den Berg G.; De Loof A.; Schoofs L.,
402 Proteomics in *Drosophila melanogaster*: first 2D database of larval hemolymph proteins.
403 *Biochem Biophys Res Commun* **2003**, 304(4), 831-838.
- 404 (16) Farraj AK.; Boykin E.; Haykal-Coates N.; Gavett SH.; Doerfler D.; Selgrade M., Th2
405 Cytokines in Skin Draining Lymph Nodes and Serum IgE Do Not Predict Airway
406 Hypersensitivity to Intranasal Isocyanate Exposure in Mice. *Toxicol Sci* **2007**, 100(1),
407 99-108.
- 408 (17) Vanoirbeek JA.; Tarkowski M.; De Vooght V.; Nemery B.; Hoet PH., Immunological
409 determinants in a mouse model of chemical-induced asthma after multiple exposures.
410 *Scand J Immunol* **2009**, 70(1), 25-33.
- 411 (18) Noel-Georis I.; Bernard A.; Falmagne P.; Wattiez R., Database of bronchoalveolar lavage
412 fluid proteins. *J Chromatogr B Analyt Technol Biomed Life Sci* **2002**, 771(1-2), 221-236.
- 413 (19) Tarkowski M.; Vanoirbeek JA.; Vanhooren HM.; De Vooght V.; Mercier CM.; Ceuppens
414 J.; Nemery B.; Hoet PH., Immunological determinants of ventilatory changes induced in
415 mice by dermal sensitization and respiratory challenge with toluene diisocyanate. *Am J*
416 *Physiol Lung Cell Mol Physiol* **2007**, 292(1), L207-L214.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(20) Howard TH.; Hartwig J.; Cunningham C., Lymphocyte-specific protein 1 expression in eukaryotic cells reproduces the morphologic and motile abnormality of NAD 47/89 neutrophils. *Blood* **1998**, 91(12), 4786-4795.

(21) Jongstra-Bilen J.; Misener VL.; Wang C.; Ginzberg H.; Auerbach A.; Joyner AL.; Joyner AL.; Downey GP.; Jongstra J., LSP1 modulates leukocyte populations in resting and inflamed peritoneum. *Blood* **2000**, 96(5), 1827-1835.

(22) Chen Z.; Lund R.; Aittokallio T.; Kosonen M.; Nevalainen O.; Lahesmaa R., Identification of novel IL-4/Stat6-regulated genes in T lymphocytes. *J Immunol* **2003**, 171(7), 3627-3635.

(23) Vuorinen K.; Ohlmeier S.; Lepparanta O.; Salmenkivi K.; Myllarniemi M.; Kinnula VL., Peroxiredoxin II expression and its association with oxidative stress and cell proliferation in human idiopathic pulmonary fibrosis. *J Histochem Cytochem* **2008**, 56(10), 951-959.

(24) Kang JS.; Yoon YD.; Ahn JH.; Kim SC.; Kim KH.; Kim HM.; Moon EY., B cell-activating factor is a novel diagnosis parameter for asthma. *Int Arch Allergy Immunol* **2006**, 141(2), 181-188.

(25) Moon EY.; Noh YW.; Han YH.; Kim SU.; Kim JM.; Yu DY.; Lim YS., T lymphocytes and dendritic cells are activated by the deletion of peroxiredoxin II (Prx II) gene. *Immunol Lett* **2006**, 102(2), 184-190.

(26) Redlich CA.; Herrick CA., Lung/skin connections in occupational lung disease. *Curr Opin Allergy Clin Immunol* **2008**, 8(2), 115-119.

- 437 (27) Bello D.; Herrick CA.; Smith TJ.; Woskie SR.; Streicher RP.; Cullen MR.; Liu Y.;
438 Redlich CA., Skin exposure to isocyanates: reasons for concern. *Environ Health Perspect*
439 **2007**, 115(3), 328-335.
- 440 (28) Hagiwara S.; Iwasaka H.; Matsumoto S.; Noguchi T., High dose antithrombin III inhibits
441 HMGB1 and improves endotoxin-induced acute lung injury in rats. *Intensive Care Med*
442 **2008**, 34(2), 361-367.
- 443 (29) Fagoonee S.; Caorsi C.; Giovarelli M.; Stoltenberg M.; Silengo L.; Altruda F.; Camussi
444 G.; Tolosano E.; Bussolati B., Lack of plasma protein hemopexin dampens mercury-
445 induced autoimmune response in mice. *J Immunol.* **2008**, 181(3), 1937-1947.
- 446 (30) Vanoirbeek JA.; De Vooght V.; Nemery B.; Hoet PH., Multiple challenges in a mouse
447 model of chemical-induced asthma lead to tolerance: ventilatory and inflammatory
448 responses are blunted, immunologic humoral responses are not. *Toxicology* **2009**, 257(3),
449 144-152.
- 450 (31) Arredouani MS.; Kasran A.; Vanoirbeek JA.; Berger FG.; Baumann H.; Ceuppens JL.,
451 Haptoglobin dampens endotoxin-induced inflammatory effects both in vitro and in vivo.
452 *Immunology* **2005**, 114(2), 263-271.
- 453 (32) Theilgaard-Monch K.; Jacobsen LC.; Nielsen MJ.; Rasmussen T.; Udby L.; Gharib M.;
454 Arkwright PD.; Gombart.; Calafat J.; Moestrup SK.; Porse BT.; Borregaard N.,
455 Haptoglobin is synthesized during granulocyte differentiation, stored in specific granules,
456 and released by neutrophils in response to activation. *Blood* **2006**, 108(1), 353-361.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(33) Piquette CA.; Robinson-Hill R.; Webster RO., Human monocyte chemotaxis to complement-derived chemotaxins is enhanced by Gc-globulin. *J Leukoc Biol* **1994**, 55(3), 349-354.

(34) Hur GY.; Choi GS.; Sheen SS.; Lee HY.; Park HJ.; Choi SJ.; Ye YM.; Park HS., Serum ferritin and transferrin levels as serologic markers of methylene diphenyl diisocyanate-induced occupational asthma. *J Allergy Clin Immunol* **2008**, 122(4), 774-780.

(35) DiMartino SJ.; Trujillo G.; McVoy LA.; Zhang J.; Kew RR., Upregulation of vitamin D binding protein (Gc-globulin) binding sites during neutrophil activation from a latent reservoir in azurophil granules. *Mol Immunol* **2007**, 44(9), 2370-2377.

(36) Humbles AA.; Lu B.; Nilsson CA.; Lilly C.; Israel E.; Fujiwara Y.; Gerard NP.; Gerard C., A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* **2000**, 406(6799), 998-1001.

(37) Krug N.; Tschernig T.; Erpenbeck VJ.; Hohlfeld JM.; Kohl J., Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. *Am J Respir Crit Care Med* **2001**, 164(10 Pt 1), 1841-1843.

(38) Kohl J.; Wills-Karp M., A dual role for complement in allergic asthma. *Curr Opin Pharmacol* **2007**, 7(3), 283-289.

(39) Taube C.; Thurman JM.; Takeda K.; Joetham A.; Miyahara N.; Carroll MC.; Dakhama A.; Giclas PC.; Holers VM.; Gelfand EW., Factor B of the alternative complement

- 477 pathway regulates development of airway hyperresponsiveness and inflammation. Proc
478 Natl Acad Sci U S A **2006**, 103(21), 8084-8089.
- 479 (40) Brogan TD.; Ryley HC.; Neale L.; Yassa J., Soluble proteins of bronchopulmonary
480 secretions from patients with cystic fibrosis, asthma, and bronchitis. Thorax **1975**, 30(1),
481 72-79.
- 482 (41) Vural H.; Uzun K.; Uz E.; Kocyigit A.; Cigli A.; Akyol O., Concentrations of copper,
483 zinc and various elements in serum of patients with bronchial asthma. J Trace Elem Med
484 Biol **2000**, 14(2), 88-91.
- 485 (42) Hur GY.; Koh DH.; Choi GS.; Park HJ.; Choi SJ.; Ye YM.; Kim KS.; Park HS., Clinical
486 and immunologic findings of methylene diphenyl diisocyanate-induced occupational
487 asthma in a car upholstery factory. Clin Exp Allergy **2008**, 38(4), 586-593.
- 488 (43) Sastre J.; Sastre B.; Fernandez-Nieto M.; Perez-Camo I.; Sanchez JJ.; Del Pozo V.,
489 Serum ferritin and transferrin levels are not serologic markers of toluene diisocyanate-
490 induced occupational asthma. J Allergy Clin Immunol **2010**, 125(3), 762-764.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Footnotes
* pI and Mw are depicted as theoretical values
† Some proteins were identified in multiple spots: see Table E2 Online Repository
‡ Two proteins were identified in same proteinspot

Fig. 1: Characteristics of TDI- and AOO-treated mice.

Airway hyperreactivity (AHR) measurements were performed 24hrs after oropharyngeal challenge **(A)** AUC of the selected TDI- and AOO-treated mice **(B)**; Auricular lymph nodes were removed and weighed **(C)**. Differential cell count of BAL **(D)**. Results for AHR and BAL cell count are presented as means $\pm$ SD. Lymph node (LN) weight is presented as individual values (1 mouse – 2 lymph nodes) and group averages (n=12). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

Fig. 2: Digital overlay images of the proteome of auricular lymph nodes, serum and BAL fluid. Yellow spots: equal expression in TDI- and AOO-treated condition, red spots: up regulation in TDI-treated condition, green spots: down regulation in TDI-treated condition.

Fig. 3: Gene Ontology was used to classify the identified proteins according to their function in the auricular lymph nodes, serum and BAL fluid. Similar categories popped up in the three different samples in the establishment of chemical-induced occupational asthma.

Fig. 4: Differentially expressed proteins (circles) from auricular lymph nodes (blue), BAL (green) and serum (red) involved in inflammation are depicted. Cytokines and chemokines (diamonds) known to be involved in asthma and are already measured previously are shown. Lines in between proteins indicate interactions (positive or negative) between proteins. Proteins that were differentially expressed in more than one sample are only depicted once due to program settings (Pathway Studio 7).

1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

518

519

520

521

Table 1: 2D-DIGE results

Auricular lymph nodes:

continued

Name	No. spots	Spot no.	Accession ID	Score	Seq Cov (%)	Peptides matched	Fold Change	pI *	Mw (Da) *
Structural									
Actin	1	3301	gi 123298587	72	30	12	1.72	5.15	32941
Tubulin, beta 5	1	1063	gi 7106439	104	52	25	1.57	4.78	50095
F-actin capping protein alpha-1 subunit	1	2352	gi 595917	114	45	9	1.45	5.34	33104
Protein disulfide isomerase	1	903	gi 112293264	73	19	10	1.37	5.53	55906
Actin-related protein 3 homolog	1	1240	gi 23956222	65	18	8	1.24	5.61	47783
Calreticulin	2	828 [†]	gi 6680836	198	56	26	-1.39	4.33	48136
Retinoblastoma binding protein 7	1	1162	gi 123223058	75	21	9	-4.84	5.22	43968
Vimentin	2	913 [†]	gi 2078001	300	73	42	-6.36	4.96	51590
Metabolism									
Pyruvate kinase	1	920	gi 31981562	166	40	30	1.45	7.18	58378
5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	1	794	gi 37572276	212	52	34	1.45	6.3	64687
Apolipoprotein A-I	1	2554	gi 160333304	152	45	16	-1.52	5.51	30597
Alpha-amylase 1	1	1105	gi 160358819	146	42	26	-5.32	6.5	58291
Stress response									
Heat shock protein 4	1	181	gi 112293266	265	52	47	1.38	5.13	94948
Tumor rejection antigen gp96	1	243	gi 6755863	229	41	47	-1.60	4.74	92703
Peroxiredoxin 2	1	2664	gi 148747558	73	30	5	-1.70	5.2	21936
Immune response									
Lymphocyte-specific protein 1	2	1127 [†]	gi 209862951	115	53	17	-5.15	4.77	36806
Binding/Transport									
Fascin	1	1079	gi 113680348	176	48	26	-2.94	6.44	55215
Sorcin	1	2737	gi 124430543	137	63	9	-2.69	5.11	20568
Heterogeneous nuclear ribonnucleoprotein H2	1	1115	gi 9845253	117	47	27	-1.41	5.89	49533
T-complex protein 1	1	873	gi 110625624	132	38	24	1.27	5.82	60867
Proteasome activator subunit 2 isoform 1	1	2292	gi 20137004	130	33	15	1.43	5.54	27268
Other									
Proteasome subunit alpha	1	2244	gi 148685116	83	55	17	1.84	6.4	26764
Serine (or cysteine) proteinase inhibitor, clade B	1	1651	gi 114158675	193	59	32	1.68	5.85	42719
Annexin A3	1	1829 [‡]	gi 5902786	194	42	16	1.37	5.33	36520
ADP ribosylarginine hydrolase	1	1829 [‡]	gi 6680658	85	32	15	1.37	5.46	40328

to be continued

522 Bronchoalveolar lavage:

continued

<i>Name</i>	<i>No. spots</i>	<i>Spot no.</i>	<i>Accession ID</i>	<i>Score</i>	<i>Seq Cov (%)</i>	<i>Peptides matched</i>	<i>Fold Change</i>	<i>pI *</i>	<i>Mw (Da) *</i>
Metabolism									
Apolipoprotein A-I	4	2090 [†]	gi 160333304	138	54	22	3.70	5.51	30597
Apolipoprotein A-IV	2	1315 [†]	gi 110347473	190	50	25	2.40	5.34	45001
Malate dehydrogenase	1	1695	gi 92087001	111	43	19	-2.21	6.16	36659
Carboxylesterase 3	2	656 [†]	gi 117553604	55	21	22	-2.91	6.17	62034
Retinal dehydrogenase	1	1024	gi 191804	90	40	17	-4.91	7.92	55060
Stress response									
Glutathione peroxidase	1	2163	gi 1586514	74	36	14	2.81	7.6	25392
Peroxiredoxin 6	1	2066	gi 3219774	146	69	17	-2.69	5.98	24925
Peroxiredoxin 2	1	2152	gi 148747558	91	46	12	-11.61	5.2	21968
Binding/Transport									
Haptoglobin	5	1442 [†]	gi 8850219	83	36	16	9.77	5.88	39241
Alpha-fetoprotein	9	706 [†]	gi 191765	174	59	20	2.90	5.47	48792
Vitamin D binding protein	4	944 [†]	gi 193446	143	43	24	2.35	5.26	54647
Hemopexin	4	734 [†]	gi 160358829	170	51	25	2.34	7.92	52026
Albumin	13	706 [†]	gi 163310765	239	53	34	2.14	5.75	70700
Transthyretin	1	2528	gi 7305599	104	61	11	2.05	5.77	15880
Transferrin	2	571 [†]	gi 20330802	311	43	29	1.87	6.94	78841
Ferritin	1	2195	gi 120524	97	59	11	-3.51	5.66	20847
Selenium binding protein 1	1	1095 [‡]	gi 22164798	113	48	19	-4.18	5.87	53051
Selenium binding protein 2	1	1095 [‡]	gi 9507079	78	38	15	-4.18	5.78	53165
Major urinary protein	1	2137 [‡]	gi 13276755	133	83	17	-7.17	4.96	20935
Major urinary protein 2	1	2137 [‡]	gi 113930712	104	71	14	-7.17	4.94	20920
Major urinary protein 8	1	2137	gi 127531	109	84	13	-7.17	4.85	17720
Major urinary protein 1	1	2189	gi 13654245	142	65	16	-9.53	5.02	20920
Complement system									
Complement C3d	1	1462	gi 89275682	80	47	13	8.83	5.43	33613
Complement C3	4	756 [†]	gi 126518317	130	25	28	2.63	6.29	187905
Complement factor B	1	375	gi 6996919	136	31	29	1.83	7.18	86319
Complement C9	1	671	gi 20141173	77	37	21	1.72	5.56	63217
Complement C8	1	780	gi 18381134	72	30	16	1.56	6.51	57044
Inflammation									
Inter alpha trypsin inhibitor	2	218 [†]	gi 16741341	80	22	23	4.46	6.05	104737
Kininogen 1	4	664 [†]	gi 12963497	134	31	18	1.96	5.74	48951
Serine (or cysteine) proteinase inhibitor, clade C	2	853 [†]	gi 18252782	104	42	22	1.76	6.1	52484
Fetuin A	1	994	gi 7304875	54	32	12	1.69	6.04	38100

to be continued

Fetuin beta	1	848	gi 148665228	92	36	20	1.59	6.75	45140
Liver carboxylesterase	2	639 [†]	gi 148679147	119	30	19	1.58	5.14	61543
Others									
Plasminogen	2	286 [†]	gi 31982113	175	43	32	4.38	6.21	93460
Beta 2 glycoprotein	3	863 [†]	gi 1938223	94	36	17	3.23	8.54	37551
Annexin 2	1	1667	gi 168983832	84	52	9	-2.43	5.21	21957
Rho-GDP-dissociation inhibitor	1	2000	gi 26344461	61	33	8	-3.46	5.12	23450
Lamin A	1	1931	gi 12835914	72	24	22	-9.50	6.54	74484
Unkown									
Uncharacterized	1	1412	gi 12844982	66	39	10	3.46	8.65	22656
Uncharacterized	1	1392	gi 26374509	64	25	6	1.73	8.23	31201
Uncharacterized	1	2242	gi 97044760	65	22	6	-5.00	7.68	44312

Serum:

<i>Name</i>	<i>No. spots</i>	<i>Spot no.</i>	<i>Accession no.</i>	<i>Score</i>	<i>Seq Cov (%)</i>	<i>Peptides matched</i>	<i>Fold Change</i>	<i>pI</i> *	<i>Mw</i> *
Structural									
Gelsolin	3	387 [†]	gi 28916693	137	25	17	-1.46	5.83	86287
Metabolism									
Apolipoprotein E	1	1561	gi 148691229	175	55	21	1.34	4.89	26994
Stress response									
Glutathione peroxidase	1	1808	gi 1586514	68	46	13	-1.22	7.60	25392
Immune response									
Thrombospondin	1	1737	gi 554390	72	18	9	-1.22	6.16	55350
Histocompatibility 2, Q region locus 10	2	1245 [†]	gi 6754132	133	40	12	-1.24	5.13	37455
Inflammation									
Serine (or cysteine) proteinase inhibitor, clade C	1	904 [‡]	gi 18252782	99	31	15	-1.23	6.1	52484
Binding/Transport									
Hemopexin	8	704 [†]	gi 160358829	183	36	20	2.28	7.92	52026
Serum amyloid P-component precursor	1	2015	gi 134198	72	33	8	1.95	5.98	26401
Vitamin D binding protein	1	904 [‡]	gi 193446	121	35	16	-1.23	5.26	54647

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

* pI and Mw are depicted as theoretical values

† Some proteins were identified in multiple spots: see Table E2 Online Repository

‡ two proteins were identified in same proteinspot

529 TOC Synopsis

So far, the exact mechanisms by which isocyanate-induced asthma develops are unclear. Therefore, (new) markers of sensitization and/or the onset of chemical-induced asthma are required.

For the first time two-dimensional difference gel electrophoresis (2D-DIGE) was introduced in a well-validated mouse model of chemical-induced asthma to compare proteome changes in different tissue-compartments of mice sensitized and challenged with toluene-2,4-diisocyanate with control mice.

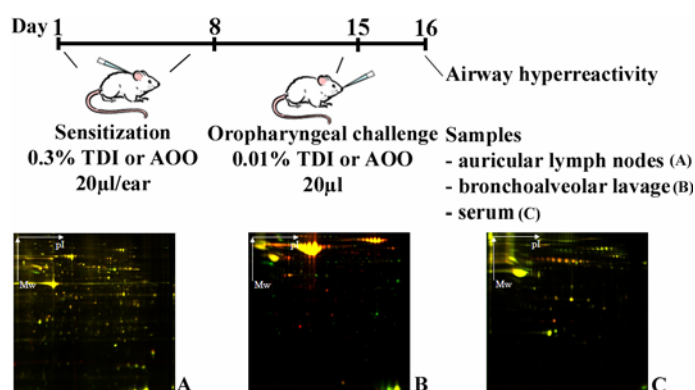
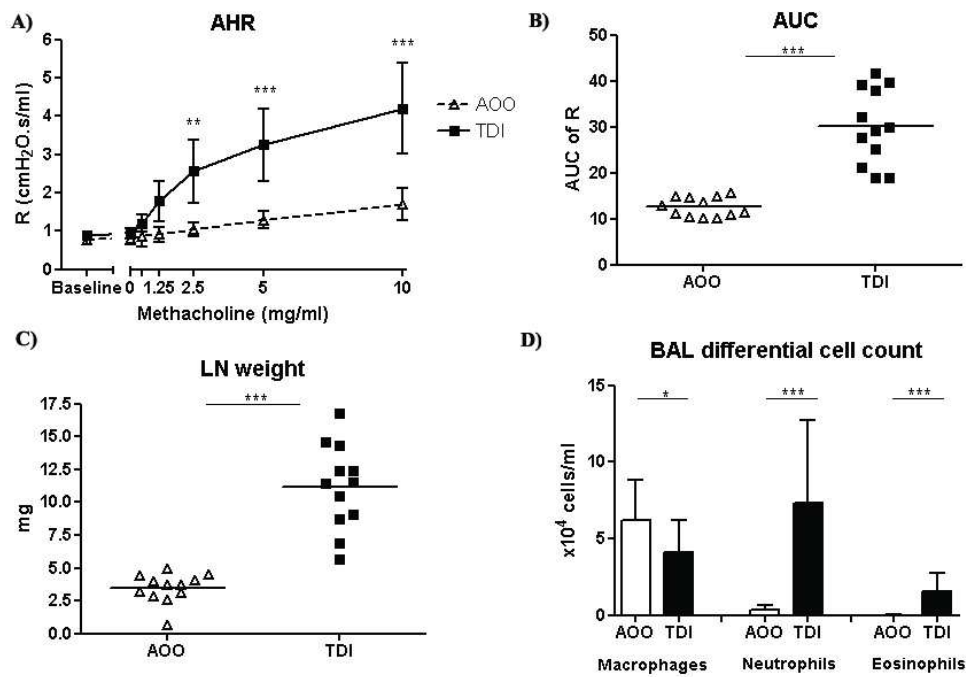
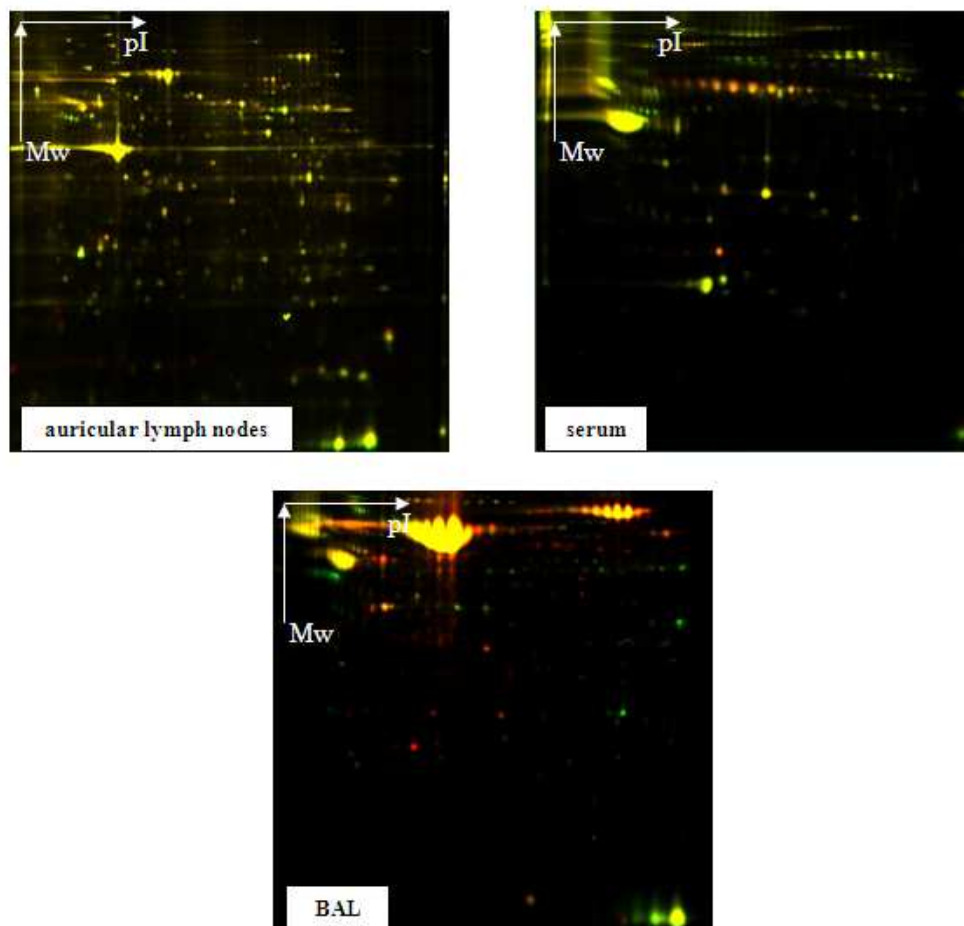


Figure 1



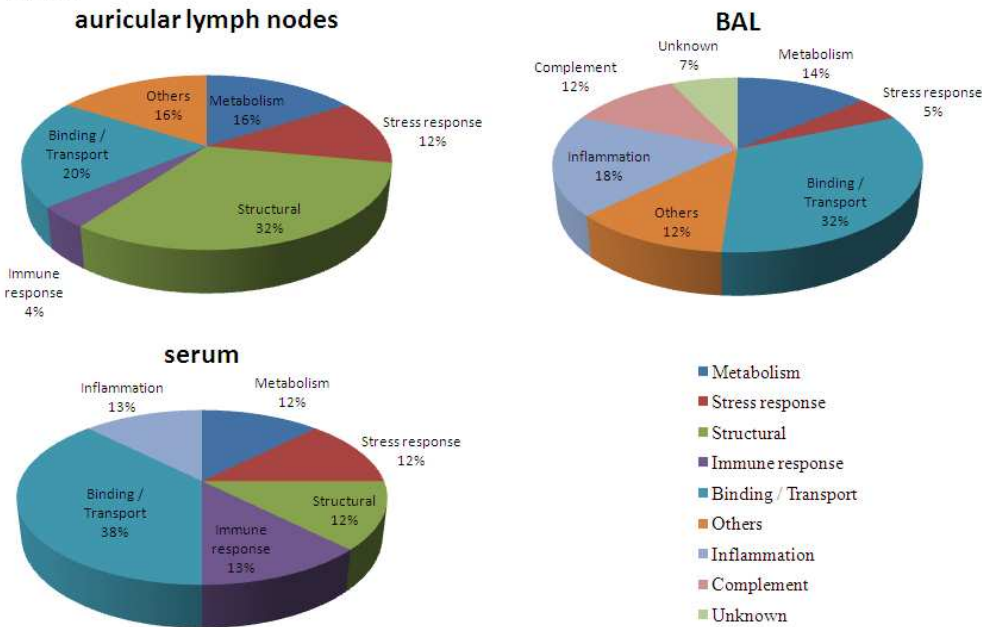
307x229mm (72 x 72 DPI)

Figure 2

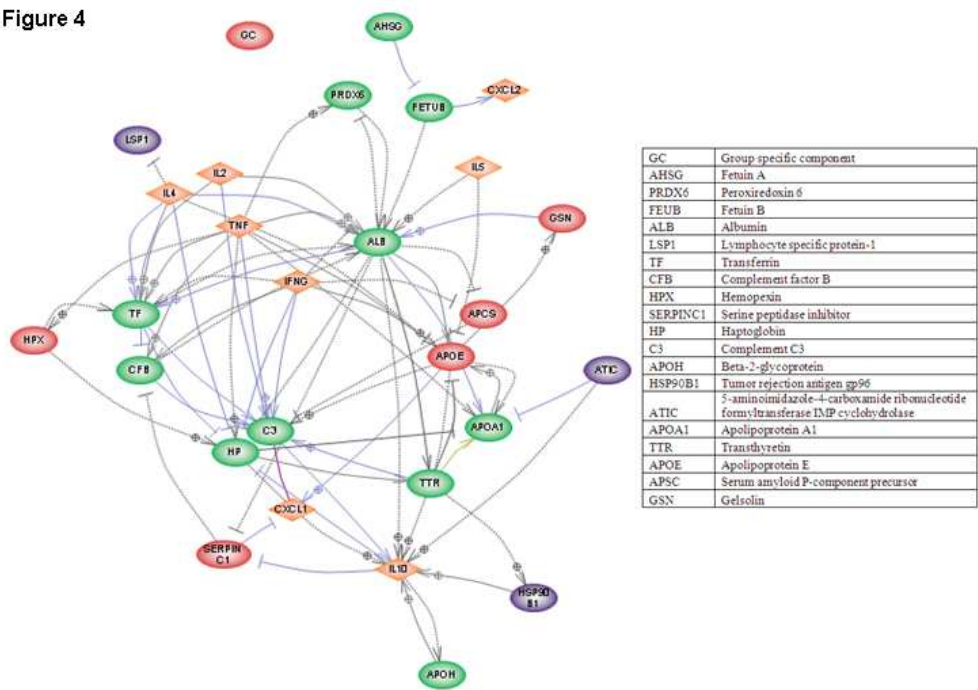


202x208mm (72 x 72 DPI)

Figure 3

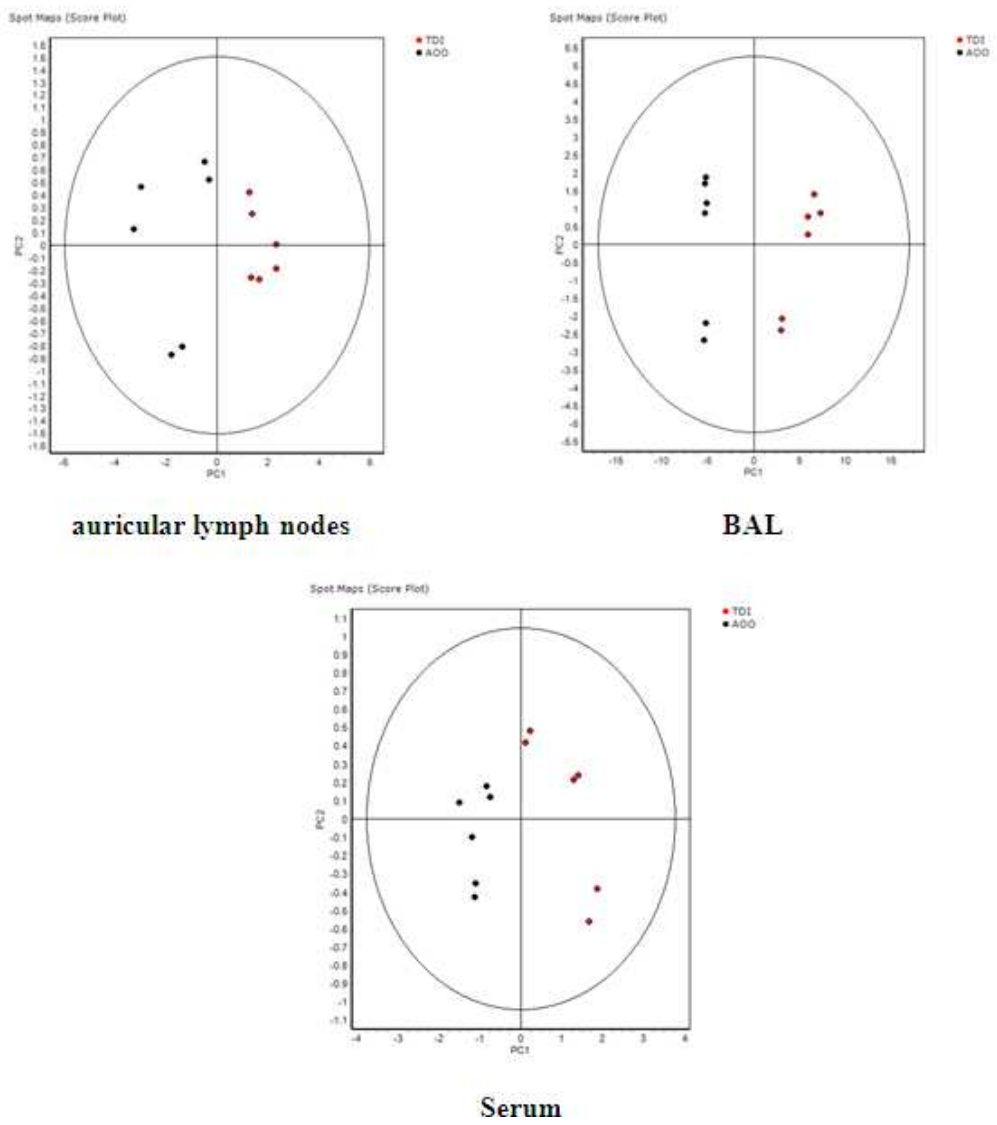


296x204mm (72 x 72 DPI)



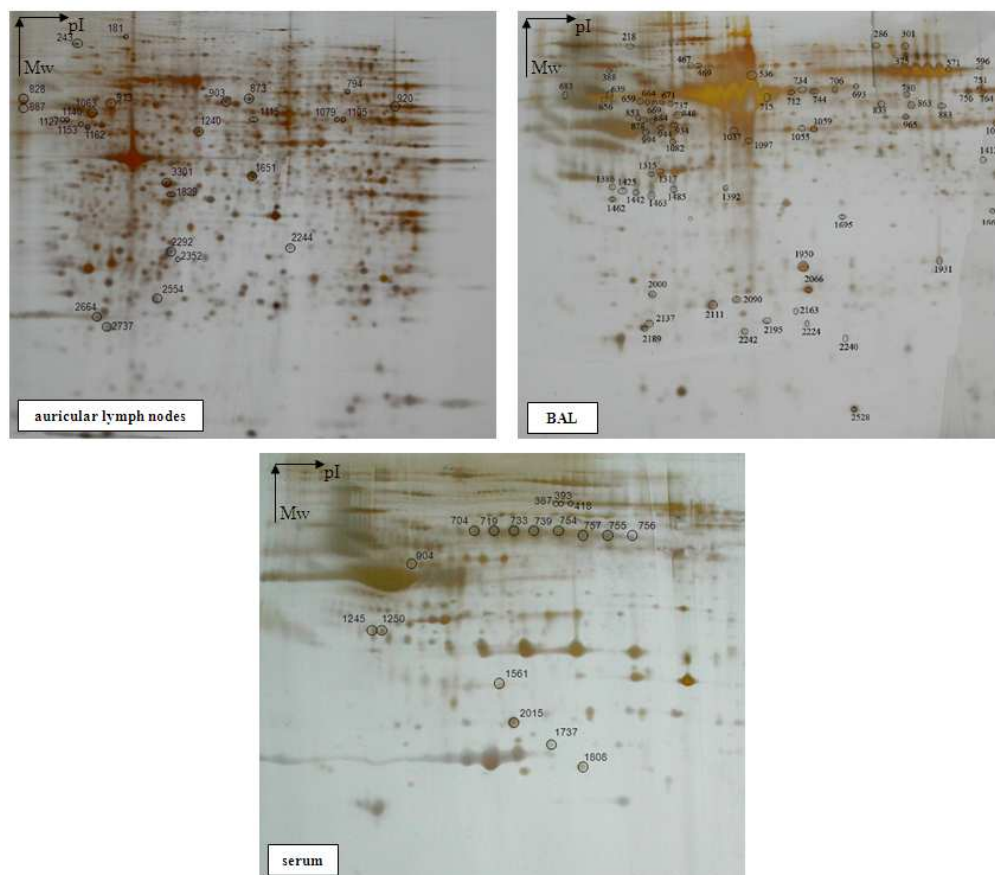
259x194mm (72 x 72 DPI)

Figure E1



195x227mm (72 x 72 DPI)

Figure E2



297x280mm (72 x 72 DPI)