

REVIEW ARTICLE

Gene expression changes in the mesenteric lymph nodes of rats after oral peanut extract exposure

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Abstract

New techniques are needed to broaden the understanding of the food allergic response. The capacity of peanut extract to influence gene expression profiles was investigated in a Brown Norway rat model for food allergy. Brown Norway rats were sensitized to peanut extract (0, 1 and 10 mg/rat/d) by daily oral gavage and were dissected after 3, 7 or 14 days of exposure. RNA extracted from mesenteric lymph nodes of individual rats were hybridized against a common reference pool on Agilent whole rat genome (4*44k) arrays. The raw data were normalized and statistically analyzed using the statistical program R. A False Discovery Rate of 10% and a Fold Ratio of $-1.5 \leq \text{Fold Ratio} \leq 1.5$ between the experimental groups and their respective control groups were applied. Differentially expressed genes were clustered into a heatmap. Functional annotation and GeneOntology term enrichment were examined. Furthermore, the involvement of the differentially expressed genes in specific cellular pathways was investigated with MetaCore. Gene expression changes, which were both dose- and time-dependent, were detected after sensitization to peanut. A total of 64 genes were differentially expressed, of which 60 were up-regulated and four were down-regulated. These changes were related to the regulation of immunological processes, most notably increased cell division. The findings indicate that responses to peanut include proliferation of immunologically relevant tissues, which can be identified by analysis of gene expression profiles. This may lay a basis for further research into possibilities for discrimination of allergenic from non-allergenic proteins.

Keywords: food allergy; peanut extract; Microarray; Brown Norway rat; mesenteric lymph node; cell division

Introduction

About 2% of the adult population and 6–8% of children below the age of 3 are affected by a food allergy (Burks and Ballmer-Weber, 2006; Untersmayr and Jensen-Jarolim, 2006), which sometimes results in fatal reactions (anaphylactic shock) (Sicherer, 2002). It is often difficult to predict whether foods that so far were not substantially consumed (novel foods) may be allergenic, and this problem may further increase due to the production of genetically modified food resulting in the introduction of new potentially allergenic proteins (Taylor, 2006).

Animal models, although not yet validated, have been proposed for assessing the potential allergenicity of novel foods. In such models, specific IgE synthesis as well as other features such as mast cell mediator release are indicators of allergenicity of the products to which the animals are exposed.

The publication of the sequence of the human genome has resulted in a dramatic increase in knowledge in the field of genomics (Gatzidou et al., 2007) and, as a consequence, during the past two decades great advances have been achieved in molecular biology. One of the

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most recent techniques accountable for these advances is the microarray technique. DNA microarrays permit the quantitative measurement of the expression levels of thousands of individual genes in different biological samples at the same time. Another benefit of this technique is that it allows for monitoring of early parameters of biological effects. Several studies have shown that it is possible to detect changes in the gene expression profile after exposure to immunomodulating agents, both *in vivo* (Ezendam et al., 2004) and *in vitro* (Schoeters et al., 2007). Schoeters et al. (2007) compared the gene expression profiles of dendritic cells (DCs) exposed to contact allergens and irritants. They found 25 genes that were altered in response to contact allergens, but not to the irritants. Most of these genes could be linked to the immune response and/or to their expression in DCs. So far, no studies have been performed that assessed the expression profile in food allergy. We aimed to test whether effects on the gene expression profile could be found in an *in vivo* setting of food allergy.

In the study described here, the effects of oral exposure to peanut extract (PE) on gene expression was investigated in a Brown Norway (BN) rat model for food allergy. PE is known to produce food allergic reactions in humans and rats. PE is the whole aqueous peanut extract and consists of several peanut allergens in the aqueous peanut matrix. Changes in expression of immunologically relevant and other genes that would be detected in this experimental setting could yield additional insight into the mechanisms of responses to proteins, including food allergens, and could eventually help to provide early markers of food allergy.

Methods

Animals

Peanut extract (PE) sensitization was studied in young Brown Norway (BN) rats. In this study Brown Norway (BN Rij:Hsd) rats obtained from Harlan Netherlands B.V. (Horst, the Netherlands) were used. These rats were bred and raised on a peanut and soy protein-free diet (because soy and peanut are cross-reactive legumes) obtained from Harlan Netherlands B.V. (2016S Teklad Global 16% Protein Rodent Diet) at our institute. Female rats of the third generation (F3) and onwards were used and fed the peanut and soy protein-free diet during the experiment. At study initiation, the rats were 3–4 weeks old. The rats were housed in an animal room maintained at $23 \pm 3^\circ\text{C}$, with a light/dark cycle of 14 hr/10 hr, and a relative humidity of 30–70% during the experiment and for at least 7 d prior to study initiation. The rats were housed in stainless-steel wire (Macrolon III) cages in groups of two and had free access to food and tap-water. The guidelines described in the Dutch “Experiments on Animals” Act (1997;

online at http://www.vet.uu.nl/nca/userfiles/other/The_Dutch_Experiments_on_Animals_Act.pdf) were applied for the care and use of the animals. Approval from the Institutional Animal Ethics Committee was obtained for all experimental procedures involving animals.

PE Sensitization

To ensure that animals were immunologically naive with respect to peanut, pre-study blood samples were always tested for allergen-specific antibodies. Rats were randomly allocated to their dosing regimens ($n=8$ per dosing regimen). Some reallocation was performed to ensure even starting weight distribution in all dosing regimens. Rats were exposed to 1 ml of 0, 1 or 10 mg/ml PE in PBS by daily *per os* gavage, using a 18-gauge stainless steel animal feeding needle for the duration of 3, 7, or 14 d for the microarrays and 42 d followed by 7 d of rest (no PE sensitization) for the mesenteric lymph node (MLN) proliferation assay. After exposure the rats were sacrificed by exsanguination from the aorta under CO_2/O_2 anesthesia. The MLNs were removed aseptically and stored at -80°C for RNA isolation. For the proliferation assay single cell suspensions were prepared from fresh MLNs for proliferation analysis of MLN cells after *ex-vivo* re-stimulation with PE.

PE-specific IgE, IgG1 and IgG2a ELISAs

PE-specific IgE, IgG₁, IgG_{2a} levels were measured to examine whether the animals were adequately sensitized. PE-specific IgE, IgG₁, and IgG_{2a} Enzyme-Linked ImmunoSorbent Assay (ELISAs) were performed as described earlier (de Jonge et al., 2007b). Serum samples were collected at sacrifice at Days 3, 7 and 14.

Ex vivo Stimulation of Spleen Cells: Allergen-Specific Cell Proliferation

Assessment of PE-specific proliferation was done to assess if the rats animals were adequately sensitized. Single cell suspensions were prepared under aseptic conditions by pressing the MLN through a $70\text{ }\mu\text{m}$ nylon cell strainer (Falcon, Franklin Lakes, NJ). Cells were washed twice and resuspended in 5 ml standard medium (RPMI 1640 + 25 mM Hepes + L-Glutamine) obtained from Gibco BRL (Breda, the Netherlands) supplemented with 10% heat inactivated fetal calf serum (FCS) obtained from Integro B.V. (Zaandam, The Netherlands) and 1% penicillin-streptomycin (Gibco) with $7.5 \times 10^{-5}\text{ M}$ 2-mercaptoethanol (2-ME) obtained from SERVA (Heidelberg, Germany). Cell number was determined with a Coulter Counter (Z2, Coulter Electronics, Mijdrecht, The Netherlands). Cell viability was determined by the Trypan blue cytotoxicity test. The concentration of the cell suspensions was adjusted to 1×10^6 viable cells/ml.

For cell proliferation, 100 μl of the cell suspension was added per well in triplicate. Cells were incubated for

96 hr with 2 mg/ml PE and [^3H]-thymidine was added for the last 18–20 hr. The optimal time to assess lymphocyte proliferation was established in previously performed studies and 96 hr was the most optimal time point.

RNA Isolation

The RNA of the MLNs was isolated using the RNeasy Lipid Tissue midi kits (Qiagen, Germantown, MD) according to the manufacturers' instructions. To start the procedure, the frozen MLNs were first finely crushed using a pestle and mortar cooled with liquid nitrogen, after which the crushed tissue was transmitted into 5 ml Qiazol Lysis Reagent (Qiagen) and homogenized to allow immediate lysis after thawing. The quantity of RNA in each sample was determined spectrophotometrically (DU 800-spectrophotometer, Beckman Coulter, Fullerton, CA) and RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies, Breda, the Netherlands). The RNA samples were stored at -80°C until used. A common reference pool was prepared by mixing 0.33 μg of RNA of all samples to be analyzed.

RNA Amplification and Labelling

First, 0.5 μg total RNA (volume: 3.25 μL) of each sample and 1 μg RNA (volume: 6.5 μL) of the common reference pool was used for the labelling with Cyanine fluorophores (Cy3 and Cy5; Agilent Technologies) using the Low RNA Input Linear Amplification kit (Agilent Technologies) according to the manufacturers' instruction, with some adjustments. To the samples, 2.5 μL T7-primer, 4.25 μL cDNA mastermix, and 15 μL transcription mastermix were added per reaction; to the reference pool, 5 μL T7-primer, 8.5 μL cDNA mastermix and 30 μL transcription mastermix were added per reaction. Briefly, the total RNA was reverse transcribed into cDNA using T7-primers and MMLV reverse transcriptase. This cDNA was transcribed into cRNA, during which the fluorophores were incorporated into the RNA; Cy3 was incorporated into the common reference pool and Cy5 was incorporated into the samples. Next, the cDNA was purified using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and the incorporation rate of the fluorophores into the cRNA was determined spectrophotometrically. Only samples with a dye-incorporation rate of $\geq 7\text{ pmol}/\mu\text{g}$ cRNA were considered suitable for further processing.

Hybridization

The labeled cRNA was prepared for hybridization using a GeneExpressionHybridizationKit (Agilent Technologies), according to manufacturers' instructions. A mix of equal amounts of cRNA from the samples and the common reference pool was hybridized to slides from the Whole Rat Genome ($4 \times 44\text{k}$) Oligo Microarray Kit (Agilent). This technique is also known as a two dye microarray; one dye for the individual samples and the other dye for the

pooled reference sample, whereby the pooled reference sample allows for standardized comparison of individual samples. In total, 23 array slides, with four samples/slide each, were generated. The arrays were hybridized for 17 hr at 65°C in a hybridization oven (Agilent). After washing the slides with the Gene Expression Wash Buffer kit (Agilent) according to the manufacturer's protocol, the slides were dipped in Stabilization and Drying Solution (Agilent) (2:1 diluted with acetonitrile) to protect the slides from environmental ozone and dried with N_2 .

Scanning

Immediately after washing, the arrays were scanned (GenePix 4000B) with a scan precision of 5 μm , which generated separate Cy3 and Cy5 images. The photomultiplier tube (PMT)-gain for Cy5 was set at 623 nm and the PMT-gain for Cy3 was set at 450 nm. Image software was used to determine Cy3 and Cy5 signal intensities for each separate spot and the background noise. Quality control was performed on the raw data: first, the scans were visually inspected in Image followed by analyses of the raw data using a specifically designed Excel-file application. In this application the raw data were visualized as a scatter plot and an M/A-plot as well as a normal probability plot to assess signal distribution.

Data Analysis

The raw data were normalized using the statistical program R (www.r-project.org), in three steps: (1) natural log-transformation, (2) quantile normalization of all scans and (3) correction of the sample spot signal for differences in the corresponding reference spot signal between arrays. For additional quality control, these normalized data were visualized by Principle Component Analysis (PCA). Next, statistical analysis of the data took place; in R significance of differences in gene expression between the experimental groups was calculated with one-way ANOVA. A False Discovery Rate (FDR) of 10% and a Fold Ratio (FR) of -1.5 δ FR or $\text{FR} \geq 1.5$ between the experimental groups and their respective control groups were applied.

Differentially expressed genes were clustered into a heatmap using GeneMaths (Applied Maths, St-Martens-Latem, Belgium). Functional annotation and GeneOntology (GO) term enrichment (www.geneontology.org) were examined with the DAVID/EASE program (david.abcc.ncifcrf.gov/). MetaCoreTM (GeneGo, San Diego, CA) was used to identify and visualize the involvement of the differentially expressed genes in specific cellular pathways, and this was related to the total amount of genes involved in the particular pathway and in all the available pathways combined.

Statistics of *ex vivo* Proliferation Assay

To determine if PE altered the *ex vivo* proliferation of MLNs within a dosing group of PE a one-way ANOVA

with Bonferroni as post-hoc test was done. Differences were considered significant if $p < 0.05$ (two-sided).

Results

Antigen-Specific Antibodies

The animals were naïve at the level of antigen-specific antibodies before study initiation. At Day 3, PE-specific

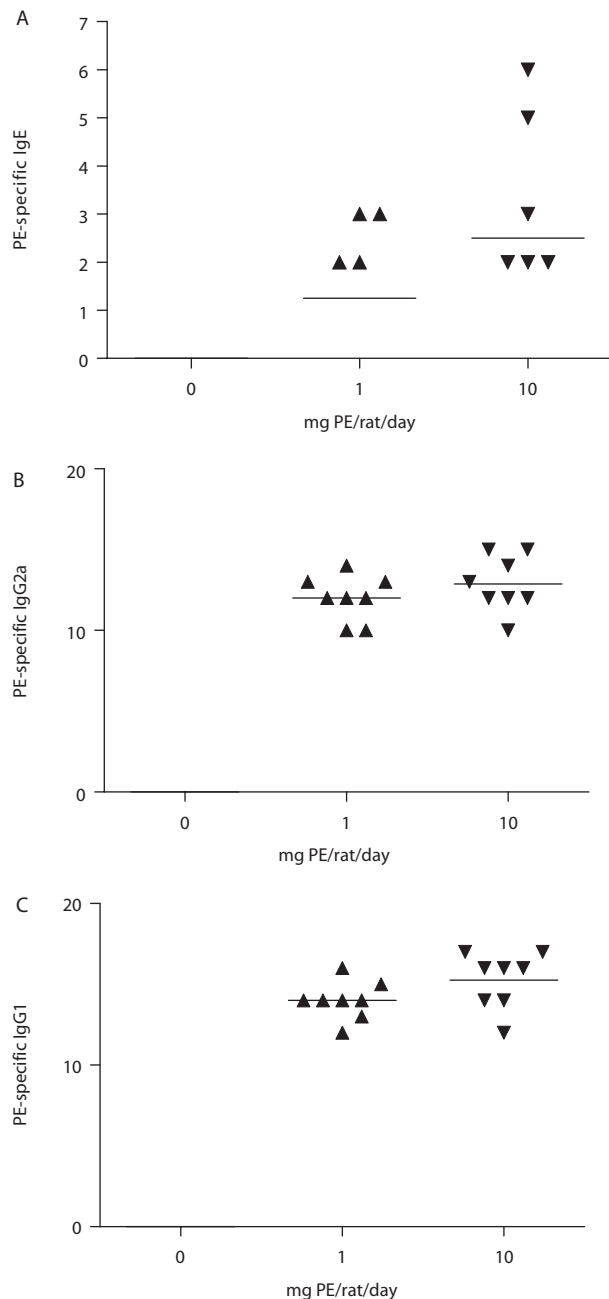


Figure 1. PE-specific IgE, IgG_{2a}, and IgG₁ titers after 14 days of exposure to PE. Groups of female rats ($n=8$ for each dosing regiment) received 0, 1 or 10 mg PE/rat/d by daily oral gavage. PE-specific Ig titers ($^2\log$) are shown at Day 14: (A) displays PE-specific IgE titers, (B) displays PE-specific IgG_{2a} titers and (C) displays PE-specific IgG₁ titers.

IgE, IgG₁, and IgG_{2a} were not detectable in both dosing groups. At Day 7, increased levels of PE-specific IgG_{2a} and IgG₁ levels were detected in the 10 mg PE group. At this time point, PE-specific IgE levels were not detectable (data not shown). At Day 14, PE-specific IgG₁ (Figure 1C) and IgG_{2a} titres (Figure 1B) are detectable in both dosing groups. PE-Specific IgE titres are detectable in 4 of the 8 rats in the 1 mg PE group and in 6 of the 8 rats in the 10 mg PE group (Figure 1A).

PE-Specific Lymphocyte Cell Proliferation of MLN Cells

Figure 2 shows that *in vivo* dosing with 10 mg PE/rat/d group increased the PE-specific *ex vivo* proliferation, as compared to the 0 mg PE/rat/d group of MLN cells. This change was not significant, due to variability especially in the 10 mg peanut/rat/d group, but a strong dose-response correlation was found ($R^2 = 0.9999$).

Gene Expression Profiles

Gene expression data of the three groups exposed to 1 mg PE/rat/d and the 3 groups exposed to 10 mg PE/rat/d were compared to the data of their time-matched controls by one-way ANOVA. An apparent time-effect was seen, as well as a clear dose-response relationship. Differential gene expression was most pronounced on Day 7 for both the 1 mg and the 10 mg PE/rat/day group. The groups exposed to 10 mg PE/rat/d showed a stronger effect on all 3 days than the groups exposed to 1 mg PE/rat/d. On Day 7 in the 10 mg PE/rat/d exposure group, 60 genes were differentially expressed with a Fold Ratio of > 1.5 ; on Day 7 in the 1 mg PE/rat/d group, 12 genes were differentially expressed with a Fold Ratio of > 1.5 . In total, 64 genes were differentially expressed with a Fold Ratio of > 1.5 in one or

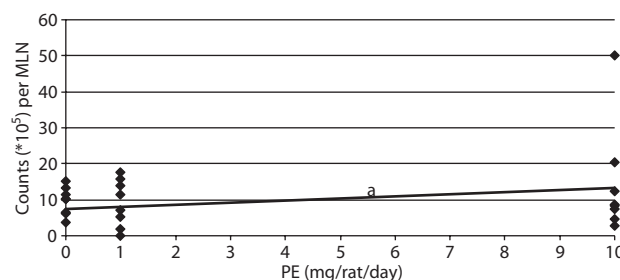


Figure 2. PE-specific proliferation of MLN cells. Groups of female rats ($n=8$ for each dosing regiment) received 0, 1 or 10 mg PE/rat/day by daily oral gavage. MLNs were dissected at day 49, single cell suspensions were prepared and 1×10^5 cells/well were incubated with 1 mg/ml PE for 96 hr. In earlier studies, we performed time series analysis and found 96 hr of re-stimulation with PE to be the optimal timepoint for lymphocyte proliferation. Lymphocyte proliferation of MLN cells is expressed in cpm ($\times 10^5$) per organ. $y = 0.5923x + 6.7682$ with a R^2 of 0.9999 define the trendline of the dose response between *ex vivo* PE-specific proliferation of MLN cells and *in vivo* PE-dosing.

Table 1. Individually regulated genes in a rat model for peanut allergy A: Cell cycle genes.

Accession number			FR					
			1 mg PE/rat/day			10 mg PE/rat/day		
			3d	7d	14d	3d	7d	14d
XM_215849	Bub1_predicted	budding uninhibited by benzimidazoles 1 homolog (<i>S. cerevisiae</i>) (predicted)	-1.03	1.49	1.07	-1.03	2.07 ^A	1.10
NM_053702	Ccna2	cyclin A2	1.13	1.51 ^A	1.07	1.18	1.92 ^A	1.05
NM_171991	Ccnb1	cyclin B1	-1.10	1.49	-1.01	-1.06	1.85 ^A	1.05
NM_019296	Cdc2a	cell division cycle 2 homolog A (<i>S. pombe</i>)	-1.10	1.69 ^A	1.11	-1.08	2.6 ^A	1.07
XM_214152	Cdkn3_predicted	cyclin-dependent kinase inhibitor 3 (predicted)	-1.07	1.69 ^A	1.00	-1.04	2.7 ^A	1.19
XM_218601	E2f8	E2F transcription factor 8	1.01	1.41	1.14	-1.05	1.94 ^A	1.21
XM_214477	Gmnn_predicted	geminin (predicted)	-1.05	1.47	-1.00	-1.11	1.67 ^A	1.05
XM_216161	Mad2l1_predicted	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast) (predicted)	-1.13	1.29	1.02	-1.08	1.59 ^A	1.10
NM_022381	Pcna	proliferating cell nuclear antigen	-1.05	1.48	1.02	1.05	1.6 ^A	-1.00
XM_236477	Ttk_predicted	Ttk protein kinase (predicted)	-1.03	1.44	1.07	-1.00	1.91 ^A	1.06
XM_213891	Aspm_predicted	asp (abnormal spindle)-like, microcephaly associated (<i>Drosophila</i>) (predicted)	-1.08	1.42	-1.04	-1.08	1.89 ^A	1.07
XM_342494	Bub1b	budding uninhibited by benzimidazoles 1 homolog, beta (<i>S. cerevisiae</i>)	1.00	1.33	1.16	1.01	1.59 ^A	1.12
XM_001077331	Ccne1	cyclin E	-1.03	1.54 ^A	1.07	1.02	2.23 ^A	1.13
XM_226624	Ell2	elongation factor RNA polymerase II 2	1.06	1.14	1.02	1.04	2.02 ^A	1.18
XM_226624	Ell2	elongation factor RNA polymerase II 2	1.09	1.15	-1.14	1.18	1.67 ^A	1.08
XM_341592	Kif20a_predicted	kinesin family member 20A (predicted)	-1.11	1.47	1.03	-1.16	1.97 ^A	1.12
NM_001025740	Rrm2_mapped	ribonucleotide reductase M2	1.00	1.50 ^A	-1.01	-1.01	2.93 ^A	1.19
NM_001025740	Rrm2_mapped	ribonucleotide reductase M2	-1.03	1.40	1.01	1.03	2.06 ^A	1.18
BG153336	Smarchb1	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily b, member 1	-1.02	1.16	1.01	-1.04	1.75 ^A	1.21
XM_215471	Solt_predicted	SoxLZ/Sox6 leucine zipper binding protein in testis (predicted)	1.10	1.42	1.02	1.06	1.93 ^A	1.00
XM_343359	Spbc24_predicted	spindle pole body component 24 homolog (<i>S. cerevisiae</i>) (predicted)	1.01	1.62 ^A	1.02	-1.01	2.06 ^A	-1.04
B: Immunological genes								
Accession number			FR					
			1mg PE/rat/day			10mg PE/rat/day		
			3d	7d	14d	3d	7d	14d
Immunological genes								
NM_130399	Ada	adenosine deaminase	1.00	1.18	-1.01	-1.02	1.95 ^A	1.09
NM_130399	Ada	adenosine deaminase	-1.00	1.33	-1.02	-1.04	1.99 ^A	1.18
XM_344042	Ig	similar to immunoglobulin light chain variable region (predicted)	1.00	1.04	-1.06	-1.02	1.86 ^A	1.05
XM_344042	Ig	similar to immunoglobulin light chain variable region (predicted)	-1.02	1.13	1.00	-1.05	1.87 ^A	1.21
XM_573321	LOC498113	similar to This CDS feature is included to show the translation of the corresponding V_region. Presently translation qualifiers on V_region features are illegal	1.02	1.81 ^A	1.36	1.11	5.21 ^A	1.88 ^A
TC548855	IgG1	immunoglobulin G1	1.06	1.09	1.21	-1.10	2.11 ^A	1.64 ^A
XM_341195	Igj	immunoglobulin joining chain	-1.06	1.49	-1.07	-1.03	2.65 ^A	1.07
S82585	Igj	immunoglobulin joining chain	1.01	1.58 ^A	-1.05	-1.03	2.84 ^A	1.12
XM_213211	Tnfrsf17_predicted	tumor necrosis factor receptor superfamily, member 17 (predicted)	1.07	1.78 ^A	-1.01	1.06	3.79 ^A	1.20
XM_343117	Siva_predicted	Siva_predicted	-1.04	1.23	1.00	1.02	1.55 ^A	1.10
NM_001012197	Tra1_predicted	tumor rejection antigen gp96 (predicted)	-1.02	1.17	-1.09	-1.10	2.46 ^A	1.03

Table 1 continued on next page

Table 1. Continued.

Accession number	Symbol	Gene name	FR					
			1 mg PE/rat/day			10 mg PE/rat/day		
			3d	7d	14d	3d	7d	14d
XM_236614	Armet_predicted	arginine-rich, mutated in early stage tumors (predicted)	-1.05	1.13	1.05	-1.04	1.90 ^A	1.13
XM_236614	Armet_predicted	arginine-rich, mutated in early stage tumors (predicted)	-1.03	1.13	1.01	-1.02	1.94 ^A	1.13
XM_343189	Ckap4_predicted	cytoskeleton-associated protein 4 (predicted)	1.08	1.16	-1.04	1.06	2.31 ^A	1.05
NM_053886	Lman1	lectin, mannose-binding, 1	1.04	1.14	-1.02	1.04	1.78 ^A	1.19
NM_053886	Lman1	lectin, mannose-binding, 1	-1.07	1.17	-1.02	1.02	1.67 ^A	1.13
NM_153628	Sec11l3	Sec11-like 3 (<i>S. cerevisiae</i>)	-1.05	1.32	-1.05	-1.04	2.17 ^A	1.20
NM_001008891	Ssr1	signal sequence receptor, alpha	-1.08	1.22	-1.14	1.06	2.09 ^A	1.16
NM_001008334	Tmem97	transmembrane protein 97	1.08	1.31	-1.37	-1.00	3.24 ^A	1.43
NM_181084	Trp53inp1	Transformation-related protein 53 inducible nuclear protein 1	-1.02	1.17	-1.02	-1.03	1.70 ^A	-1.01
XM_225257	Txndc5_predicted	thioredoxin domain containing 5 (predicted)	1.04	1.28	-1.04	1.11	2.02 ^A	1.09
NM_001034952	Pqlc3	PQ loop repeat containing 3	-1.04	1.18	1.05	1.05	1.85 ^A	1.12
NM_053849	Pdia4	protein disulfide isomerase family A, member 4	1.07	1.13	-1.05	-1.00	2.21 ^A	1.01
NM_001024240	RGD1310251	similar to RIKEN cDNA 2010001M09	-1.06	1.55 ^A	1.02	-1.08	3.90 ^A	1.33
XM_001058771	LOC681870	hypothetical protein LOC681870	-1.14	1.55 ^A	1.05	-1.03	1.88 ^A	-1.01
NM_001025683	RGD1311946	similar to RIKEN cDNA 1810055G02	-1.02	1.19	-1.04	-1.10	1.81 ^A	1.10
XM_579997	RGD1561797_predicted	RGD1561797 (predicted)	-1.08	1.37	1.05	-1.04	1.77 ^A	1.18
NM_001014041	LOC310721	similar to 4930431B09Rik protein	-1.04	1.13	-1.11	1.01	1.75 ^A	1.14
XM_001058844	LOC680782	similar to signal peptidase complex subunit 3 homolog	-1.05	1.13	-1.01	-1.01	1.75 ^A	1.06
AY310157	RGD1311563	similar to Oligosaccharyl transferase 3 CG7748-PA	1.02	1.14	-1.01	-1.01	1.63	-1.06
NM_198776	MGC72973	beta-glo	-1.22	1.24	-1.55 ^A	-1.78 ^A	1.32	-1.07
NM_001013853	LOC287167	globin, alpha	-1.28	1.08	-1.51 ^A	-1.68 ^A	1.14	-1.05
X53003	Acaca	acetyl-coenzyme A carboxylase alpha	1.02	-1.14	-1.26	1.04	-1.23	-1.63 ^A
NM_012543	Dbp	D site albumin promoter binding protein	-1.07	-1.22	1.15	1.14	-1.48	1.54 ^A
NM_017297	Kcnj5	potassium inwardly rectifying channel, subfamily J, member 5	-1.08	-1.33	1.00	-1.10	-1.64 ^A	1.03

Gene expression data are displayed as the Fold Ratio (FR) of the individual genes at the three time points: Days (d) 3, 7 or 14.

^ADifferentially expressed genes.

more of the groups exposed to PE, of which there were 50 unique identifiable genes.

Table 1 shows these genes with the FRs for the different exposure groups. In this table, the genes are itemized into three groups: (Table 1A) cell cycle genes, (Table 1B) immunological genes and (Table 1C) other genes. Figure 3 shows the clustering of these regulated genes. GO term enrichment analysis showed that cluster A, which consists of consistently up-regulated genes for both dosing regimens, was significantly enriched for cell cycle-related and immunological genes. In addition, MetaCore analysis of the differentially regulated genes resulted in the identification of several pathways in which the proteins coded by these genes were implicated.

The six most regulated pathways identified by MetaCore were involved in cell cycle, confirming the GO-term enrichment analysis. These pathways were (number of genes regulated of the total genes included in this pathway during analysis are placed between

brackets): role anaphase-promoting complex (APC) in cell cycle regulation (8 of 53), nucleocytoplasmic transport of cyclin-dependent kinase (CDK)/cyclins (4 of 22), cell cycle (generic schema; 4 of 26), the meta-phase checkpoint (4 of 36), ataxia telangiectasia mutated (ATM)/ ataxia telangiectasia and RAD3-related (ATR) regulation of G2/M checkpoint (3 of 29) and chromosome condensation in prometaphase (3 of 33).

Figure 4 shows the highest ranking pathway obtained by MetaCore analysis. It represents the role of the APC in cell cycle regulation and illustrates the expression changes for cell cycle related genes. The genes involved in the six most significant pathways were: Cdk1 (cyclin-dependent kinase; cdc2a: cell division cycle 2a), cyclin A, cyclin E and SPBC24 (Spbc24), which were up-regulated at Day 7 in BN rats exposed to 1 mg PE/rat/day or 10 mg PE/rat/day; and cyclin B1, MAD2 (mitotic arrest deficient; Mad2l1), Cks1 (Cdc kinase subunit 1; RGD1561797), geminin, BUB1 (budding uninhibited by benzimidazoles 1 homolog, beta; Bub1) and BUBR1 (Bub1b) which were up-regulated at Day 7

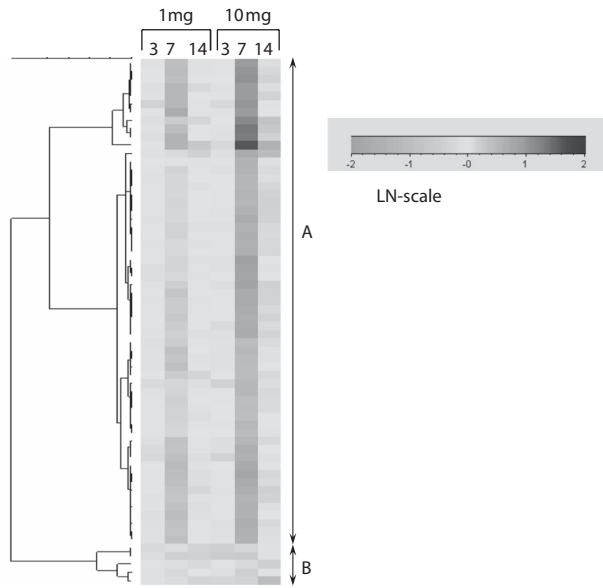


Figure 3. Hierarchical clustering of genes regulated by PE. Gene expression data are displayed as a clustered heat map (in LN-scale ranging from -2 to $+2$). The color bar and the corresponding Fold Ratio indicate gene expression: red represents up-regulation, green down-regulation and yellow no difference. Cluster A consists of consistently up-regulated genes for both dosing regimens and cluster B consists of less consistent regulated genes.

after exposure to 10 mg PE/rat/d, but not after exposure to 1 mg PE/rat/d. All these genes were proliferation related.

No immunological pathways based on sufficient numbers of regulated genes were found using MetaCore analysis, but several individual immunologically relevant genes were regulated by PE in the food allergy model: adenosine deaminase (ADA), immunoglobulin light chain variable region (Ig), IgG1, Igj, tumor necrosis factor receptor (TNFR) superfamily, member 17 (Tnfrsf17), Siva and tumor rejection antigen gp96 (Tra1). These genes were all up-regulated in the exposure group receiving 10 mg PE/rat/d at Day 7, Tra1 was also up-regulated in the 1 mg PE/rat/day group at Day 7.

ADA is an enzyme that catalyzes the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. ADA is required for the maturation and function of lymphocytes, especially T-lymphocytes in which its main biologic activity is detected (Mishra et al., 2005), and for the maturation of human blood monocytes to macrophages.

Tnfrsf17 is a member of the TNFR superfamily that is important for B-lymphocyte development. It is preferentially expressed in mature B lymphocytes, especially that of B-memory cells (Treml et al., 2006), and autoimmune response. Several TNF Receptor-Associated Factors are able to bind to Tnfrsf17, hereby transducing cell-signals for cell survival and proliferation (Hatzoglou et al., 2000).

Siva (apoptosis-inducing factor) plays an important role in the apoptotic pathway of thymocytes. Association

of Siva with members of the TNFR family sends pro-apoptotic signals into the cell. It negatively regulates the nuclear factor- κ B (NF- κ B) activity, which has anti-apoptotic properties. Hereby, Siva promotes the activation-induced cell death, resulting in the down-regulation of immune responses, which is essential for T-lymphocyte homeostasis (Gudi et al., 2006).

Tra1, also known as heat shock protein gp96, has protective functions during cellular stress and plays an important role in the maintenance of protein homeostasis. Furthermore, gp96 exerts potent stimulatory activity on both innate and adaptive immunity. Gp96 is especially involved in the immune response to tumor antigens by chaperoning tumor antigen-derived immunogenic peptides and deliver them to antigen-presenting cells for specific T-lymphocyte stimulation. Gp96 also mediates the maturation of DCs by up-regulating the expression of MHC Class II and co-stimulatory molecules (Rivoltini et al., 2003; Zhang et al., 2006).

Discussion and conclusions

We investigated the immunologic response to PE at the level of gene expression in a BN rat model for food allergy. This peanut model has been tested for various conventional immune parameters (de Jonge et al. 2007a, 2007b). In an earlier study (de Jonge et al., 2007b), we used PE in combination with the adjuvant cholera toxin as a positive control, and found that careful breeding for multiple generations on feed that excluded the protein of interest (PE) and cross-reactive proteins (soy) resulted in strong allergic responses in non-adjuvated animals that were similar to animals that were adjuvated with cholera toxin. In the current article, we repeat earlier results indicating that oral exposure to PE leads to specific antibody responses and specific lymphocyte proliferative responses, and in addition that oral exposure to PE results in gene expression profiles in the MLN that were associated with cell division and immunological processes.

Gene expression profiles demonstrate a dose-response relationship, exposure to 10 mg PE induced a more pronounced differential expression than exposure to 1 mg PE. In previous experiments it has been shown that PE-specific IgE levels (de Jonge et al., 2007a), and the number of eosinophilic and basophilic granulocytes (de Jonge et al., 2007b) were higher in the BN rats exposed to 10 mg PE compared to those exposed to 1 mg PE. In addition, the *ex vivo* proliferation rates of the MLNs of BN rats exposed to 10 mg PE were also higher than those of the 1 mg PE group.

Besides a dose-dependent effect, a time-dependent effect was also demonstrated. In both the 1 and 10 mg PE groups, the strongest differential gene expression was shown on Day 7. This might be explained by the observation that the T-lymphocyte response after the encounter with an antigen peaks after 7 days (Haring and

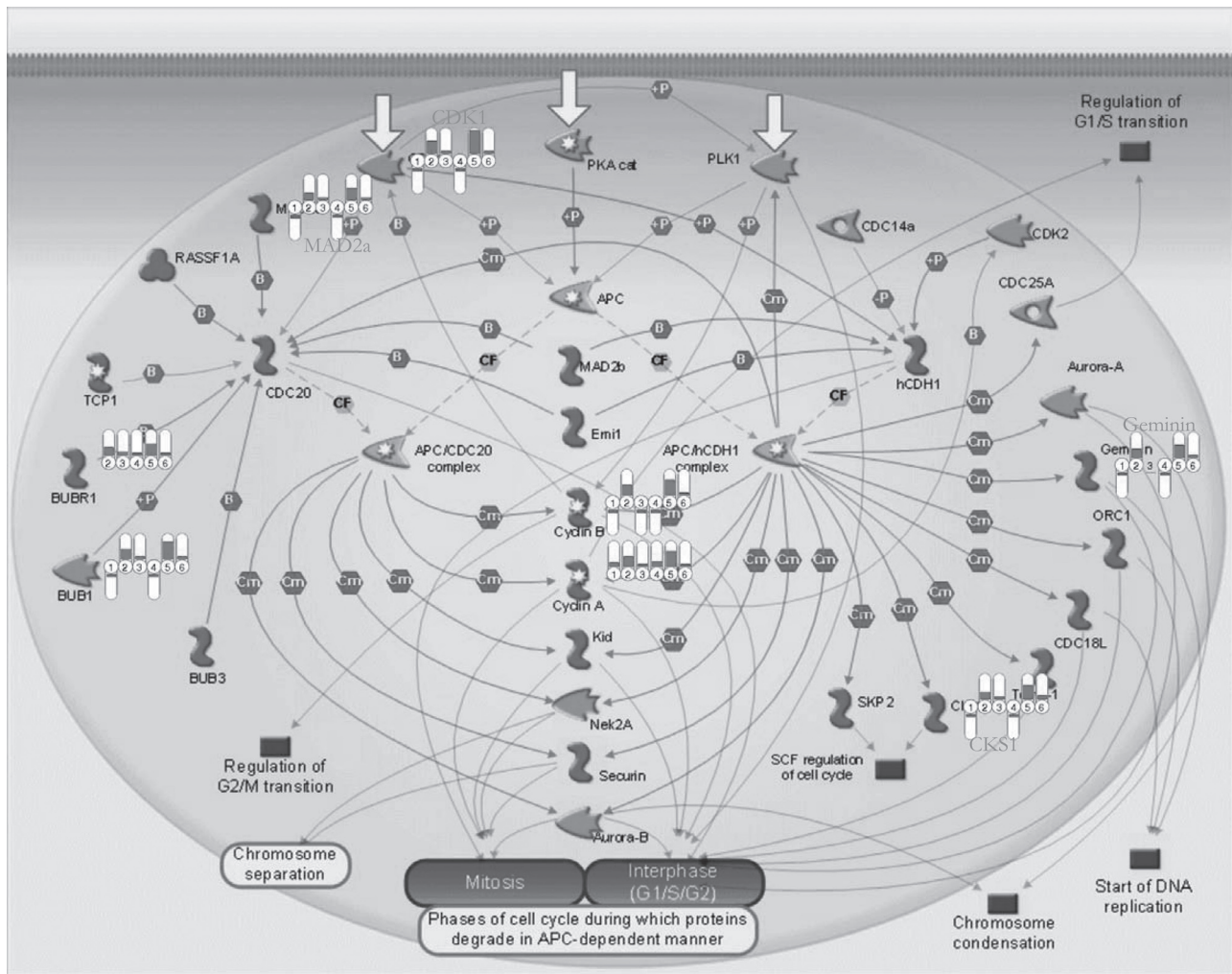


Figure 4. Effect of PE on role of anaphase-promoting complex (APC) in cell cycle regulation. Expression changes of several cell cycle-related genes are displayed in a pathway. The red (up-regulation) and green (down-regulation) bars represent the raw gene expression data. For each regulated gene, from left to right the first three bars and the last three bars represent Days 3, 7 and 14 of the 1 mg PE and the 10 mg PE groups, respectively. A legend of symbols can be found online ([http://www.genego.com/files/MC legend.pdf](http://www.genego.com/files/MC%20legend.pdf)).

Harty, 2006). Furthermore, analysis of Ig levels by ELISA showed that the PE-specific $^2\log$ IgG_{2a} and IgG₁ titers after PE-sensitization were higher on Day 14 than on Day 7. It should be mentioned that Days 3 and 7 are, in fact, too early to detect specific IgE responses, and even Day 14 is not optimal (de Jonge et al., 2007b). The early response in the MLN can be explained by the fact that the MLN is an immunologic organ that is at the frontline of the gut-associated immune response, whereas the production of food allergen-specific antibodies by B-lymphocytes takes place at a later moment in the response. Furthermore, changes in gene expression precede biological changes. Lastly, some regulation or some degree of oral tolerance could have occurred on Day 14.

In the MLN, several genes involved in immunological processes were found to be upregulated. Increased expression of genes encoding for the immunoglobulin components Ig (the variable light region) and Igj (the

joining segment) are in accordance with the observed increased levels of PE-specific IgG₁ in serum. Also, recent studies indicate that free light chains, not assembled into an immunoglobulin, are involved in the pathogenesis of allergies (Redegeld et al., 2004). Several genes, ADA, Tnfrs17 and Siva, known to be involved in lymphocyte functioning (Hatzoglou et al., 2000; Mishra et al., 2005; Gudi et al., 2006; Trem et al., 2006), were found to be up-regulated by PE. Gp96, is involved in innate and adaptive immune response (Rivoltin et al., 2003; Zhang et al., 2006).

Besides genes directly involved in immunological processes, a large number of cell cycle-related genes was affected by PE and the most regulated pathways were all involved in cell proliferation. In accordance with this are the dose-dependent effects of PE on lymphocyte proliferation in the MLN. In this conventional assay high variability was observed. This might indicate a higher sensitivity of

investigating numerous genes by the microarray technique as compared to conventional testing of cell proliferation.

The genes that we found stimulated in the current experiment could be involved in the allergic response against peanut, although it cannot be excluded that other antigen-specific immune responses, including oral tolerance, are also associated with regulation of these genes. That the genes found in this experiment are indicative of an immunological response of rats to an orally applied protein extract (in this case PE) is supported by the fact that almost all regulated genes were involved in cell proliferation or general immunological processes, but these genes do not discriminate between different types of immunity, i.e., do not specifically indicate allergy.

The only gene that was up-regulated and that is strongly associated with allergy, was the gene encoding for IgG₁ (Knippels et al., 1998; Faquim-Mauro et al., 1999). Further research is needed to determine whether the effect at the gene expression level found in our BN rat model was limited to an immunologic response, or could also be indicative of an allergic response. Subsequent studies should therefore include immunologically active, but non-allergenic proteins alongside known allergens such as peanut.

To date, no other studies have been conducted that determined the gene expression profiles in food allergy models. However, microarray data of respiratory allergy studies are available. Benson et al. (2006) performed a network-based analysis of DNA microarray data from allergen-challenged CD4⁺ T-lymphocytes from patients with seasonal allergic rhinitis. They found a strong up-regulation of expression of TNFRSF4, one of the genes of the TNF superfamily, which is involved in regulation of apoptosis and T_H2 proliferation. Interestingly, in our study we found the TNFRSF17 gene of the same superfamily to be up-regulated most strongly of all immunologic genes (Table 1). Although both studies differ: (a) one is a study into respiratory allergy, the other study is that of a food allergic model; (b) different species were studied; and, (c) Benson et al. (2006) studied allergen-specific T-lymphocytes, whereas we studied a pool of MLN cells. With both studies in mind, one could further substantiate the claim that the proliferation of allergen-challenged T_H2 cells is associated with an increase in TNFRSF-family signaling. However, an association of TNFRSF17 with T_H2 responses has not been made to date. In addition, in a mouse model for experimental asthma, gene expression profiling of lung tissue was performed (Zimmermann et al., 2003). We compared the regulated genes of this study with the results of our study and found overlap, especially at the levels of immunological genes (11 overlapping genes) and cell cycle genes (19 overlapping genes). This corroborates that both immunological and cell cycle genes are involved in allergen response reactions.

Gene expression profiles assessed in this food allergy model were compared to previous results of studies on

immunotoxicogenomics in rodents, performed at the National Institute of Public Health and the Environment. For this, Numerical Overlap Analysis of GenericGene Groups (NOAGGG) analysis, developed in house, was used. It was demonstrated that exposure to both hexachlorobenzene (HCB) (Ezendam et al., 2004) and bis(tri-*n*-butyltin)oxide (TBTO) (Baken et al., 2007a) affected several genes implicated in the cell cycle, and some of the genes induced by these compounds overlap with genes that were up-regulated in the food allergy model. TBTO, which is a known immunosuppressive chemical, down-regulated genes involved in cell proliferation (Baken et al., 2007a), whereas HCB, a pollutant known to stimulate the immune system, up-regulated cell cycle genes (Ezendam et al., 2004). Hence, when we compare gene expression profiles of known immunotoxic compounds with PE, it appears that a common target of immunosuppressive as well as immunostimulatory compounds is the cell cycle.

In conclusion, gene expression changes related to the regulation of immunological processes, most notably increased cell division were detected after oral exposure to peanut. This finding confirms that in the response to a foreign protein proliferation of immunologically relevant tissues is of great importance, and that such effects can be detected by analysis of gene expression profiles. Further studies using other food allergens, and proteins that are capable of inducing immune responses, yet fail to induce food allergic sensitization, will likely shed more light on specific genes and pathways indicative of food allergens and might thus be of value for both the mechanistic insight in the food allergic process as well as potential predictive screening purposes for potential allergens.

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Declaration of interest: The authors report no conflicts of interest.

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