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Molecular Characterization of Trimellitic Anhydride–induced Respiratory Allergy in Brown Norway Rats

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Abstract

To contribute to the hazard identification of low molecular weight (LMW) respiratory allergens, respiratory allergy induced by trimellitic anhydride (TMA) was characterized by whole genome analysis of lung tissue and blood proteomics in Brown Norway rats. Dermal sensitization (50% and 25% w/v) with TMA and an inhalation challenge of 15 mg/m^3 TMA-induced apneas, laryngeal inflammation, increased numbers of eosinophils, neutrophils and macrophages in bronchoalveolar lavage (BAL), and increased immunoglobulin E levels in serum and lung tissue. Whole genome analysis of lung, sampled 24 hours after challenge, showed expression changes of not only genes belonging to several Gene Ontology groups with up-regulation of inflammatory-associated genes and those associated with lung remodeling but also genes involved in downsizing these processes. Blood proteomics reflected activation of inflammation-inhibiting pathways. Unsensitized animals challenged with TMA exhibited also an increased number of macrophages in BAL, but gene expression in the above-mentioned gene pathways was unchanged or down-regulated. The authors conclude that parameters for lung remodeling can be a valuable tool in hazard identification of LMW respiratory allergens.

Keywords: trimellitic anhydride; respiratory allergy; genes; lung remodeling; inflammation downsizing mechanisms; toll-like receptor pathway.

Introduction

Asthma is present in 10% to 15% of the adult population, and it is estimated that up to 15% of all cases in industrialized settings are associated with workplace exposure (Bauer et al. 1994; Bauer et al. 1995). Diisocyanates and acid anhydrides are among the most common low molecular weight (LMW) classes of chemicals responsible for occupational asthma. The symptoms of LMW asthma are similar to those induced by high molecular weight (HMW) compounds. Asthma in man is defined as variable or intermittent airway obstruction associated with bronchial hyperresponsiveness (BHR) and is considered to be an inflammatory disorder. There is a relationship between magnitude of airway inflammation and BHR, but factors such as airway remodeling also influence hyperresponsiveness (Haley and Drazen 1998). The development of HMW asthma is associated with elevated immunoglobulin E (IgE) levels, the predominance of Th2 cytokine production and eosinophilic inflammation of the airways. However, IgE may not be essential for allergen-induced BHR and airway inflammation, and thus far, the physiological role of IgE in the airways has not been fully elucidated (Moffatt 2005). Asthma induced by the diisocyanates has a low association with atopy, low prevalence with specific IgE antibodies and a mixed Th1-Th2 response (Matheson et al. 2005; Jones et al. 2006). For acid anhydride allergens like trimellitic anhydride (TMA), the relationship between disease status and IgE is stronger but on a group not on an individual basis (man: Liss et al. 1993; rodents: Arts et al. 1998; Ban and Hettich 2001; Dearman et al. 2002).

Because of these uncertainties in the pathogenesis of asthma, there are, at present, no validated animal models to test the potential of a chemical to induce respiratory allergy. Candidate models for hazard identification investigate respiratory allergic potential in rodents on the basis of serum IgE or cytokine fingerprinting in skin and skin-draining lymph nodes following dermal application of the chemical (Dearman, Skinner, and Humphreys 2003). However, immune responses measured in blood, skin, and skin-draining lymph nodes may differ in various ways from those measured in the respiratory tract (Gould et al. 2006; Fritz et al. 2008) and particularly the lungs, for asthma is a disease of the smaller bronchi/bronchioles. Following intranasal exposure to diisocyanates, the cytokine profiles in the lymph nodes were not predictive of differences in responses to inhaled methacholine to measure BHR (Selgrade et al. 2006).

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Abbreviations: BN, Brown Norway; BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; DNCB, dinitrochlorobenzene; ELISA, Enzyme-Linked-Immuno-Sorbent Assay; ECM, extracellular matrix components; HMW, high molecular weight; IgE, immunoglobulin E; LMW, low molecular weight; TMA, trimellitic anhydride.

Some animal toxicity models include a challenge phase, in which changes are measured in breathing parameters and respiratory-tract inflammation on an inhalation challenge in sensitized animals (reviewed by Pauluhn and Mohr 2005; Arts and Kuper 2007; Kimber et al. 2007). These models resemble human disease in some but not all aspects. The mechanistic basis for species differences in response to a chemical may be found at any level of biological organization. Microarray technology enables us to compare the responses of test animals with those of humans at the global transcriptome level (Mattes 2006). Changes in mRNA (microarrays) that occur in the asthmatic lungs of man specifically refer to cytokines and chemokines and, to some extent, to genes related to lung remodeling (Ober and Hoffjan 2006; Bloemen et al. 2007), but there is a need to identify groups of genes related to the causative allergen (Holsapple et al. 2006). This will contribute to cross-species comparative toxicogenomics for safety assessment and to the identification of novel targets for in vitro toxicity tests.

This article aims to improve the hazard identification of respiratory allergens by molecular characterization of respiratory allergy in Brown Norway (BN) rats induced by the LMW respiratory allergen TMA, using a protocol that successfully identified chemical respiratory allergens in the high IgE-responding BN rat (Pauluhn et al. 2002; Zhang et al. 2004; Arts and Kuper 2007). Whole genome analysis (microarrays) of lung tissue was performed, and the results were related to functional (breathing) variables, allergic inflammation, and proteomics in blood.

MATERIALS AND METHODS

Animals and Maintenance

Female, seven-to-eight-week-old, inbred BN rats were purchased from Charles River Deutschland GmbH (Sulzfeld, Germany). The animals were acclimatized for seven days before the start of the study. They were kept under conventional laboratory conditions and received the institute's grain-based, open-formula diet and unfluoridated tap water ad libitum. All animal procedures were approved by the TNO Committee of Animal Welfare.

Treatment Schedules and Groups

The study was conducted with four groups of rats according to the scheme depicted in Table 1. Blood was collected one day before the start of the study. Female BN rats received 150 µl of TMA (purity 97%; Aldrich, Brussels, Belgium; 50% w/v) in a 4:1 (v/v) mixture of acetone and olive oil (AOO) as the vehicle in a 4:1 (v/v) mixture of acetone (Merck, Darmstadt, Germany) and refined olive oil (AOO; Sigma Diagnostics, St. Louis, MO, USA) as the vehicle on each flank (approximately 12 cm^2 each). Flanks had been shaved with an electrical razor at least two to three days earlier. Seven days after the first sensitization, they received 75 µl of TMA (25% w/v) on the dorsum of both ears. Controls received vehicle AOO. On day 21, basal breathing parameters (frequency, tidal volume, and pattern) were assessed, followed by TMA inhalatory challenge and assessment of the same breathing parameters during and after challenge. Animals were challenged by inhalation of a minimally irritating (based on breathing frequency) concentration of 15 mg/m^3 of TMA for 15 minutes. On day 22, breathing parameters were assessed again, and afterward, necropsy was performed.

Atmosphere Generation and Analysis

During inhalation challenge, an all-glass nebulizer of the institute's design was used to generate the test atmospheres from freshly prepared solutions of TMA in acetone. The acetone concentration was kept between 3,000 and 5,000 ppm (~7–12 g/m^3), which levels are far below the level inducing sensory irritation (Alarie 1973; De Ceaurriz et al. 1981; Schaper and Brost 1991) and which indeed did not induce changes in breathing pattern in either sensitized or unsensitized rats (Arts et al. 1998). The challenge concentration of TMA was based on a previous study performed in BN rats (Arts et al. 2004). Atmospheric concentrations of TMA were determined gravimetrically by filter sampling, and those of acetone, by calculations based on the nominal concentration, assuming the usual 100% generation efficiency for this vapor. The particle size distribution of TMA in the test atmosphere was determined using a ten-stage cascade impactor (Andersen, Atlanta, GA, USA). Due to the small sampling air-flow rate and the large total volume required for analytical and particle-size determinations, samples were not collected during the challenge itself but immediately prior to or after challenge. The mass median aerodynamic diameter of the aerosolized TMA particles was between 0.9 and 1.2 µm with a geometric standard deviation between 2.5 and 2.9.

Inhalation Challenge and Measurements of Breathing Parameters

Rats were individually restrained in Battelle tubes, and each tube was placed into one of four whole-body plethysmographs connected to the central exposure unit. In this experimental setup, two TMA-sensitized (+/+) and two vehicle-treated (–/+) rats, at a time, were first exposed to fresh air for at least 25 minutes (prechallenge period) and then to the TMA atmosphere for exactly 25 minutes (challenge period), followed by a recovery (postchallenge) period of 30 minutes.

As a surrogate for changes in lung function, breathing parameters (frequency, pattern, and tidal volume; Arts and Kuper 2007) were monitored by means of recording the pressure signal in the plethysmograph before, during, and after challenge. Before challenge, breathing parameters were monitored approximately 20 seconds each minute, starting 6 minutes prior to challenge. During challenge, breathing parameters were monitored continuously, whereas after challenge, they were monitored approximately 20 seconds during each minute for the first 10 minutes, followed by 20 seconds each 3 minutes

TABLE 1.—Treatment schedule.

Group designation[a]	Sensitization Day 0: 300 µl on flanks - Day 7: 150 µl on ears	Challenge Day 21: 15 min. inhalation	Day 22: Necropsy
A. –/– Unsensitized/unchallenged	–	–	Lung function[b] Serum for proteomics and total IgE
B. +/– Sensitized/unchallenged	50% TMA–25% TMA	–	Liver, kidneys, and left lung weights BAL of right lung lobes
C. –/+ Unsensitized/challenged	Vehicle	15 mg/m3 TMA	Nasal passages, larynx, and trachea fixed in formalin
D. +/+ Sensitized/challenged (allergic group)	50% TMA–25% TMA	15 mg/m3 TMA	Left lung snap frozen for transcriptomics, immunohistochemistry, IgE, and cytokine levels

[a] Six female BN rats per group.
[b] Lung function parameters were determined before, during, and after challenge.

for 9 minutes, and followed by 20 seconds each 5 minutes for 10 minutes. Furthermore, breathing parameters were monitored approximately 20 seconds each minute for 6 minutes, 24 hours after challenge. Mean values were thus obtained based on six preexposure, fifteen exposure, fifteen postexposure (immediately after), and six postexposure (24 hours after) values.

Clinical Signs, Serum Collection, Body and Organ Weights, and Necropsy

The animals were observed daily and weighed shortly before the TMA dermal application, at weekly intervals thereafter, and just prior to necropsy. Individual serum samples were prepared from blood withdrawn via the orbital plexus prior to sensitization and via the abdominal aorta at necropsy. For blood proteomics, protease inhibitor cocktail was added to freshly obtained serum samples. The samples were stored at –80°C until analysis. For IgE analysis, the serum samples were stored at –20°C until analysis by Enzyme-Linked-Immuno-Sorbent Assay (ELISA). At necropsy, animals were anaesthetized with pentobarbital, killed by exsanguination from the abdominal aorta and examined grossly for abnormalities. Liver, kidneys, and the unlavaged left lung were weighed (after bronchoaleolar lavage had been performed on the right lung lobes; see below). The left lung was inflated with 50% Tissue Tek in saline, quick frozen on dry ice and kept at –70°C for microarray analysis or immunohistochemistry. The nasal tissues, trachea, and larynx were collected and fixated in neutral, phosphate-buffered 4% (v/v) formaldehyde for histopathological evaluation.

Bronchoalveolar Lavage

At necropsy, the right lung lobes were lavaged two times with a volume of 23 ml saline per kg bw, after binding of the left (main) lobe, which was used for histopathological evaluation (see below). The total amount of retracted lavage fluid was weighed and retained on ice. The bronchoalveolar cells were isolated from the supernatant by centrifugation (250 g) during 5 minutes at 4°C and resuspended in 0.5 ml saline to assess total cell and differential cell numbers. Total cell numbers were counted using an automated haematology analyzer (K-800, Sysmex, Toa, Kobe, Japan). The percentage of viable cells was determined using an acridine orange/ethidium bromide staining method in combination with fluorescence microscopic evaluation. For differential cell counts, cytospins were prepared and stained with May-Grunwald Giemsa. At least 200 cells were counted per animal to determine absolute numbers and percentages of macrophages or monocytes, lymphocytes, neutrophils, and eosinophils.

Serum and Lung Homogenate IgE Levels

Total IgE levels in serum or lung homogenate of snap-frozen left lung tissue were analyzed by means of an ELISA as described earlier (Arts et al. 1997). The concentration of IgE in the samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as µg/ml serum.

Histopathology and Immunohistochemistry

The formalin-fixed nasal tissues, larynx, and trachea were embedded in paraffin wax and sectioned at 5 µm. Nasal tissues were cut at six levels according to Woutersen et al. (1994). The larynx was cut longitudinally through the epiglottis. The cranial part of the trachea was cut transversally and the caudal part longitudinally, together with the bifurcation and the two extrapulmonary bronchi. The sections were stained with hematoxylin and eosin.

Cryostat sections were made from the deep-frozen left lungs of three animals per group (the other three were used for microarray analysis; see below) and from the lavaged right lung lobes. The 7 µm sections of the right lung lobes were stained with hematoxylin and used only for counting the number of small, medium, and large granulomas (Figure 1A). The sections of the three left lungs were stained for epsilon chain of rat IgE (MARE; Oxford Biotechnology, Oxford, UK; 1:500), CD4 (W3/25; Serotec, Oxford, UK; 1:800), CD8 (OX8; Serotec; 1:800), or CD161 (10/78; Serotec; 1:800), using a two-step

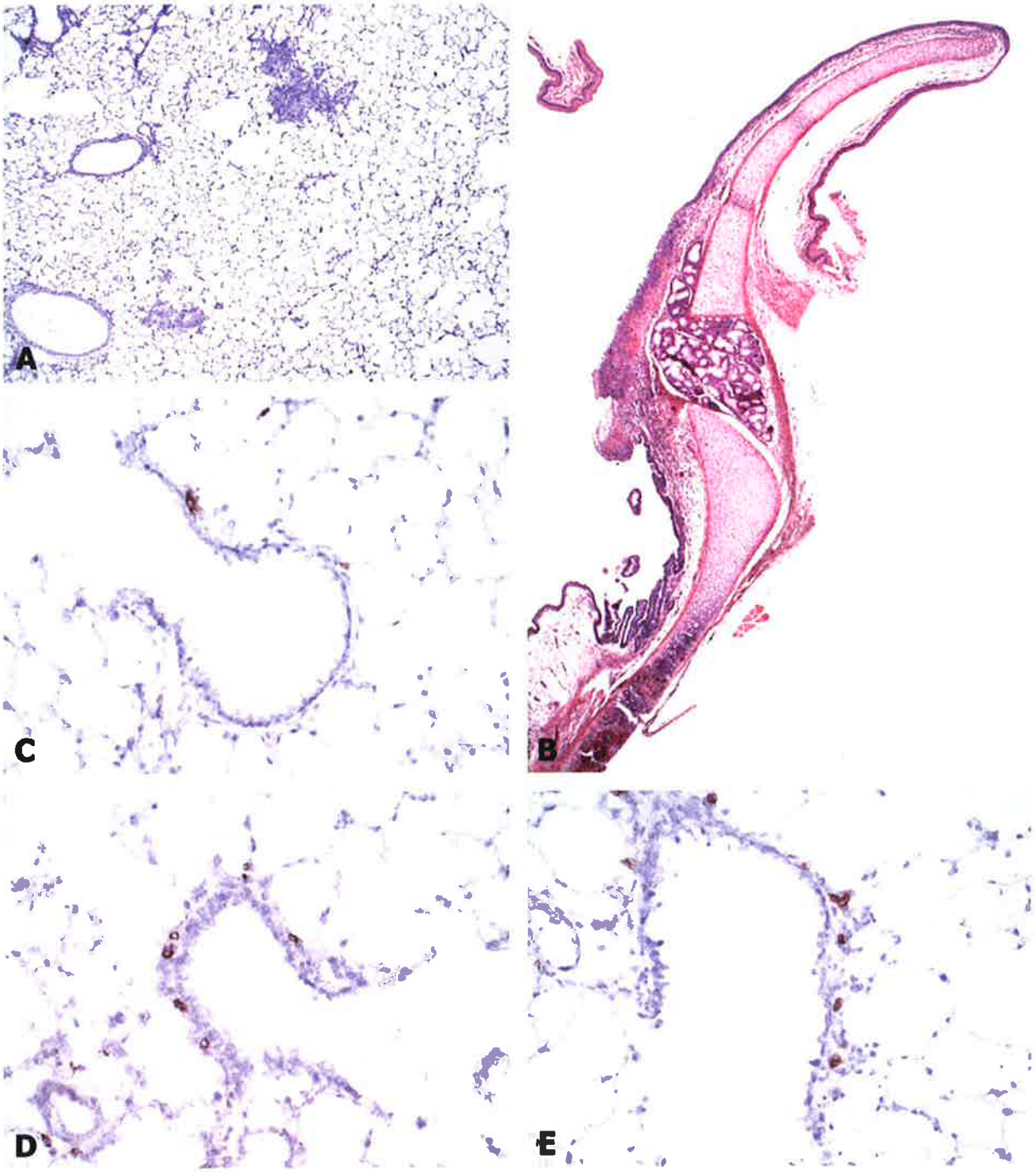

FIGURE 1.—Histopathology and immunohistochemistry. (A) A small and medium granuloma in the lung of a naive (–/–) BN rat. Hematoxylin-stained cryostat section; original magnification x25. (B) Laryngitis at the base of the epiglottis. H&E-stained, paraffin-embedded section; original magnification x25. (C) Subepithelial IgE-positive cell around the bronchioles of a naive (–/–) BN rat. IgE and hematoxylin-stained cryostat section; original magnification x50. (D and E) IgE-positive cells around bronchioles of a sensitized/challenged (+/+) BN rat. IgE and hematoxylin-stained cryostat section; original magnification x50.

indirect immunolabeling. After drying for one night at room temperature, the sections were fixed in cold acetone for 7 minutes at 4°C. Unspecific binding was blocked with 25% normal goat serum in PBS for 15 minutes. The sections were incubated for 60 minutes with the monoclonal antibodies listed above. Control sections were processed in the same way, except for the specific antibodies. For the second step, the sections were incubated with powervision poly HRP-anti-mouse IgG mixed with normal rat serum (100:5, first preincubation for 60 minutes) for 30 minutes. The sections were then treated with the chromogen NovaRed for 8 minutes and were additionally contrasted with hematoxylin for 20 seconds. All incubations were carried out at room temperature in a humid chamber and were followed by a three-times washing step with PBS. Positive cells were counted in the lung parenchyma at 5 square fields per left lung at 250x magnification, using a square grid of 0.4 mm^2, or counted as number of cells per terminal bronchiole transsection in the left lung (5 transsections per lung) and as number of cells per 0.2 mm epithelium of main intrapulmonary bronchioles/bronchi (3 sites per bronchiole).

Microarray Analysis

Total RNA was isolated from frozen left lung tissue of three rats per group, as described previously (Kuper et al. 2008). The RNA was labeled and hybridized to Affymetrix rat 230–2.0 GeneChip, containing probesets for 31,000 rat genes at ServiceXS (Leiden, the Netherlands). Data were loaded into GCOS software (Affymetrix, USA). GCOS data were transferred to GeneSpring (Agilent/Silicon Genetics, USA) for GC-RMA normalization. Log-transformed, normalized data were analyzed using Rosetta Resolver (RosettaBio, USA), Spotfire, and Excel (Microsoft, USA). Data from GCOS were annotated with Gene Ontology (GO) information via the SOURCE Web site (Stanford University, Palo Alto, CA, USA). Automated annotation updates were also performed with BRB ArrayTools. A subset of "expressed genes" was selected by filtering on expression level > 15 in at least three samples (18,624 probesets). To explore the variability in gene-expression groups, the explorative multivariate methods principal components analysis and hierarchical clustering were performed with Spotfire, Rosetta, and GeneSpring (demo version). Selection of differentially expressed genes was done by *t*-tests or by filtering on more than 25% up- or down-regulation in all three rats in a group.

A multiple testing correction (estimation of False Discovery Rate [FDR]) was incorporated into the statistical analysis of the microarray data. An analysis of variance (ANOVA) was performed to test for significant differences in expression among the four treatment groups. In addition, *t*-tests were performed to test for differences among the groups.

The microarray data set was also analyzed on the pathway or gene-group level. To infer gene groups that are significantly co-expressed we used *T*-profiler (Boorsma et al. 2005). *T*-profiler uses the unpaired *t*-test to score changes in average activity of predefined groups of genes. The gene groups are defined on the basis of GO categorization and have to contain at least six gene members (Ashburner et al. 2000; Boyle et al. 2004). *T*-profiler uses the log(2) ratios of gene expression between the treatment and the reference as input. After applying the unpaired *t*-test for each gene group, a p value was calculated from t using the t-distribution with $n - 2$ degrees of freedom, where n is the total number of genes tested. The p value was corrected for multiple testing by multiplying it by the number of gene groups that are being tested in parallel (Bonferonni correction) leading to an E value. To make the procedure more robust against outliers, the highest and lowest value of each gene group was discarded. This is similar to the jackknife procedure. A major advantage of *T*-profiler is that no parameters have to be tuned; the complete transcriptome is used for the calculation. The analysis is supported by the *T*-profiler Web site (http://www.t-profiler.org).

Blood Proteomics

Sample Preparation for Proteomic Analysis: The serum samples, obtained at necropsy, were pooled per group (–/–; +/–; –/+; +/+). Albumine and immunoglobulin G were removed (Albumine en IgG removal kit). Unless indicated otherwise, all reagents and equipment for blood proteomics were obtained from Amersham biosciences (Uppsala, Sweden). Per sample, 37.5 µl of serum was incubated for 1.5 hours at room temperature with 750 µl of antibody affinity resin from the kit. The mixture was then centrifuged for 5 minutes at 11,000 rpm and 4°C. The proteins in the supernatant were precipitated by adding four volumes of ice-cold acetone (1.6 ml). After 2 hours of incubation at –20°C, the mixture was centrifuged at 13,000 rpm and 4°C. The pellet was air dried and dissolved in 100 µl of DIGE buffer (8 M Urea, 4% w/v CHAPS, and 30 mM Tris), and the pH was adjusted to 8.5. The protein content was determined using the modified method of Bradford (Bio-Rad, Veenendaal, the Netherlands). Each sample (200 µg) was labeled with 4 µl of Cy 5 CyDye™ DIGE fluors minimal dye (400 µM). After 30 minutes, the incubation was stopped by adding 1 µl of 10 mM Lysine. The labeled samples were further diluted with an equal volume of 2x sample buffer containing 8 M urea, 4% w/v CHAPS, 130 mM DTT (Sigma), and 2% Pharmalyte™ 3-10. The internal standard included 140 µg of each sample (4 samples in total), labeled with CyDye 5. The two labeled samples were mixed, and the volume was adjusted to 350 µl with rehydration buffer containing 8 M urea, 4% w/v CHAPS, 2 mM tributyl phosphine (Fluka, Buchs SG, Switzerland), and 1% (v/v) immobilized pH gradient (IPG) ampholytes pH 4-7. Per gel, one sample (CyDye 3) and one internal standard (CyDye 5) were run. The gels, sixteen in total (4 per group), were processed in two separate 2-D experiments in which a duplicate of each group was performed simultaneously. The first dimension was carried out on an IPGphor system using pH 4-7 IPG gel strips of 18 cm. The isoelectric focusing was performed at 20°C under the following conditions: 12 hours at 30 V, 30 minutes at 150 V, 1 hour at 300 V, 1 hour at 1,500 V, and 6 hours at 8,000 V. After isoelectric focusing, the IPG strips were equilibrated for 30 minutes in 6 M urea, 30% (v/v) glycerol, 5 mM tributyl

phosphine, and 2% (m/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8. The second dimensional separations were carried out on custom-made 12% SDS-polyacrylamide gels and a Hoefer DALT electrophoresis system.

Gel Image Analysis: The gels were scanned using the Typhoon 9400 laser scanner at two different settings (Cy3, green laser 532 nm and 580 bp 30 filter; Cy5, red laser 633 nm and 670 bp 30 filter). Two images per gel were obtained (32 in total). One gel from group A was rejected and therefore omitted in further analyses. The scanned images were analyzed using Progenesis Workstation 2004 with the special cross-stain analysis module for analyzing multilabeled gels (Nonlinear Dynamics, Newcastle upon Tyne, UK). Spots were automatically detected and visually checked for undetected or incorrectly detected spots. The protein spots detected in each image were automatically linked between the two images per gel. All gels were matched to a digitized reference gel, containing all the protein spots. Per image the intensity levels were normalized by dividing the spot volume through the total intensity of all the spots in the image and multiplying it by the average of the total spot intensity of the gel images. Subsequently, the Cy3-labeled spot volumes were divided by the spot volume of the corresponding protein spot in the internal standard (Cy5) image. The obtained data set containing the expression ratios of each spot in each sample was analyzed by principal components discriminant analysis (PC-DA).

In-Gel Digestion and nLC-MS-MS: Preparative gels were run with 1 mg of protein and stained with RuBPS fluorescent staining. Spots were excised and sliced into small pieces. The gel pieces were washed twice with 100 mM ammonium bicarbonate and acetonitrile. Next, the pieces were dried in a vacuum centrifuge and digested overnight with 25 ng/µl trypsin (sequencing grade, Promega Benelux, Leiden, the Netherlands) in 100 mM ammonium bicarbonate and 2 mM dithiothreitol at 37°C. The peptide fragments were extracted twice with 5 µl water:acetonitrile:formic acid (5:14:1). After drying in a vacuum centrifuge, the lyophilized digest was dissolved in 25 µl of 4 M Urea buffered at pH 8.0 with 25 mM Tris. Nanobore chromatography was performed on an Ultimate nano LC system from LC Packings (Amsterdam, the Netherlands). Ten microliters of the peptide mixture was injected on a 300 µm ID X 0.5 mm Pepmap C_{18} trap column (LC Packings) and washed at 30 µL/min for 10 minutes with 0.05% trifluoroacetic acid in water before the RP trap was switched online in back-flush mode to a 75 µm X 150 mm Pepmap C_{18} nano LC column. Gradient elution of peptides was achieved at 300 nL per minute going from 95% mobile phase A (water:acetonitrile:formic acid, 97.9:2:0.08 v/v/v) and 5% mobile phase B (water:acetonitrile:formic acid, 19.9:80:0.1 v/v/v) to 45% B in 35 minutes and then to 60% B in 10 minutes. The nano LC column was coupled to a LCQ DECA ion trap MS (Thermo Electron, San Jose, CA, USA) via a nanoelectrospray interface from Proxeon (Odense, Denmark). Electrospray was performed by applying 1.3 kV to the electrospray pico tip (20 µm ID, 10 µm tip ID, distal coated from New Objective, Cambridge, MA, USA) via a Pt wire; ions were introduced in the mass spectrometer through a heated capillary kept at 180°C. The ion trap was operated in data-dependent mode, selecting top two ions for MS/MS scans at 35% collision energy units. Protein identification was performed by searching MS/MS spectra against the Swissprot database using Mascot search engine (www.matrixscience.com). Search parameters used were as follows: peptide mass tolerance 0.8 Da; MS/MS tolerance 1.2 Da; allowed missed cleavages 1; enzyme trypsin; taxonomy rattus; fixed modification, carbamidomethyl (C) variable modifications, oxidation (M), deamidation (NQ).

Statistics and Data Analysis

Body weights were analyzed by one-way analysis of covariance, followed by the two-sided Dunnett's multiple comparison test. Organ weights, immunoglobulin levels, BAL biochemical parameters, and absolute cell numbers were determined by Anova-Dunnett's test. Relative cell numbers in BAL were analyzed by Kruskall-Wallis nonparametric ANOVA followed by a Mann/Whitney *U* test. Differences were considered statistically significant if $p < .05$. Analyses were performed by the usage of Graphpad Prism (Version 3.0, San Diego, CA, USA).

Statistical analysis of the microarray data are described under "microarray analysis." The statistics and multivariate data analysis of the proteomics data were as follows: The differences in spot ratios were analyzed by ANOVA followed by Student's *t*-test, using Excel (Microsoft, Redmond, WA, USA) and PC-DA (PLS toolbox 3.0.2; 2003, Eigenvector Research in Matlab; Version 7.0, R14; Service Pack 1; 2004, The MathWorks).

RESULTS

Clinical Signs, Body and Organ Weights, and Lung Function

Distinct clinical signs were absent. All animals gained approximately 27 grams between days 0 and 21; from day 21 to 22, animals of the sensitized/challenged (+/+) group showed a statistically significantly reduced body weight when compared with controls (Table 2). The weights of kidneys and liver were unaffected (data not shown). The mean absolute and relative weights of the left lung were increased by the challenge: the challenged groups (–/+ and +/+) differed statistically significantly from the unchallenged groups (–/– and +/–; Table 2). There was no additional effect of sensitization: the mean weights of the –/+ group did not differ significantly from that of the +/+ group.

TMA challenge in sensitized rats induced apneic periods—that is, irregularly lengthened pauses between varying number of breaths. Breathing frequency decreased to 50% of the original rate, starting within 1 or 2 minutes of the challenge, and returned to normal after the challenge but was increased at 24 hours after challenge (data not shown).

TABLE 2.—Body and left lung weights.

Group[a]	Body weight, day 22 (g)	Left lung (g)	Left lung (g/kg BW)
–/–	171.1 ± 1.4	0.51 ± 0.02	2.96 ± 0.08
+/–	175.4 ± 1.0	0.43 ± 0.02	2.47 ± 0.14
–/+	170.5 ± 2.4	0.57 ± 0.05[&&]	3.35 ± 0.35[&&]
+/+	166.4 ± 3.9[*]	0.64 ± 0.03[#,&&]	3.84 ± 0.11[#,&&]

[a] Six female BN rats per group.
[*]$p < .05$. Analysis of covariance and Dunnett's tests (two-sided): +/+ compared with –/–.
[#]$p < .05$. Analysis of variance and Dunnett's tests (two-sided), +/+ compared with –/–.
[&&]$p < .01$. Brown-Forsythe analysis of variance.

TABLE 3.—Cell counts in bronchoalveolar lavage.

Group[a]	Total cells (x10^6)	Eosinophils (x10^6)	Neutrophils (x10^6)	Lymphocytes (x10^6)	Macrophages (x10^6)
–/–	2.73 ± 0.20	0.75 ± 0.16	0.04 ± 0.01	0.05 ± 0.01	1.90 ± 0.07
+/–	1.52 ± 0.33[#]	0.31 ± 0.18	0.01 ± 0.01	0.02 ± 0.01	1.17 ± 0.15
–/+	3.08 ± 0.29	0.39 ± 0.17	0.05 ± 0.03	0.05 ± 0.02	2.58 ± 0.56[&&]
+/+	8.68 ± 1.56[##]	4.90 ± 1.16[**]	0.65 ± 0.13[**]	0.10 ± 0.03	3.12 ± 0.38[*,&&]

[a] Six female rats per group.
[*]$p < .05$. [**]$p < .01$. Analysis of variance and Dunnett's tests (two-sided).
[#]$p < .05$. [##]$p < .01$. Kruskall-Wallis analysis of variance and Mann-Whitney *U*-tests (two-sided).
[&&]$p < 0.01$. Brown-Forsythe analysis of variance.

Serum and Lung Homogenate IgE Levels

Serum IgE levels were markedly increased in the sensitized (+/– and +/+) groups (4215.7 ± 616.6 and 2948.6 ± 532.4 µg/ml, respectively) when compared with the unsensitized (–/– and –/+) groups (346.4 ± 79.6 and 405.6 ± 116.8 ng/ml, respectively). IgE in homogenate of unlavaged left lung tissue was increased in the sensitized/challenged (+/+) group, compared with the unsensitized/challenged (–/+) group (Figure 2).

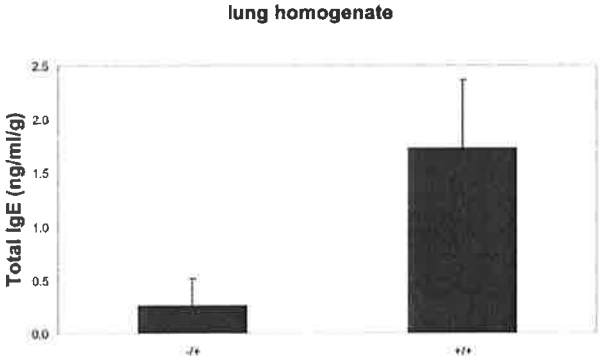

FIGURE 2.—Total IgE in lung homogenate of the unsensitized/challenged (–/+) group and the sensitized/challenged (+/+) group.

BAL

There was a slight reduction in total cell counts in the sensitized/unchallenged (+/–) group. The mean total cell number in BAL in the sensitized/challenged (+/+) group was about three times higher than that in the other three groups (Table 3). The increased total cell number in the +/+ group was largely due to an increase in the number of eosinophils and macrophages; the number of neutrophils was also increased. In the unsensitized/challenged (–/+) group, the total cell number tended to increase, and the increase was due to an increase in the number of macrophages. The increase in macrophages in the challenged (+/+ and –/+) groups was due to the challenge, and there was no additional effect of sensitization (the –/+ group did not statistically significantly differ from the +/+ group; Table 3).

Histopathology and Immunohistochemistry

The sensitized/challenged (+/+) rats exhibited a moderate laryngitis (Figure 1B), consisting of mucosal aggregates of predominantly macrophages with varying numbers of eosinophils and lymphocytes, with epithelial damage. No such changes were observed in rats of the three control groups. Histopathological changes were not observed in the nasal passages and trachea. The granulomatous inflammation in the lungs, specific for BN rats (Germann et al. 1998), did not differ between the groups but was slightly more pronounced in two naive (–/–) animals (animal nos. A1 and A3) and one sensitized/unchallenged (+/–) animal (animal no. B21).

Counts of immunohistochemically stained lung sections revealed an increase in IgE- and CD4-positive cells in the lung parenchyma of sensitized/challenged (+/+) rats (Table 4). The IgE staining was mostly membrane bound, and the cytology of these cells resembled that of lymphocytes (i.e., small, round cells). They were, therefore, considered to be predominantly B cells. In addition, there was an increase in IgE-positive cells around terminal bronchioles of sensitized/challenged (+/+) rats (Table 4; Figures 1C–E). Their staining was membrane bound or cytoplasmic, and they were considered to be B cells and plasma cells. The CD8, CD161 counts did not show differences between the groups (data not shown).

Microarrays

Data Exploration and Differentially Expressed Genes–Single Gene Comparison: Principal components analysis and hierarchical clustering showed a separation of the sensitized/challenged (+/+) group from the other three groups. Figure 3 depicts a part of the dendrogam (related to inflammation) after hierarchical clustering and illustrates the marked response in lung gene expression of the sensitized/challenged (+/+) group compared with the three control groups, despite the variability in granulomatous inflammation in the lungs, which was slightly more pronounced in animals A1, A3, and B21. Single-gene comparison between the groups (at a FDR threshold of 10%) resulted in the sensitized/challenged (+/+) group in a high number of differentially expressed genes above the number of differentially

TABLE 4.—Number of immunohistochemically stained cells in lung parenchyma[a] or bronchi/bronchioles.[b]

Group[c]	IgE+ cells bronchial epithelium	IgE+ cells terminal bronchioles	IgE+ cells in lung parenchyma	CD4+ cells in lung parenchyma
–/–	15.2 ± 5.2	1.1 ± 0.5	3.7 ± 1.2	2.1 ± 0.8
+/–	12.7 ± 5.6	1.3 ± 0.3	5.8 ± 0.7	3.5 ± 0.8
–/+	17.5 ± 1.5	0.5 ± 0.5	2.9 ± 1.5	2.7 ± 0.7
+/+	15.3 ± 3.3	8.7 ± 3.2$^{\#\#}$	10.9 ± 2.3*	8.1 ± 1.4**

[a] Mean number of positive cells per square field of 0.4 mm^2 lung parenchyma ± sem. For each animal, five square fields were counted per left lung at 250x magnification.

[b] Mean number of cells per 0.2 mm epithelium (main intrapulmonary bronchioles/bronchi; 3 sites per animal), or mean number of cells per terminal bronchiole transsection in the left lung (5 transsections per animal).

[c] Three animals per group.

$^{\#\#}p < .01$. Log transformation. Allergy effect: sensitized and challenged (+/+) versus the three control (–/– , +/–, and –/+) groups.

$^{*}p < .05$. $^{**}p < .01$. Analysis of post hoc Dunnet's variance test. Allergy (+/+) versus naive (–/–) group. No statistical differences between –/–, +/–, or +/– groups.

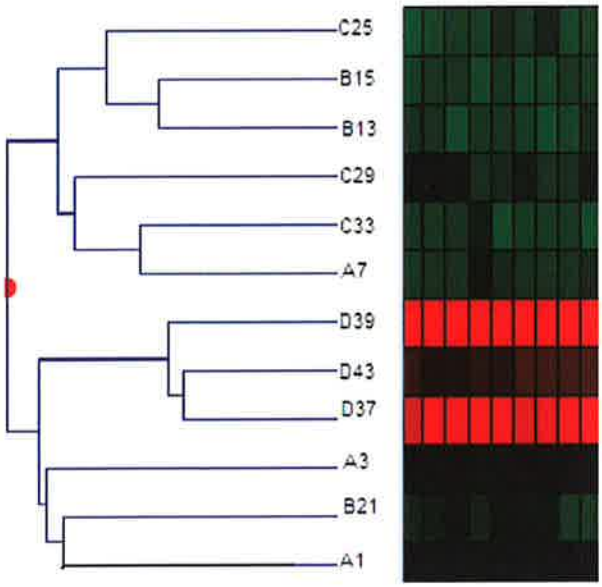

FIGURE 3.—Dendrogram of microarray analysis of lung tissue, which visualizes the clustering based on correlations between the animals (vertically) and a few genes related to inflammation (horizontally). Challenge after sensitization (+/+ group; group D animals) elicited a marked response in these genes. Gene-expression pattern is slightly different in two naive animals (–/–; A1 and A3) and one sensitized/unchallenged animal (+/–; B21), due to background granulomatous inflammation.

expressed genes by chance (Table 5). The genes expressed most distinctly in the sensitized/challenged group are listed in Table 6. These genes are associated with inflammation, including extracellular remodeling, as well as inflammation inhibition, but none of them is directly related to IgE. The effect of sensitization—that is, the number of genes expressed differently between the naive (–/–) and the dermally sensitized (+/–) group was low, if any, as was the number of genes expressed differently between the naive (–/–) and unsensitized/challenged (–/+) group. The last group reflects the possible toxic or irritating effects of the TMA challenge as well as the very early stage of sensitization by inhalation. Genes in this group were mostly down-regulated, but their differential expression was low. The mean fold changes were highest for the chemokines Ccl4, Ccl17, and Ccl20.

TABLE 5.—Number of differentially expressed genes in left lung tissue, 24 hours after challenge with TMA.

Group comparison[a]	$p < .01$	$p < .001$
D–A		
+/+ vs. –/–	668	70
D–C		
+/+ vs. –/+	1,122	143
B–A		
+/– vs. –/–	110	10
C–A		
–/+ vs. –/–	119	7

[a] Three female rats per group; The number of differentially expressed genes by chance is 186 at $p < .01$.

Gene Group Analysis (T-Profiler): Seven GO groups/pathways of genes were expressed highly significantly different between the +/+ group and the other three groups (Table 7). The GO gene groups/pathways were "chemokine activity," "chemotaxis," "inflammatory response," "extracellular space," "extracellular region," "cytokine-cytokine receptor interaction," and "Toll-like receptor signaling pathway" (Figure 4). The GO gene groups/pathways were all up-regulated and had a *t* value of about 6.00 or more and *e* values of < .0001 in the +/+ group. In the +/– and –/+ group, most of these GO gene groups/pathways tended to be down-regulated; the only GO gene pathway that was significantly up-regulated was the Jak-STAT signaling pathway in the –/+ group.

Blood Proteomics

In total, 360 different spots were detected on each internal standard gel. The score plot showed a distinct protein pattern between the groups, with the –/– (A) and +/– (B) groups placed close to each other in the score plot. The +/+ (D) group is placed at a greater distance. Sixteen proteins were differentially expressed between the +/+ group and the three control groups (ANOVA followed by Student's *t*-test) and were selected for identification. Figure 5 shows the differences between the expression levels of the sixteen proteins, as represented by the mean spot-intensity ratios on the DIGE gels. All spots could be identified, demonstrating six proteins (Table 8). Spots 660, 662, 665, 697, and 705 were found to consist of two

TABLE 6.—Differentially expressed genes in lung tissue, 24 hours after challenge with TMA, of sensitized/challenged (+/+; allergic) rats.

Description	Gene name	Accession no.	GO gene group/pathway	Mean log2 ratio (+/+ vs. –/+)
Strongly expressed genes[a]				
Cytokine (chemokine)-monocyte chemotactic protein 3	Ccl7/MCP3	BF419899	Chemokine activity; extracellular space; inflammatory response	8.20*
Cytokine (chemokine)-macrophage inflammatory protein 1beta	Ccl4/MIP 1beta	U06434	Chemokine activity; extracellular space; inflammatory response; Toll-like receptor signaling; cytokine-cytokine receptor interaction	8.07***
Cytokine (chemokine)-monocyte chemotactic protein 1	Ccl2/MCP1	NM_03153	Chemokine activity; extracellular space; inflammatory response; cytokine-cytokine receptor interaction	7.74*
Cytokine (chemokine)	Cxcl11	BF281987	Chemokine activity; Toll-like receptor signaling; immune response; cytokine-cytokine receptor interaction	7.24*
Immune response/inhibitor-serine protease inhibitor 2c	Spin2c	NM_03153		6.43***
Cytokine (chemokine)	Ccl17/TARC	NM_05715	Chemokine activity; immune response; cytokine-cytokine receptor interaction	5.46*
Cytokine (chemokine)-macrophage inflammatory protein 1alpha	Ccl3/MIP1alpha	U22414	Chemokine activity; extracellular space; Toll-like receptor signaling; cytokine-cytokine receptor interaction	5.36***
Tissue factor pathway inhibitor 2	Tfpi2	AL179507		5.30***
Cytokine (interleukin)	IL-6/Ifnb2	NM_01258	Extracellular space; Toll-like receptor signaling; cytokine-cytokine receptor interaction; Jak-STAT signaling	5.26*
Cytokine (chemokine)-macrophage inflammatory protein 2	Cxcl2/MIP2	NM_05364	Chemokine activity; extracellular space; cytokine-cytokine receptor interaction	4.87**
Cytokine (chemokine)	Ccl22/MDC	AF163477	Chemokine activity; immune response; cytokine-cytokine receptor interaction	4.86*
Ubiquitin D	Ubd	NM_05329		4.77*
Interferon gamma inducible gene	Pumag	BL296811		4.56*
Prostaglandin-endoperoxidase synthase 2	Ptgs2/Cox2	U03389		4.49*
Tissue inhibitor of metalloproteinase 1	Timp1	NM_05381		3.56***
Cytokine (chemokine)	Ccl20/MIP3alpha	AF053312	Chemokine activity; extracellular space; inflammatory response; cytokine-cytokine receptor interaction	3.51**
Cytokine receptor (interleukin receptor)	IL-13ra2	NM_13353	Cytokine-cytokine receptor interaction; Jak-STAT signaling	3.08*
Metabolism-Arginase 1	Arg1	NM_017134		3.01***
Six transmembrane epithelial antigen of the prostate (predicted)	—	BE109939		2.56***
Cytokine (interleukin)	IL-1beta	NM_031512	Extracellular space; Toll-like receptor signaling; cytokine-cytokine receptor interaction	2.52***
Angiopoietin-like protein 4	Angptl4	AA818262		2.51**
Slightly/moderately expressed genes[b]				
Superoxide dismutase 2, mitochondrial	Sod2	AA892254		2.44***
Superoxide dismutase 2, mitochondrial	—	BG671549		2.39***
Protein C receptor, endothelial (predicted)	—	Al137406		2.30***
Cell adhesion-Chitinase 3-like 1 (cell adhesion)	Chi3l1	AA945643		2.09***
Inhibitor of apoptosis protein 1	Birc3	NM_023987		1.88***
Rho family GTPase 1 (predicted)	Rnd1	Al144754		1.85***
Solute carrier family 39 (metal ion transporter) member 8 (predicted)	—	BM388442		1.70***
Amiloride binding protein 1	Abp	NM_022935		1.56***
Glial fibrillary acidic protein	GFAP	NM_01700		1.56***
Inflammation, attractant for neutrophils-Gene model 1960 (NCBI)	Gm1960	D87927		1.54***

[a] Mean fold change > 2.50; significance of +/+ versus –/+ and versus all three groups together at $p < .05$, $p < .01$, or $p < .001$.
[b] Mean fold change 1.50–2.50; significance of +/+ versus –/+ and versus all three groups together at $p < .001$.
$^{*}p < .05$. $^{**}p < .01$. $^{***}p < .001$.

TABLE 7.—*T* values of differentially expressed groups of genes (Gene Ontology) in left lung tissue of BN rats treated with TMA.

Gene Ontology pathway	+/+ vs. –/– D–A	+/+ vs. +/– D–B	+/+ vs. –/+ D–C	+/– vs. –/– B–A	+/– vs. –/+ B–C	–/+ vs. –/– C–A
Chemokine activity	11.00***	14.87***	17.11***	–6.20**	–1.96	–6.24**
Chemotaxis	7.10**	8.08***	8.87***	–1.73	0.79	–1.56
Extracellular space	6.44***	10.40***	10.95***	–5.90**	0.75	–4.62*
Extracellular region	5.81**	8.74***	8.87***	–2.44	0.86	–3.36
Cytokine-cytokine receptor interaction	9.18***	9.63***	10.08***	–0.40	–1.01	0.28
Inflammatory response	7.01***	9.67***	9.77***	–4.69*	2.22	–2.41
Toll-like receptor signaling pathway	6.95***	6.73***	7.56***	0.82	–0.76	0.54
Immune response	5.65***	6.65***	7.33***	–1.73	–0.38	–1.16
Jak-STAT signaling	6.67***	4.00*	3.44	3.85*	–2.00	4.72**

*E value < .05. **E value < .001. ***E value < .0001.

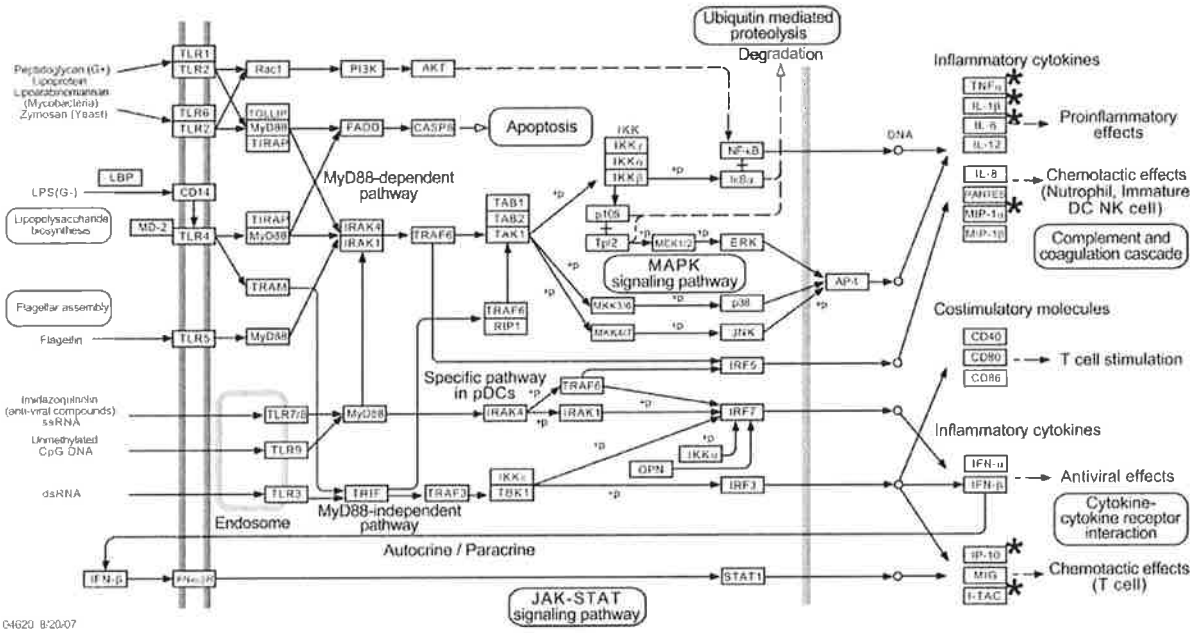

FIGURE 4.—Toll-like receptor signaling pathway from *Kyoto Encyclopedia of Genes and Genomes* (www.genome.ad.jp/kegg; Kanehisa et al. 2006). The genes that were up-regulated significantly in the sensitized/challenged group, when compared with the three control groups, are marked with an asterisk.

proteins, Serine-like protease inhibitor 6 and Alpha-1 antiproteinase. The individual contribution of the two proteins to the spot intensity was not investigated. Many spots were spot trains, which indicates posttranslational modifications like phosphorylation or glycosylation (Swissprot database). This could also explain the difference between experimental and theoretical molecular mass.

DISCUSSION

To contribute to the hazard identification of LMW respiratory allergens, respiratory allergy induced by TMA in the Th2-prone BN rat was characterized by whole genome analysis (microarrays) of lung tissue and proteomics in blood. In confirmation with previous studies, TMA-induced apneas and allergic laryngitis, increased inflammatory cell numbers in BAL, altered breathing frequency, and elevated IgE levels in serum and lung (Arts et al. 1998; Arts et al. 2004; Valstar et al. 2006).

The identification of inflammatory changes in the bronchial or bronchiolar mucosa and lung parenchyma of the allergic (sensitized/challenged or +/+) BN rats was hampered by the variable background inflammation in the lungs of BN rats (see Results section; Arts et al. 1998; Germann et al. 1998). This is reflected by the interanimal variability in expression of a number of

TABLE 8.—List of proteins in peripheral blood, differentially expressed in sensitized/challenged (+/+; group D) rats compared with rats from the other groups (–/– or A; +/– or B; –/+ or C).

Spot no.	Protein identification	Accession no.	Experimental MW	Experimental Ip	Theoretical MW	Theoretical Ip	Peptides identified	Coverage (%)	Total score[a]
551	T-kininogen I	P01048	65.4	4.9	45.8	5.9	5	10	207
566	T-kininogen I	P01048	64.4	5	45.8	5.9	5	12	266
572	T-kininogen II	P08932	64.3	4.8	47.8	6.1	7	16	268
578	T-kininogen II	P08932	63.9	4.8	47.8	6.1	9	18	351
579	T-kininogen II	P08932	63.2	4.9	47.8	6.1	9	21	316
588	Serine-like protease inh 3	P05544	64.4	4.7	43.5	5.6	4	13	206
660	Alpha-1antiproteinas	P17475	55.7	4.6	43.7	5.6	9	22	357
	Serine-like protease inh 6	P09006	55.7	4.6	43.6	5.6	8	22	331
662	Alpha-1antiproteinas	P17475	55.9	4.6	43.7	5.6	4	9	109
	Serine-like protease inh 6	P09006	55.9	4.6	43.7	5.6	7	21	367
665	Alpha-1antiproteinas	P17475	55.5	4.7	43.7	5.6	4	17	229
	Serine-like protease inh 6	P09006	55.5	4.7	43.6	5.6	8	25	444
697	Alpha-1antiproteinas	P17475	53.7	4.7	43.7	5.6	9	23	312
	Serine-like protease inh 6	P09006	53.7	4.7	43.6	5.6	15	35	704
705	Alpha-1antiproteinas	P17475	52.7	4.8	43.7	5.6	4	13	212
	Serine-like protease inh 6	P09006	52.7	4.8	43.6	5.6	5	15	221
827	haptoglobin	P06866	37.4	6.1	38.6	6.1	2	4	66
830	haptoglobin	P06866	36.9	5.1	38.6	6.1	3	6	76
834	haptoglobin	P06866	36.4	5.3	38.6	6.1	6	15	203
837	haptoglobin	P06866	35.8	5.5	38.6	6.1	8	21	274
1005	Ig kappa chain C region, B allele	P01835	26.6	4.7	11.8	5.0	1	13	182

Principal components discriminant analysis and analysis of variance ($p < .05$), followed by Student's t-test ($p < .05$).

[a] Total score is the sum of the individual peptide scores. Individual peptide score > 39 indicate identity or extensive homology ($p < .05$) according to mascot database search program.

inflammatory-associated genes in the lung (Figure 3). Nevertheless, a single TMA challenge in TMA-sensitized animals (+/+) led to a strong differential expression of single genes and GO gene groups/pathways in lung tissue (Tables 6–8).

There was a considerable consistency between the genes significantly up-regulated in the TMA-sensitized and TMA-challenged BN rats in the present study and data found in asthmatic humans: almost all cytokines, for which the genes were up-regulated in BN rats (Table 6), have been found in increased amounts in sputum, BAL, and exhaled air of asthmatic patients (reviewed by Bloemen et al. 2007). Moreover, the Toll-like receptor pathway, activated in the sensitized/challenged (+/+) BN rats, is considered to be a pathway that is commonly activated in a number of inflammatory conditions, including asthma, in man (Bauer, Hangel, and Yu 2007). However, of the list of about 100 genes associated with asthma in man (Ober and Hoffjan 2006), only Timp1 and Ccl2/MCP1 were up-regulated sufficiently in the BN rats to be included in Table 6.

Interestingly, a single challenge by TMA in the sensitized rats was sufficient to activate genes related to lung remodeling, which is featured by smooth muscle hypertrophy, transformation of fibroblasts into myofibroblasts, angiogenesis, and deposition of subepithelial collagen (Holgate et al. 2000). This early onset of lung remodeling is in agreement with the observation in man that remodeling is a consistent component of early asthma, rather than being a secondary phenomenon, developing late in the disease. Remodeling changes might even be of higher diagnostic value than eosinophilic infiltration (Davies et al. 2003). The most strongly up-regulated genes in the BN rat, involved in remodeling, were for arginase and glial fibrillary acid protein, which may lead to local synthesis of collagen (Zimmermann et al. 2003) and is linked to transformation of fibroblasts into myofibroblasts (Zhao and Burt 2007), respectively. In the present study there was indirect evidence for involvement of MMP9, namely, strong up-regulation of IL-1beta and IL-6, which are able to stimulate MMP-9 secretion (Lin et al. 2007), and the presence of high numbers of eosinophils in BAL, which are a source of MMP9 (Kay, Phipps, and Robinson 2004; Trivedi and Lloyd 2007). MMP9 selectively degrades extracellular matrix components (ECM) like type IV and V collagen (Vignola et al. 2003). An increase in MMP9 has been demonstrated in patients with acute severe asthma or following allergen challenge (Belleguic et al. 2002), supporting the idea that ECM and basement membrane degeneration by MMP's and other proteinases may occur in the early phase of asthma. In addition, the GO pathways "extracellular space" and "extracellular region" were highly significantly expressed in the sensitized/challenged (+/+) rats, compared with the three control groups. The contact allergen

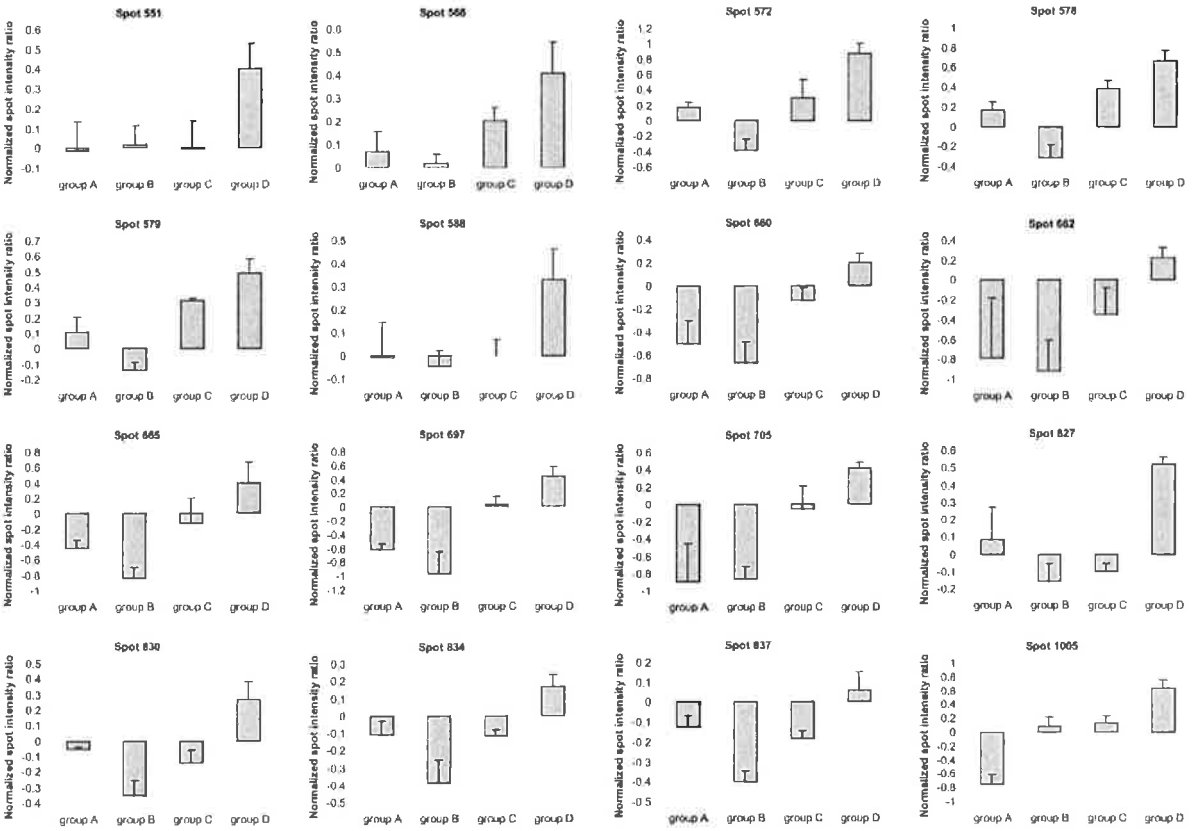

FIGURE 5.—Expression ratios of the protein spots, which showed differential expression between sensitized/challenged (+/+; group D) rats and the three control groups (–/– or naive group A; +/– or sensitized/unchallenged group B; –/+ or unsensitized/challenged group C; PC-DA, ANOVA $p < .05$, followed by Student's t-test $p < .05$). The expression levels are given as ratios (spot-intensity sample/spot-intensity internal standard on the DIGE gels). The ratios are expressed as the mean of four expression ratios ± *SD* (except group A, $n = 3$). The proteins represented by the spots are described in Table 8.

dinitrochlorobenzene, tested according to an identical protocol, induced expression changes in a few allergy-associated genes for chemokines but did not up-regulate any of the genes for lung remodeling (Kuper et al. 2008). Thus, early lung remodeling may be useful in identification of compounds, which can induce asthma.

The single challenge with TMA in sensitized BN rats not only induced lung inflammation and lung remodeling but also activated mechanisms that down-regulate these processes. The increased IMP-1 production may have accompanied the increase in MMP to inhibit ECM degradation. Genes for serine protease inhibitors, especially 2c, were up-regulated as well. Interestingly, 24 hours after challenge, serine protease inhibitor 3 and 6 proteins were markedly increased in peripheral blood of the TMA-sensitized and TMA-challenged rats. Serine-like protease inhibitor 6 has also been found in the BAL of BN rats, 24 hours after intratracheal exposure to lipopolysaccharide (Signor et al. 2004). The serine-like protease inhibitors are considered to be involved in tissue repair and directed to down-regulate inflammation (Ohkubo et al. 1991). It should be kept in mind as well that respiratory allergy-associated molecules may have dual actions–that is, stimulation but also inhibition of allergic manifestations. For example, IL-1beta can induce NF-kappaB, which, under certain circumstances, inhibits myofibroblast phenotype (Kuang et al. 2007), whereas IL-6 can act as an anti-inflammatory agent. Together, it supports the idea that sustained or chronic allergic diseases result not only from excessive or deranged activation of lung cells but also from deficiencies at the level of suppressing molecules (Ravetch and Lanier 2000).

The development of respiratory allergy, especially when induced by HMW allergens, is considered to be regulated predominantly by Th2-polarized immune reactions in which IgE plays a dominant role (reviewed by Boverhof et al. 2008). With TMA-induced respiratory allergy in BN rats, several parameters pointed to such a Th2-polarized mechanism. First, the increases in eosinophils in BAL, CD4+ lymphocytes in lung parenchyma, IgE+ cells in lung parenchyma and around terminal bronchioles, and in total IgE in serum and lung (and BAL;

Valstar et al. 2006) are associated with Th2-mediated immune responses. Second, the strongly up-regulated gene for arginase-1 is thought to be the result of activation by the Th2-related cytokines Il-4 and Il-13 (Zimmermann et al. 2003), and it indicates that macrophages are activated via the alternative pathway (Mantovani et al. 2004). Macrophages, activated via this pathway, are involved in Th2-mediated allergy and are associated with changes in gene-expression profiles for the chemokines Ccl2, Ccl3, Ccl4, Ccl17, and Ccl22, which are also strongly up-regulated in the TMA-sensitized and TMA-challenged BN rats (Table 6). Such changes are not associated with classical macrophage activation. Classical activation of macrophages probably occurs in the unsensitized/challenged (–/+) BN rats. These animals also exhibited increased numbers of macrophages in BAL, albeit slightly less than the sensitized/challenged animals, but the above-mentioned genes were down-regulated, if at all. Arginase-1 is also linked with STAT6-dependent pathways, which are considered to be crucial in the induction of asthma (Zimmerman et al. 2004).

Although the GO groups/pathways for chemokines were highly significant in the sensitized/challenged rats when compared with the three control groups, no differential expression was observed of genes for those chemokines that are being used in cytokine profiling tests to try to distinguish typical respiratory from typical contact allergens (Dearman, Skinner, and Humphreys 2003) or those directly related to the IgE-cascade of events, including the Fc receptors for IgE that are determinants of the allergic reaction (Kraft and Novak 2006). This is probably related to the time of the tissue sampling—that is, 24 hours after challenge.

Whole genome analysis of lung tissue from TMA-sensitized and TMA-challenged BN rats provided a host of information, which at present cannot be put entirely in proper perspective with respect to chemical respiratory allergy. Nevertheless, it is clear that a single TMA challenge provokes a predominantly Th2-mediated gene profile in the lungs of TMA-sensitized BN rats. The gene-expression profile in lung tissue of the sensitized/challenged rats, 24 hours after challenge, is in agreement with the pulmonary inflammation—that is, increases in eosinophils, CD4+ lymphocytes, and activation of macrophages via the alternative pathway. In addition, the gene-expression profile points to early activation of lung remodeling. At the same time, inflammation downsizing mechanisms were activated, which is reflected by proteomics in peripheral blood. It is concluded that parameters for lung remodeling may not only be of diagnostic value for early asthma in humans but also be a useful tool for the hazard identification of respiratory allergens in test models.

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References

Alarie, Y. (1973). Sensory irritation by airborne chemicals. *CRC Crit Rev Toxicol* **2,** 299-363.

Ashburner, M., Ball, C.A., Blake, J.A,, Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., and Eppig, J.T. (2000). Gene Ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nature Genet.* **25,** 25-29.

Arts, J. H. E., De Koning, M. W., Bloksma, N., and Kuper, C. F. (2004) Respiratory allergy to trimellitic anhydride in rats: Concentration-response relationships during elicitation. *Inhal Toxicol* **16**, 1–11.

Arts, J. H. E., Dröge, S. C. M., Spanhaak, S., Bloksma, N., Penninks, A. H., and Kuper, C. F. (1997). Local lymph node activation and IgE responses in Brown Norway and Wistar rats after dermal application of sensitizing and non-sensitizing chemicals. *Toxicology* **117**, 229–37.

Arts, J. H. E., and Kuper, C. F. (2007). Animal models to test respiratory allergy to low molecular weight chemicals: A guidance. *Methods* **41**, 61–71.

Arts, J. H. E., Kuper, C. F., Spoor, S., and Bloksma, N. (1998). Airway morphology and function of rats following dermal sensitization and respiratory challenge with low molecular weight chemicals. *Toxicology* **152**, 66–76.

Ban, B., and Hettich, D. (2001). Relationship between IgE positive cell numbers and serum total IgE levels in mice treated with trimellitic anhydride and dinitrochlorobenzene. *Toxicol Lett* **118**, 129–37.

Bauer, X., Czuppon, A. B., Rauluk, I., Zimmermann, F. B., Schmitt, B., Egen-Korthaus, M., Tenkhoff, N., and Degens, P. O. (1995). A clinical and immunological study on 92 workers occupationally exposed to anhydrides. *Int. Arch Occup Environ Health* **67**, 395–403.

Bauer, S., Hangel, D., and Yu, P. (2007). Immunobiology of toll-like receptors in allergic disease. *Immunobiology* **212**, 521–33.

Bauer, X., Marek, W., Ammon, J., Czuppon, A. B., Marczynski, B., Raulf-Heimsoth, M., Roemmelt, H., and Fruhmann, G. (1994). Respiratory and other hazards of isocyanates. *Int Arch Occ Environ Health* **66**, 141–52.

Belleguic, C., Corbel, M., Germain, N., Lena, H., Boichot, E., Delaval, P. H., and Lagente, V. (2002). Increased release of matrix metalloproteinase-9 in the plasma of acute severe asthmatic patients. *Clin Exp Allergy* **32**, 217–23.

Bloemen, K., Verstraelen, S., Van den Heuvel, R., Witters, H., Nelissen, I., and Schoeters, G. (2007). The allergic cascade: Review of the most important molecules in the asthmatic lung. *Immunol Lett* **113**, 6–18.

Boorsma, A., Foat, B.C., Vis, D., Klis, F., and Bussemaker, H.J. (2005). T-profiler: scoring the activity of predefined groups of genes using gene expression data. *Nucleic Acid Res* **33,** W592-W595.

Boverhof, D. R., Dillington, R., Bhaskar Gollapudi, B., Hotchkiss, J. A., Krieger, S. M., Poole, A., Wiescinski, C. M., and Woolhiser, M. R. (2008). Respiratory sensitization and allergy: Current research approaches and needs. *Toxicol Appl Pharmacol* **226**, 1–13.

Boyle, E.I., Weng, S., Gollub, J., Jin, H., Botstein, D., Cherry, J.M., and Sherlock, G., (2004). GO: TermFinder - open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics* **20,** 3710-3715.

Davies, D. E., Wicks, J., Powell, R. M., Puddicombe, S. M., and Holgate, S. T. (2003). Airway remodeling in asthma: New insights. *J. Allerg Clin Immunol* **111**, 215–26.

Dearman, R. J., Skinner, R. A., and Humphreys, N. J. (2003). Methods for the identification of chemical respiratory allergens in rodents: Comparisons of cytokine profiling with induced changes in serum IgE. *J Appl Toxicol* **23**, 199–207.

Dearman, R. J., Warbrick, E. V., Skinner, R., and Kimber, I. (2002). Cytokine fingerprinting of chemical allergens: Species comparisons and statistical analyses. *Fd. Chem Toxicol* **40**, 107–18.

De Ceaurriz, J.C., Micillino, J.C., Bonnet, P., and Guenier, J.P. (1981). Sensory irritation caused by various industrial airborne chemicals. *Toxicol Lett* **9,** 137-143.

Fritz, J. H., Le Bourhis, L., Magalhaes, J. G., and Philpott, D. J. (2008). Innate immune recognition at the epithelial barrier drives adaptive immunity: APCs take the back seat. *Trends in Immunol* **29**, 41–49.

Germann, P. G., Haefner, D., Hanauer, G., and Drommer, W. (1998). Incidence and severity of granulomatous pneumonia in Brown Norway (BN) rats: Breeder related variations. *J Exp Anim Sci* **39**, 22–33.

Gould, H. J., Takhar, P., Harries, H. E., Durham, S. R., and Corrigan, C. J. (2006). Germinal-centre reactions in allergic inflammation. *Trends Immunol* **27**, 446–52.

Haley, K. J., and Drazen, J. M. (1998). Inflammation and airway function in asthma. What you see is not necessarily what you get. *Am J Respir Crit Care Med* **157**, 1–3.

Holgate, S. T., Davies, D. E., Lackie, P. M., Wilson, S. J., Puddicombe, S. M., and Lordan, J. L. (2000). Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol* **105**, 193–204.

Holsapple, M. P., Jones, D., Kawabata, T. T., Kimber, I., Sarlo, K., Selgrade, M. J. K., Shah, J., and Woolhiser, M. R. (2006). Assessing the potential to induce respiratory hypersensitivity. *Toxico Sci* **91**, 4–13.

Jones, M. G., Floyd, A., Nouri-Aria, K. T., Jacobson, M. R., Durham, S. R., Newman Taylor, A. J., and Cullinan, P. (2006). Is occupational asthma to diisocyanates a non-IgE-mediated disease? *J Allergy Clin Immunol* **117**, 663–69.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K. F., Itoh, M., Kawashima, S., Katayama, T., Araki, M., and Hirakawa, M. (2006). From genomics to chemical genomics: New developments in KEGG. *Nucleic Acids Res* **34**, D354–57.

Kay, A. B., Phipps, S., and Robinson, D. S. (2004). A role for eosinophils in airway remodelling in asthma. *Trends in Immunol* **25**, 477–82.

Kimber, I., Agius, R., Basketter, D. A., Corsini, E., Cullinan, P., Dearman, R. J., Gimenez-Arnau, E., Greenwell, L., Hartung, T., Kuper, C. F., Maestrelli, P., Roggen, E., and Rovida, C. (2007). Chemical respiratory allergy: Opportunities for hazard identification and characterization. *ATLA* **35**, 243–65.

Kraft, S., and Novak, N. (2006). Fc receptors as determinants of allergic reactions. *Trends in Immunol* **27**, 88–95.

Kuang, P. P., Berk, J. L., Rishikof, D. C., Foster, J. A., Humphries, D. E., Ricupero, D. A., and Goldstein, R. H. (2007). NF-κB induced by IL-1β inhibits elastin transcription and myofibroblast phenotype. *Am J Physiol Cell Physiol* **283**, C58–65.

Kuper, C. F., Stierum, R. H., Boorsma, A., Schijf, M. A., Prinsen, M., Bruijntjes, J. P., Bloksma, N., and Arts, J. H. E. (2008). The contact allergen dinitrochlorobenzene (DNCB) and respiratory allergy in the Th2-prone Brown Norway rat. *Toxicology* **246**, 213–21.

Lin, T., Zhang, W., Fan, Y., and Mulholland, M. (2007). Interleukin-1beta and interleukin-6 stimulate matrix metalloproteinase-9 secretion in cultured myenteric glia. *J Surg Res* **137**, 38–45.

Liss, G. M., Bernstein, D., Genesove, L., Roos, J. O., and Lim, J. (1993). Assessment of risk factors for IgE-mediated sensitisation to tetrachlorophthalic anhydride. *J Allergy Clin Immunol* **92**, 237–47.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunol* **25**, 677–86.

Matheson, J. M., Johnson, V. J., Vallyathan, V., and Luster, M. I. (2005). Exposure and immunological determinants in a murine model for toluene diisocyanate (TDI) asthma. *Toxicol Sci* **84**, 89–98.

Mattes, W. B. (2006). Cross-species comparative toxicogenomics as an aid to safety assessment. *Expert Opin Drug Metab Toxicol* **2**, 859–74.

Moffatt, J. D. (2005). What targets have knock-outs revealed in asthma? *Pharmacol Therap* **107**, 343–57.

Ober, C., and Hoffjan, S. (2006). Asthma genetics (2006). The long and winding road to gene discovery. *Genes and Immunity* **7**, 95–100.

Ohkubo, K., Ogata, S., Misumi, Y., Takami, N., and Ikehara, Y. (1991). Molecular cloning and characterization of rat contrapsin-like protease inhibitor and related proteins. *J Biochem* **109**, 243–50.

Pauluhn, J., and Mohr, U. (2005). Experimental approaches to evaluate respiratory allergy in animal models. *Exp Toxicol Pathol* **56**, 203–34.

Pauluhn, J., Eidmann, P., Freyberger, A., Wasinska-Kempka, G., and Vohr, H.W. (2002). Respiratory hypersensitivity to trimellitic anhydride in Brown Norway rats: a comparison of end-points. *J Applied Toxicol* **22,** 89-97.

Ravetch, J. V., and Lanier, L. L. (2000). Immune inhibitory receptors. *Science* **290**, 84–89.

Schaper, M., and Brost, M.A. (1991). Respiratory effects of trimellitic anhydride aerosols in mice. *Arch Toxicol* **65,** 671-677.

Selgrade, M. K., Boykin, E. H., Haykal-Coates, N., Woolhiser, M. R., Wiescinski, C., Andrews, D. L., Farraj, A. K., Doerfler, D. L., and Gavett, S. H. (2006). Inconsistencies between cytokine profiles, antibody responses, and respiratory hyperresponsiveness following dermal exposure to isocyanates. *Toxicol Sci* **94**, 108–17.

Signor, L., Tigani, B., Beckmann, N., Falchetto, R., and Stoeckli, M. (2004). Two-dimensional electrophoresis protein profiling and identification in rat bronchoalveolar lavage fluid following allergen and endotoxin challenge. *Proteomics* **4**, 2101–10.

Trivedi, S. G., and Lloyd, C. M. (2007). Eosinophils in the pathogenesis of allergic airway disease. A review. *Cell Mol Life Sci* **57**, 1–21.

Valstar, D. L., Schijf, M. A., Arts, J. H. E., Kuper, C. F., Nijkamp, F. P., Storm, G., Bloksma, N., and Henricks, P. A. (2006). Alveolar macrophages suppress non-specific inflammation caused by inhalation challenge with trimellitic anhydride conjugated to albumin. *Arch Toxicol* **80**, 561–71.

Vignola, M. A., Mirabella, F., Costanzo, G., DiGiorgi, R., Gjomarkaj, M., Bellia, V., and Bonsignori, G. (2003). Airway remodeling in asthma. *Chest* **123**, 417–30.

Woutersen, R.A., van Garderen-Hoetmer, A., Slootweg, P.J., and Feron, V.J. (1994). Upper respiratory tract carcinogenesis in experimental animals and humans. In: Carcinogenesis, Target Organ Toxicology Series (Waalkens, M.P. and Ward, J.M., eds), Raven Press, New York, pp. 27-36.

Zhang, X. D., Fedan, J. S., Lewis, D. M., and Siegel, P. D. (2004). Asthmalike biphasic airway responses in Brown Norway rats sensitized by dermal exposure to dry trimellitic anhydride powder. *J Allergy Clin Immunol* **113**, 320–26.

Zhao, L., and Burt, A. D. (2007). The diffuse stellate system. *J Molec Histol* **38**, 53–64.

Zimmermann, N., King, N. E., Laport, J., Yang, M., Mishra, A., Pope, S. M., Muntel, E. E., Witte, D. P., Pegg, A. A., and Foster, P. S. (2003). Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J Clin Invest* **111**, 1863–74.

Zimmermann, N., Mishra, A., King, N. E., Fulkerson, P. C., Doepker, M. P., Nikolaidis, N. M., Kindinger, L. E., Moulton, E. A., Aronow, B. J., and Rothenberg, M. E. (2004). Transcript signatures in experimental asthma: Identification of STAT6-dependent and –independent pathways. *J Immunol* **172**, 1815–24.