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Spleen transcriptome profiles of BALB/c mouse in response to egg ovomucoid sensitisation and challenge

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Despite its growing prevalence in westernised countries, our current understanding of the molecular mechanism(s) of food allergy remains incomplete. In this study, transcriptome profile of spleen from BALB/c mice was analysed in response to egg ovomucoid (OVM) sensitisation and challenge. Microarray analysis revealed 87 genes (± 1.5 fold, $p \leq 0.05$); 28 up-regulated and 59 down-regulated. Gene ontology analysis revealed several biological processes related to hypersensitivity, inflammation or immune response. Biological network analysis illustrated connections among differentially expressed genes and several immune or hypersensitivity-related processes. Expression of five genes from microarray experiment was validated by real-time RT-PCR. Microarray analysis identified several differentially expressed genes that were not previously characterised in food allergic conditions. Results from this study may help to understand the underlying molecular events that take place in the spleen after OVM-stimulation, and may contribute as reference for future studies that may want to identify biomarkers of egg allergy.

Keywords: food allergy; microarray; ovomucoid; real-time RT-PCR; spleen; transcriptome

Introduction

Food allergy is a serious health concern that affects approximately 5% of the infants and young children in the western countries (Sicherer & Sampson, 2010). There are approximately 200 allergenic proteins that are primarily distributed in approximately 180 foods (Malarkey, 2003). These allergenic foods are mainly grouped into eight categories that include peanuts, tree nuts, cow's milk, hen's egg white, fish, shellfish, wheat and soy, and they typically cause about 90% of food allergy reactions (Malarkey, 2003). Allergy to these common foods always leads to considerable anxiety and fear of accidentally ingesting allergen-containing food products, thus, negatively affects individuals health-related quality of life. Currently, the only

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effective means to prevent food allergy is to avoid contact with allergenic foods; this can, however, may increase the risk of developing nutritional deficiency for children with multiple food allergies (Lieberman & Sicherer, 2011; Nowak-Węrzyn & Sampson, 2006).

Allergy to hen's egg is the second most common food allergy, affecting approximately 0.5–2.5% of children (Caubet, Kondo, Urisu, & Nowak-Węrzyn, 2011). Five different allergenic proteins have been identified from hen's egg white and these include ovomucoid (Gal d 1, OVM) that represents approximately 11% of the egg white protein, ovalbumin (Gal d 2, OVA), ovotransferrin (Gal d 3), lysozyme (Gal d 4) and ovomucin (Gal d 5) with OVM being the most immunodominant allergen (Caubet et al., 2011; Tey & Heine, 2009). Studies suggest that children with food allergy including egg allergy may also suffer from other types of allergies, for example, atopic dermatitis, rhinitis and asthma (Caubet et al., 2011; Celakovska, Ettlerova, Ettler, & Vaneckova, 2011; Lack, 2008). Therefore, accurate diagnosis of food allergy is essential; however, having multiple allergies often make it difficult for proper diagnosis of food allergy. The approaches that are currently available for diagnosis of food allergy included individual clinical history, physical examination, skin prick tests, detection of total and specific IgE and oral food challenges (Lock & Unsworth, 2011). Detection of food-specific IgE and skin prick tests are often used in combination with individual clinical history to diagnose food allergy. These tests, however, do not have very high predictive values and are not suitable for all patients (Caubet et al., 2011; Lock & Unsworth, 2011; Nicolaou & Custovic, 2011). Although double-blind placebo-controlled food challenges are considered to be the gold standard for diagnosis of food allergy, such tests are time-consuming, labour intensive, expensive and patients may have a risk of developing anaphylaxis (Lock & Unsworth, 2011; Nicolaou & Custovic, 2011; Yum et al., 2011). Therefore, it is imperative that new tools to be developed for clinicians to diagnose food allergy.

The spleen is the largest filter of the blood in the body and unique among the lymphoid organs. Within the spleen, erythrocytes and iron are recycled, blood-borne pathogens are captured and destroyed, and both innate and adaptive immune responses can be mounted in response to circulating antigens (Mebius & Kraal, 2005). In this study, for the first time we investigated the transcriptome profiles of spleen from BALB/c mice that had been sensitised and challenged with egg OVM. The aim of this study was to understand the effects of OVM on the gene expression profiles of an animal model. This study may help to better understand the genes and biological pathways that are involved in regulating the allergic response to OVM sensitisation and challenge in mammalian system.

Materials and methods

Animals

Animals used in this experiment were the same animals used in the study described by Hobson et al. (2007). Briefly, 30 female BALB/c mice of 6–8 weeks of age were purchased from Charles River (Montreal, QC, Canada) and divided randomly into control ($n = 15$) and experimental ($n = 15$) groups. Mice were maintained on an egg free diet in the pathogen-free conditions at the University of Guelph Central Animal Facility. Animals were maintained between 22–24°C and at a relative humidity of 40–

50%. The light-to-dark cycle was maintained at 12 h from 7 AM to 7 PM. All the animal experiments were approved by the University of Guelph Animal Care Committee according the Canadian Council on Animal Care guidelines.

Sensitisation and challenge of mice and harvest of spleen sample

Mice were sensitised and challenged as described by Hobson et al. (2007). Briefly, mice in the experimental group were sensitised by intra-gastric gavage (i.g.) twice every week for 5 weeks with OVM (1 mg/100 µl) and 10 µg of cholera toxin (CT) adjuvant. An amino acid (Sigma, St. Louis, MO) solution of 10 µg/µl (100 µl per mouse with 10 µg of CT) was used as a negative control. The amino acids were mixed accordingly to match the OVM amino acid composition. Five weeks after the first sensitisation, mice were challenged by i.p. injection with 1 mg of OVM in 50 µl of sterile water and aluminium hydroxide (50 µl, 2% Alhydrogel) adjuvant (Superfos Biosector, Denmark), to induce an immediate type I hypersensitivity reaction. The anaphylactic responses were visualised and scored depending on the response of the individual mice in each of the treated and control group. Mice were euthanised 40 min post-challenge with CO₂ and partial spleen tissue samples were collected in RNeasy lysis solution for microarray experiment (Ambion Inc., TX, USA). Spleen samples in RNeasy lysis solution were kept at 4°C overnight prior to long term storage at -80°C.

Immunological profiling of the mice

Immunological profiling (histamine, OVM-specific IgE and IgG) of all of the mice used in this experiment were carried out and reported by Hobson et al. (2007) to confirm the type I hypersensitivity response.

Total RNA extraction

Spleen tissues (15–20 mg) stored in RNeasy lysis solution were homogenised using Polytron Aggregate 7 mm homogeniser (Kinematica AG, Switzerland) in 600 µl of RNeasy lysis (RLT) buffer (QIAGEN Canada Inc., Mississauga, ON) with 6 µl of β-Mercaptoethanol added just before homogenisation. Total RNA from spleen tissues was isolated by using QIAGEN RNeasy Mini Kit (QIAGEN Canada Inc., Mississauga, ON), according to manufacturer's instruction. The concentration and quality of the RNA samples were determined by using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

Microarray hybridisation and scanning

Total RNA extracted from the spleens of control mice ($n = 15$) were randomly pooled into three control pools. In a similar way, total RNA extracted from the spleens of OVM sensitised and challenged mice ($n = 15$) were pooled randomly into three experimental RNA pools. Quality and concentrations of these pooled total RNA samples were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and a NanoDrop[®] ND-1000 spectrophotometer (Thermo Fisher

Scientific Inc., Wilmington, DE, USA). Six (3 control and 3 OVM challenged groups) GeneChip® Mouse Genome 430 2.0 arrays (Affymetrix Inc., CA, USA) were used for this study. Pooled RNA labelling, hybridisation, washing and scanning were performed according to manufacturer's instruction at the London Regional Genomics Centre (London, ON, Canada). All microarray data files from this study are available at the EMBL-EBI ArrayExpress database (<http://www.ebi.ac.uk/microarray-as/ae/>). ArrayExpress accession number for these files is E-MEXP-2796.

Analysis of microarray data

Microarray data were analysed by using the open-source statistical software, R (cran.rproject.org) and R software packages from the Bioconductor Project (www.bioconductor.org). All microarrays used in this experiment were of comparable qualities that were assessed by using the affyQCReport package. RNA integrity was confirmed by the Affymetrix RNA degradation controls (GAPDH and β -actin, 3'-5' ratios). Background subtraction, normalisation and summarisation of probe set intensities were carried out using the Gene Chip Robust Multi-Array Average (GCRMA) package for R from Bioconductor. This method corrects the background, normalises and summarises the data using the sequence-specific probe affinities and the total GC contents of the probes, and attains more accurate gene expression values. The log-2 transformed gene expression intensities obtained after background subtraction, normalisation and probe summarisation using GCRMA were further analysed using Significant Analysis of Microarray (SAM) software (Tusher, Tibshirani, & Chu, 2001) to obtain differentially expressed genes. Two tailed unpaired *t*-test was performed to determine statistical significance of the differentially expressed genes. The genes in the OVM sensitised and challenged group that had expression ratios of at least 1.5 fold (± 1.5 fold change) when compared to that of the control group with *p*-values of ≤ 0.05 were considered to be differentially expressed and statistically significant. Functional annotations of the differentially expressed genes were identified using NetAffx (Affymetrix, Santa Clara, California) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Dennis et al., 2003; Huang da, Sherman, & Lempicki, 2009). Gene Ontology (GO) analysis was carried out using GOMiner, and the GO biological processes that had Fisher's exact *p*-values of ≤ 0.05 were considered to be significantly enriched (Zeeberg et al., 2003). Pathway and biological network analysis was performed using the PathwayStudio 4.0 software (Ariadne Genomics Inc., MD, USA).

Real-time RT-PCR

The same pooled total RNA samples that were used for microarray experiment were used for real-time RT-PCR experiment. Total RNA was reverse-transcribed with SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) to prepare cDNA, according to manufacturer's instruction. Concentrations of the cDNA were determined using Quant-iT™ PicoGreen® Kit (Molecular Probe by Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction with a Victor 1420 Multilabel Counter machine (Perkin Elmer, MA, USA). Primers for the real-time RT-PCR were generated using Primer3 software (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Before conducting the real-time RT-PCR, regular PCR was

performed on each of the selected gene using Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) on a GeneAmp PCR System 9700 machine (Applied Biosystems, CA, USA) to check the primer efficacy and to optimise the amount of template and primers to be used for real-time RT-PCR. Real-time RT-PCR was conducted using the Platinum[®] SYBR[®] Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction in an ABI Prism 7000 Sequence Detection System machine (Applied Biosystems, CA, USA). Standard curves were prepared from serially diluted standard cDNAs prepared by pooling cDNAs from all of the control and treated cDNA samples. The Best-Keeper[©] (version 1) software (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004) was used to identify the most stably expressed housekeeping gene to be used as an internal reference. Data (CT values) generated from real-time RT-PCR experiment for the selected genes were analysed using Relative Expression Software Tool-384[©] (REST-384) version 1 and pair wise fixed reallocation randomisation test was performed to determine the fold changes and statistical significance (Pfaffl, Horgan, & Dempfle, 2002). The gene IDs, gene symbols and sequences of the forward and reverse primers are summarised in Table 1.

Results

Significantly differentially expressed genes and their biological functions

The genes that were differentially expressed with a fold change of at least ± 1.5 in the spleen of OVM sensitised and challenged BALB/c mice with a statistical significance

Table 1. Sequences of the forward and reverse primers for the differentially expressed and housekeeping genes considered for the real-time RT-PCR experiment.

Accession ID	Gene symbol	Direction	Primer sequences
<i>OVM allergy specific differentially regulated genes</i>			
BC008237	<i>Igh4</i>	Forward	5' ACCCTGTTCTCTGCAAATGAC
BC008237	<i>Igh4</i>	Reverse	5' GGGCCAGTGGATAGACTGAT
NM_031168	<i>Il6</i>	Forward	5' AACGATGATGCACTTGCAGA
NM_031168	<i>Il6</i>	Reverse	5' TGTTCTTCATGTACTCCAGGTAGC
M94967	<i>Ptgs2</i>	Forward	5' TTCTTTGCCAGCACTTCAC
M94967	<i>Ptgs2</i>	Reverse	5' ATCCTTGAAAAGGCGCAGT
NM_013459	<i>Adn</i>	Forward	5' TGCACAGCTCCGTGTACTTC
NM_013459	<i>Adn</i>	Reverse	5' CACGGAAGCCATGTAGGG
BB213876	<i>Car 3</i>	Forward	5' TCTTATGACCCTGGCTCTGC
BB213876	<i>Car 3</i>	Reverse	5' AGAGGAGCCCCAGTGAAGAT
<i>Housekeeping/reference genes</i>			
NM_007393	<i>Actb</i>	Forward	5' CAGCTTCTTTGCAGCTCCTT
NM_007393	<i>Actb</i>	Reverse	5' ACGATGGAGGGGAATACAGC
M60978	<i>Gapdh</i>	Forward	5' ACAAAGGAAACGTGGAACAT
M60978	<i>Gapdh</i>	Reverse	5' TGCCTCGATGGACAGATAGA
NM_013556	<i>Hprt1</i>	Forward	5' CCGGCTTCCTCCTCAGAC
NM_013556	<i>Hprt1</i>	Reverse	5' TTTCCAAATCCTCGGCATA
BF715219	<i>B2m</i>	Forward	5' CCTGGTCTTTCTGGTGCTTG
BF715219	<i>B2m</i>	Reverse	5' TATGTTCGGCTTCCCATTCT

of $p \leq 0.05$ were considered to be responsive to OVM sensitisation and challenge. With these selection criteria, 94 individual probes were found to be differentially expressed. On the basis of NetAffx (Affymetrix) annotation and NCBI's Unigene database, these 94 probes represented 87 unique genes; 28 genes were up-regulated and 59 genes were down-regulated (Table 2).

Sixty-seven of these 87 genes were classified into nine major functional categories that included immune/inflammatory response, cell cycle regulation, cell signaling, metabolic process, tissue/muscle development, transcription and translation regulation, protein metabolism/regulation, ion and other small molecule transport, and DNA binding/chromatin organisation (Table 2). The rest of the 20 genes were classified as 'other' category of which 14 genes do not have any known functional annotations. Two of these differentially expressed genes were represented by multiple probes, for example, immunoglobulin heavy chain 4 or *Igh4* (also known as immunoglobulin heavy chain 6/*Igh6* or immunoglobulin heavy constant gamma 1/*Ighg1/IgG1*) was represented by five probes and prostaglandin-endoperoxide synthase 2 or *Ptgs2* (also known as *Cox2*) was represented by two probes, and all of the probes for these two genes were found to be up-regulated. Other interesting differentially expressed genes included interleukin 6 (*Il6*; 1.78 fold; $p \leq 0.02$), adipisin (*Adn*) or complement factor D (*Cfd*; 1.71 fold; $p \leq 0.03$), carbonic anhydrase 3 (*Car3*; 7.56 fold; $p \leq 0.02$), chemokine (C-X-C motif) ligand 10 (*Cxcl10*; -1.55 fold, $p \leq 0.02$) and serine (or cysteine) peptidase inhibitor, clade H, member 1 (*Serpinh1*; -1.99; $p \leq 0.01$).

GO and network analysis of the differentially expressed genes

Gene Ontology analysis was conducted to better understand the biological functions of the genes differentially expressed in response to OVM sensitisation and challenge. GO analysis revealed at least 83 significantly enriched biological processes that were related with immune, inflammatory or hypersensitivity responses. Some of these top enriched immune responsive GO biological processes included 'immune system process' [GO:0002376], 'immune response' [GO:0006955], 'immune effector process' [GO:0002252] and 'response to wounding' [GO:0009611] (Figure 1). Detailed analysis further revealed that majority of these immune response or hypersensitivity-related GO biological processes were enriched with a group of 11 genes that included *Adn*, *Cd47*, *Cenpf*, *Csf3r*, *Cxcl10*, *Dab2*, *Gna13*, *Igh4*, *Il6*, *Oas1a* and *Spnal* (Table 3).

A biological network was generated with the differentially expressed genes (Figure 2). This network identified several affected biological pathways and disease processes, and illustrated the involvement these differentially expressed genes including *Igh4*, *Ptgs2*, *Il6*, *Serpinh1* and *Cxcl10* with hypersensitivity, immune response, inflammatory or asthmatic conditions.

Validation of the differentially expressed genes by real-time RT-PCR

Real-time RT-PCR was conducted to validate the differential expression of five representative genes from microarray experiment. Four genes β -actin (*Actb*), beta-2-microglobulin (*B2m*), hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were initially selected to

Table 2. Genes significantly differentially expressed in the spleen of BALB/c mice in response to OVM sensitisation and challenge, and their biological functions.

Accession ID	Gene name	Gene symbol	Fold change	p-Values	Biological function
<i>Immune/inflammatory response</i>					
K00686	Immunoglobulin heavy chain 4	Igh4	3.38	0.01	Adaptive immune response
BB254163	CD47 antigen	Cd47	1.89	0.01	Phagocytosis
NM_013459	Adipsin (Complement factor d)	Adn (Cfd)	1.71	0.03	Acute inflammatory response
BB740660	Spectrin alpha 1	Spna1	1.72	0.02	Immune system development
BC018470	2'-5' oligoadenylate synthetase 1A	Oas1a	-1.53	0.04	Immune response
NM_007782	Colony stimulating factor 3 receptor (granulocyte)	Csf3r	1.55	0.04	Chemotaxis
NM_021274	Chemokine (C-X-C motif) ligand 10	Cxcl10	-1.55	0.02	Chemotaxis
NM_031168	Interleukin 6	Il6	1.78	0.02	Acute inflammatory response
NM_011558	T-cell receptor gamma, variable 2	Tcrg-V2	-1.65	0.01	T-cell receptor
NM_010742	Lymphocyte antigen 6 complex, locus D	Ly6d	-1.63	0.03	B-cell development
<i>Cell cycle regulation</i>					
NM_009829	Cyclin D2	Ccnd2	-1.61	0.02	G1/S transition of mitotic cell cycle
BE848253	Centromere protein F	Cenpf	1.58	0.03	G2 phase of mitotic cell cycle
NM_008613	Meiosis-specific nuclear structural protein 1	Mns1	-1.81	0.05	M phase
AV316937	Shugoshin-like 2	Sgol2	1.59	0.03	M phase
BC006588	Disabled homolog 2	Dab2	1.76	0.03	Cell morphogenesis
BE368753	Growth arrest and dna-damage-inducible, gamma interacting protein 1	Gadd45gip1	-1.51	0.03	Cell cycle
AW743721	VDNA-damage regulated autophagy modulator 2	Dram2	-1.56	0.05	Apoptosis
<i>Cell signaling</i>					
BM211430	A kinase (PRKA) anchor protein 13	Akap13	1.60	0.03	Regulation of RAS protein signal transduction

Table 2 (Continued)

Accession ID	Gene name	Gene symbol	Fold change	<i>p</i> -Values	Biological function
BB136172	DEP domain containing 1B	Depdc1b	1.71	0.03	Intracellular signaling cascade
NM_133753	ERBB receptor feedback inhibitor 1	Errfi1	1.52	0.04	Stress-activated protein kinase signaling pathway
BG066967	RAB20, member RAS oncogene family	Rab20	−1.65	0.02	Small gtpase mediated signal transduction
NM_033616	Component of Sp100-RS	Csprs	−1.64	0.03	G-protein coupled receptor protein signaling pathway
<i>Metabolic process</i>					
AU017520	Oviductal glycoprotein 1	Ovgp1	2.19	0.04	Polysaccharide metabolic process
NM_007833	Decorin	Dcn	1.57	0.05	Aminoglycan metabolic process
AV307219	Galactose mutarotase	Galm	−1.51	0.01	Hexose metabolic process
NM_013743	Pyruvate dehydrogenase kinase, isoenzyme 4	Pdk4	1.59	0.05	Glucose metabolic process
BM235734	Malic enzyme 2, NAD(+)-dependent, mitochondrial	Me2	−1.61	0.02	Dicarboxylic acid metabolic process
NM_007606	Carbonic anhydrase 3	Car3	7.56	0.02	One-carbon metabolic process
<i>Tissue/muscle development</i>					
M94967	Prostaglandin-endoperoxide synthase 2	Ptgs2	2.28	0.05	Skeletal system development
BG094302	Guanine nucleotide binding protein, alpha 13	Gna13	1.81	0.04	Angiogenesis
BB283590	Zinc finger protein X-linked	Zfx	−1.57	0.04	Ovarian follicle development
NM_029791	Bicaudal D homolog 2	Bicd2	−1.61	0.03	Microtubule-based process
AK013867	Kinesin family member 18B	Kif18b	1.99	0.05	Microtubule-based process
AW208944	Proteasome 26S subunit, ATPase, 6	Psmc6	−1.64	0.01	Macromolecule catabolic process
BB329489	Serine (or cysteine) peptidase inhibitor, clade H, member 1	Serpinh1	−1.99	0.01	Extracellular matrix organisation

Transcription and translation regulation

Table 2 (Continued)

Accession ID	Gene name	Gene symbol	Fold change	<i>p</i> -Values	Biological function
NM_008321	Inhibitor of DNA binding 3	Id3	-1.62	0.01	Regulation of DNA replication
AK014353	KH domain containing, RNA binding, signal transduction associated 3	Khdrbs3	-1.59	0.02	Regulation of transcription
AW488885	Kruppel-like factor 9	Klf9	1.51	0.01	Intracellular signaling cascade
BB550311	Polymerase (RNA) II (DNA directed) polypeptide A	Polr2a	-1.68	0.01	Transcription
BM246377	Transcription factor Dp 2	Tfdp2	1.56	0.04	Regulation of transcription
AV172851	Zinc finger protein 455	Zfp455	-1.69	0.05	Regulation of transcription
AK007652	Mitochondrial ribosomal protein L44	Mrpl44	-1.62	0.03	RNA processing
BC021443	Small nuclear ribonucleoprotein 48 (U11/U12)	Snrnp48	-1.54	0.02	mRNA processing
BM238943	Glutamyl-prolyl-tRNA synthetase	Eprs	-1.53	0.03	Translation
BB204225	Tyrosyl-tRNA synthetase 2	Yars2	-1.93	0.02	Translation
AV031454	Mitochondrial ribosomal protein S2	Mrps2	-1.60	0.01	Translation
BB283415	Ribosomal protein L30	Rpl30	1.68	0.02	Translation
AV097965	Tu translation elongation factor, mitochondrial	Tufm	-1.52	0.01	Translational elongation
<i>Protein metabolism/regulation</i>					
AL024092	Chaperonin containing Tcp1, subunit 3 (gamma)	Cct3	-1.64	0.02	Protein folding
C79445	Tubulin, beta 2a, pseudogene 2	Tubb2a-ps2	-1.78	0.01	Protein complex assembly
BC008532	Bone marrow stromal cell antigen 2	Bst2	-1.82	0.03	Protein amino acid phosphorylation
AK008118	Protein arginine N-methyltransferase 3	Prmt3	-1.63	0.04	Protein amino acid methylation
AK018606	SUMO/sentrin specific peptidase 8	Senp8	-1.55	0.03	Proteolysis
BB746075	Dipeptidylpeptidase 7	Dpp7	-1.51	0.05	Proteolysis

Table 2 (Continued)

Accession ID	Gene name	Gene symbol	Fold change	<i>p</i> -Values	Biological function
AI314028	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	P4ha1	-2.02	0.03	Cellular amino acid derivative metabolic process
BC012406	Anterior pharynx defective 1a homolog (<i>C. elegans</i>)	Aph1a	1.51	0.01	Urogenital system development
NM_008281	Hepsin	Hpn	-1.58	0.02	Proteolysis
<i>Ion and other small molecule transport</i>					
BB456860	FK506 binding protein 4	Fkbp4	-1.72	0.02	Transition metal ion transport
BB449960	Transcobalamin 2	Tcn2	-1.52	0.04	Transition metal ion transport
BG065288	INO80 complex subunit E	Ino80e	-1.52	0.03	Regulation of calcium ion-dependent exocytosis
AK014572	Solute carrier family 6, member 9	Slc6a9	1.56	0.03	Neurotransmitter transport
NM_053086	Nucleolar and coiled-body phosphoprotein 1	Nolc1	-1.51	0.01	Lipid transport
BC018188	ERGIC and golgi 2	Ergic2	-1.62	0.01	ER to Golgi vesicle-mediated transport
<i>DNA binding/chromatin organisation</i>					
AU015341	Chromodomain helicase DNA binding protein 8	Chd8	-1.66	0.04	In utero embryonic development
BM230202	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	Smarca2	-1.61	0.05	DNA packaging
AI987819	High mobility group 20A	Hmg20a	-1.54	0.02	Chromatin organisation
AK002491	RIKEN cDNA 0610010K14 gene	0610010K14Rik	-1.70	0.02	DNA binding
<i>Other</i>					
NM_020282	NAD(P)H dehydrogenase, quinone 2	Nqo2	-1.52	0.05	Oxidation reduction
BM218877	A kinase (PRKA) anchor protein 5	Akap5	-1.58	0.01	Protein kinase binding

Table 2 (Continued)

Accession ID	Gene name	Gene symbol	Fold change	<i>p</i> -Values	Biological function
BG070246	DNA segment, Chr 13, ERATO Doi 608, expressed	D13Ert608e	-1.54	0.01	—
AV302436	RIKEN cDNA 2410127L17 gene	2410127L17Rik	-1.62	0.03	—
BB140256	RIKEN cDNA D930015E06 gene	D930015E06Rik	1.50	0.05	—
BM119088	TLC domain containing 1	Tlcd1	1.57	0.01	—
BB314680	TatD DNase domain containing 2	Tatdn2	1.54	0.01	Endonuclease activity
BQ174624	UBX domain protein 2B	Ubxn2b	-1.63	0.03	Adapter protein for Golgi and endoplasmic reticulum biogenesis
BB326394	Butyrophilin-like 9	Btnl9	-1.70	0.05	—
BB051286	Coiled-coil domain containing 86	Ccdc86	-1.59	0.04	—
BB812902	Deleted in lymphocytic leukaemia, 2	Dleu2	-1.57	0.01	—
BC021511	Glutamine and serine rich 1	Qser1	-1.52	0.03	—
AI642973	Myc target 1	Myct1	-1.67	0.05	—
BC019407	Occludin/ELL domain containing 1	Ocell	-1.63	0.02	—
AK012261	Family with sequence similarity 118, member B	Fam118b	-1.52	0.04	—
BC005560	X-linked lymphocyte-regulated complex	Xlr	-1.68	0.01	—
BB667930	Stefin a2 like 1	Stfa2l1	3.45	0.02	Cysteine-type endopeptidase inhibitor
NM_016737	Stress-induced phosphoprotein 1	Stip1	-1.66	0.01	Binding
BB745549	Transmembrane protein 30a	Tmem30a	-1.58	0.05	—
BM232203	—	—	-1.69	0.03	—

identify the most stably expressed house-keeping gene for normalisation of the experimental genes. Beta-actin turned out to be the most stably expressed house-keeping gene and therefore, considered as the internal reference. The genes selected for validation included *Igh4*, *Ptgs2*, *Il6*, *Car3* and *Adn*, and were selected based on their fold change, statistical significance and biological relevance to allergy. The fold changes and *p*-values of all of the selected genes from the microarray experiment were in agreement with those from the real-time RT-PCR experiment (Table 4).

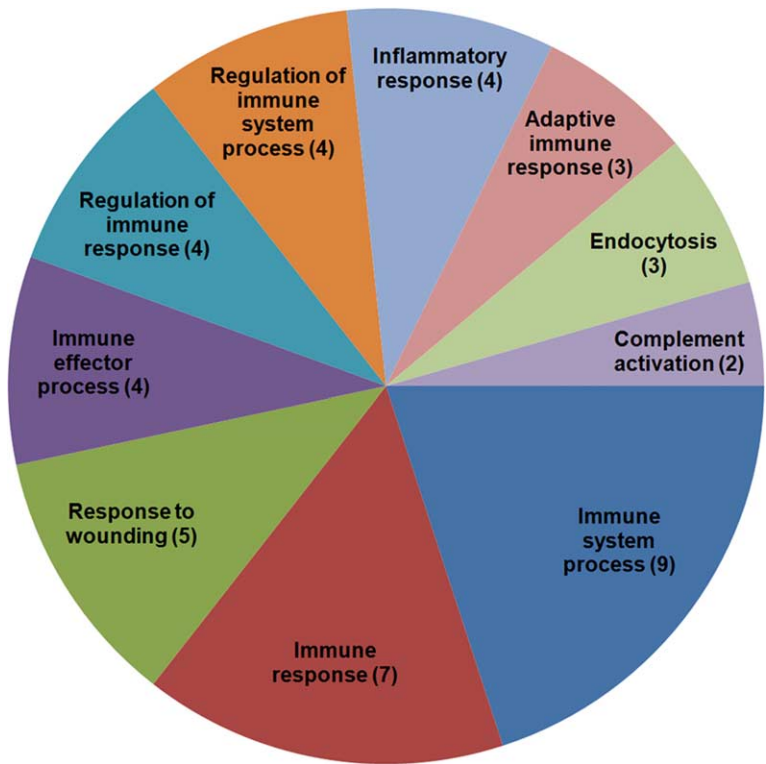


Figure 1. Immune or hypersensitivity-related GO biological processes enriched with the differentially expressed genes. Numbers in the parenthesis in each of GO process indicate the number of differentially expressed genes enriched with each of the process.

Discussion

In this study, we sought to investigate the transcriptome profiles of the spleen from BALB/c mice that was sensitised and challenged with egg OVM, the dominant allergen of hen's egg. The spleen was considered because it is the largest filter of the blood and allows erythrocyte phagocytosis, iron recycling, capture and immune responses to circulating antigens. In this study, female BALB/c mice were sensitised by intra-gastric (i.g.) gavage and challenged by intra-peritoneal (i.p.) injection with OVM and CT. Microarray analysis revealed some known and some previously uncharacterised genes in food allergic conditions in the spleen of these OVM sensitised and challenged mice that will add to the molecular mechanisms of food allergy in the mammalian system.

Among the differentially expressed genes up-regulation of *Igh4*, *Ptgs2*, *Cd47* and *Il6*, and simultaneous down-regulation of *Cxcl10* in BALB/c mice after OVM sensitisation and challenge indicated the activation of Th2 and inhibition of Th1 pathways respectively, as the role of these genes in immune responsive processes is well documented (Chacon et al., 2005; Conti et al., 2002; Mix, Goertsches, & Zett, 2006; Romagnani et al., 2005; Sick, Niederhoffer, Takeda, Landry, & Gies, 2009). In order to identify the biological functions of other differentially expressed genes GO analysis was performed. GO analysis revealed a group of 11 genes that played

Table 3. Enrichment of several top immune response-related GO biological processes and the corresponding immune responsive genes.

Gene symbol	Gene name	Fold change	<i>p</i> -Value	Enriched GO biological processes
<i>And</i>	Adipsin	1.71	0.03	Immune system process [GO:0002376]; Immune response [GO:0006955]; Response to wounding [GO:0009611]; Immune effector process [GO:0002252]; Regulation of immune response [GO:0050776]; Regulation of immune system process [GO:0002682]; Inflammatory response [GO:0006954]; Complement activation [GO:0006956]
<i>Cd47</i>	CD47 antigen	1.89	0.01	Immune system process [GO:0002376]; Immune response [GO:0006955]; Immune effector process [GO:0002252]; Endocytosis [GO:0006897]
<i>Cenpf</i>	Centromere protein F	1.58	0.03	Immune system process [GO:0002376]; Immune response [GO:0006955]; Immune effector process [GO:0002252]; Regulation of immune response [GO:0050776]; Regulation of immune system process [GO:0002682]; Adaptive immune response [GO:0002250]
<i>Csf3r</i>	Colony stimulating factor 3 receptor	1.55	0.04	Immune system process [GO:0002376]
<i>Cxcl10</i>	Chemokine (C-X-C motif) ligand 10	−1.55	0.02	Immune system process [GO:0002376]; Immune response [GO:0006955]; Response to wounding [GO:0009611]; Inflammatory response [GO:0006954]
<i>Dab2</i>	Disabled homolog 2	1.76	0.03	Endocytosis [GO:0006897]
<i>Gna13</i>	Guanine nucleotide binding protein, alpha 13	1.81	0.04	Response to wounding [GO:0009611]
<i>Igh4</i>	Immunoglobulin heavy chain 4 (serum IgG1)	3.38	0.01	Immune system process [GO:0002376]; Immune response [GO:0006955]; Response to wounding [GO:0009611]; Immune effector process [GO:0002252]; Regulation of immune response [GO:0050776]; Regulation of immune system process [GO:0002682]; Inflammatory response [GO:0006954]; Adaptive immune response [GO:0002250]; Endocytosis [GO:0006897]; Complement activation [GO:0006956]

Table 3 (Continued)

Gene symbol	Gene name	Fold change	<i>p</i> -Value	Enriched GO biological processes
<i>Il6</i>	Interleukin 6	1.78	0.02	Immune system process [GO:0002376]; Immune response [GO:0006955]; Response to wounding [GO:0009611]; Regulation of immune response [GO:0050776]; Regulation of immune system process [GO:0002682]; Inflammatory response [GO:0006954]; Adaptive immune response [GO:0002250]
<i>Oas1a</i>	2'-5' oligoadenylate synthetase 1A	-1.53	0.04	Immune system process [GO:0002376]; Immune response [GO:0006955]
<i>Spna1</i>	Spectrin alpha 1	1.72	0.02	Immune system process [GO:0002376]

significant roles in the enrichment of majority of the immune response-related GO biological processes. Enrichment of these immune or hypersensitivity response-related GO processes suggested the possible involvement of these differentially expressed genes in the OVM-induced food allergy.

The network analysis (Figure 2) was conducted to understand the role of differentially expressed genes in more detail and their biological relevance with the OVM sensitisation and challenge in BALB/c mice. Genes that demonstrated most connections in this network were *Il6* and *Ptgs2*. Involvement of these two genes in hypersensitivity, inflammation and other immune responsive processes is well documented. For example, the multifunctional cytokine IL-6 is known to be involved in immune regulation, hematopoiesis, inflammation, oncogenesis, mast cell maturation and up-regulation of histamine production (Conti et al., 2002; Gabay, 2006; Naka, Nishimoto, & Kishimoto, 2002). Additionally, IL-6 along with TGF- β has recently been implicated to play a pivotal role in the differentiation of pro-inflammatory Th17 cells and induction of autoimmune diseases (Basso, Cheroutre, & Mucida, 2009; Kimura & Kishimoto, 2011). Few recent studies demonstrated the involvement of IL-6 in the process of ovalbumin- and peanut-induced food allergy (Valeur et al., 2009; Wang et al., 2010). Another previous study reported an increased secretion of IL-6 along with other cytokines including IL-4, IL-13 and IFN- γ from T cell lines stimulated with OVM (Holen, Bolann, & Elsayed, 2001). Therefore, up-regulation of *Il6* in our study in both microarray and real-time RT-PCR experiments supported its possible involvement in OVM-induced food allergy. Similarly, prostaglandin-endoperoxide synthase 2 (*Ptgs2*), also known as cyclooxygenase 2 (*Cox2*) is the inducible isoform of COX enzyme that is involved in the production of prostaglandins associated with the inflammatory process (Chacon et al., 2005). The role of *Ptgs2* in asthma and airway smooth muscle inflammation is well documented (Carey, Germolec, Langenbach, & Zeldin, 2003; Chacon et al., 2005). Prostaglandin E2 synthesised by *Ptgs2* can inhibit the production of the Th1 cytokines and supports a Th2 cytokine response that may lead to allergic stress (Carey et al., 2003). Furthermore, Knoferl et al. (2001), reported the activation of *Ptgs2* and up-regulation of cytokine IL-6 in Kupffer cell of C3H/HeN mice after

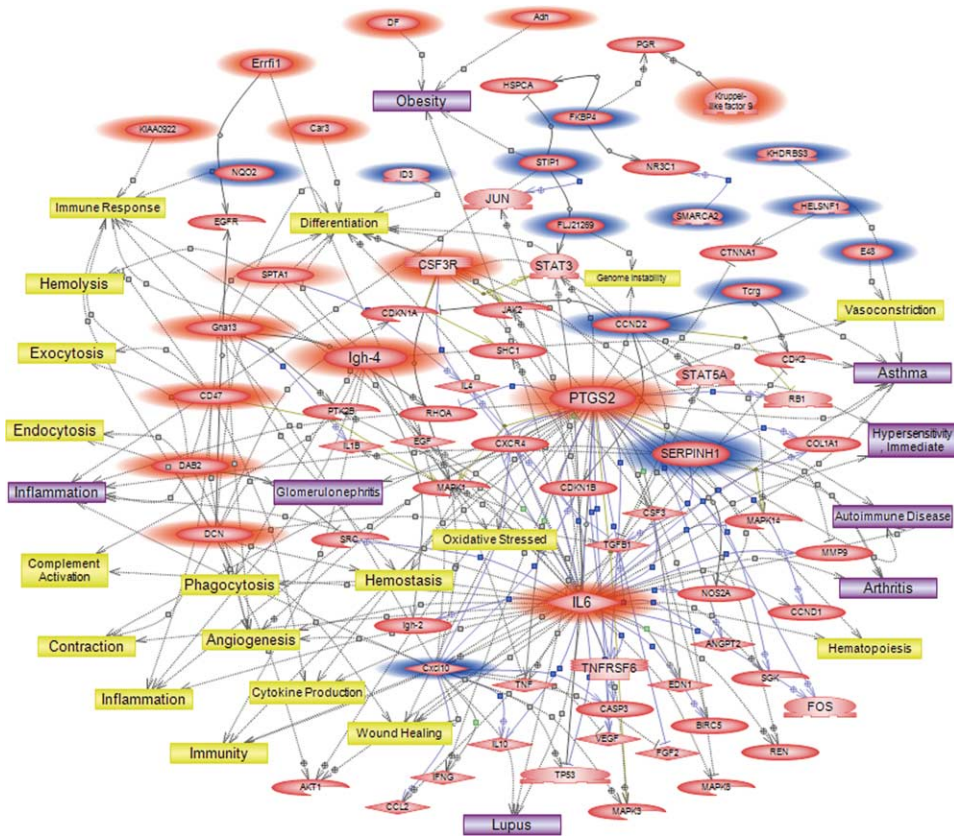


Figure 2. Biological network of the genes that were significantly differentially expressed in the spleen of BALB/c mice in response to OVM sensitisation and challenge. The genes indicated with a red shadow were up-regulated and with a blue shadow were down-regulated. The downstream effects of the genes are indicated in rectangular boxes. Yellow boxes represent the cell processes and the purple boxes represent the diseases. Inducing or up-regulatory effects between two genes are indicated with '→' symbol, and inhibitory or down-regulatory effects are indicated with '⊥' symbol.

trauma-haemorrhage and subsequent sepsis. Such co-ordinated up-regulation of *Ptgs2* and *Il6* was also observed in our study in both microarray and real-time RT-PCR experiments that indicated the involvement of these two genes in the process of OVM-induced food allergy.

Among other genes, serine or cysteine peptidase inhibitor, clade H, member 1 (*Serpinh1*) demonstrated significant connections in the biological network, where *Serpinh1* indicated an inhibitory effect on the gene nitric oxide synthase 2 (*Nos2a*) (Figure 2). Nitric oxide synthase 2 is known to be involved in the production of nitric oxide (NO) under oxidative stress that in turn generates strong oxidising agents. These reactive nitrogen species amplify the inflammatory response in asthma and may act as a pro-inflammatory mediator (Ricciardolo, Nijkamp, & Folkerts, 2006). In our study, *Serpinh1* was down-regulated; as a result, it was unable to exert its inhibitory effect on *Nos2a*, thus, *Nos2a* was able to induce its pro-inflammatory

Table 4. Differential expression of five representative genes from microarray experiment was validated by real-time RT-PCR.

Accession ID	Gene symbol	Gene name	Real-time RT-PCR		Microarray	
			Fold change	p-Value	Fold change	p-Value
NM_013459	<i>Adn</i>	Adipsin	7.13	0.01	1.71	0.03
BB213876	<i>Car3</i>	Carbonic anhydrase 3	4.57	0.01	7.56	0.02
BC008237	<i>Igh4</i>	Immunoglobulin heavy chain 4	3.66	0.02	3.38	0.01
NM_031168	<i>Il6</i>	Interleukin 6	4.12	0.01	1.78	0.02
M94967	<i>Ptgs2</i>	Prostaglandin-endoperoxide synthase 2	21.51	0.01	2.28	0.05

effects. Such co-ordination was in agreement with the induction of allergic response and provided an explanation for the down-regulation of *Serpinh1* in our study. Another down-regulated gene *Cxcl10* demonstrated considerable connections in the network. Chemokine CXCL10 is an interferon-gamma-inducible (IFN- γ) protein and a member of the α -chemokine subfamily. It is involved in delayed-type hypersensitivity, induction of IFN- γ gene expression and recruitment of Th1 cells into inflamed tissues (Azzurri et al., 2005; Romagnani et al., 2005). The Th1 cytokine IFN- γ is known exert antagonistic effects to the Th2 cytokines IL-4, IL-5 and IL-13 that are the hallmarks of type I hypersensitivity response (Morafo et al., 2003). Thus, the down-regulation of *Cxcl10* in this study was in agreement with previous findings related to type I hypersensitivity response. Therefore, this biological network illustrated the involvement of the differentially expressed genes with hypersensitivity, immune response, inflammation and asthma, and provided an opportunity to better understand the biological roles of these genes in the process of OVM-induced food allergy.

Gene *Igh4* was up-regulated 3.38 fold in microarray experiment and 3.66 fold in real-time RT-PCR experiment ($p < 0.05$; Table 4). In mice, induction of IgG1 isotype indicates the activation of Th2 cytokines that are required for immunoglobulin class switching during an allergic response (Mix et al., 2006). Immunoglobulin IgG1 had previously been reported to be up-regulated in murine models of food allergy, along with the up-regulation of immunoglobulin IgE and Th2 cytokines (Adel-Patient, Bernard, Ah-Leung, Creminon, & Wal, 2005; Akiyama et al., 2001). Such up-regulation of *Igh4* in our study indicated the immunogenicity of OVM and possible activation of Th2 cytokines in response to OVM sensitisation and challenge (Dearman & Kimber, 2005).

Gene *Adn* is also known as complement factor D (*Cfd*), a serine protease that is indispensable for complement activation and opsonisation. Complement factor D catalyses the initial proteolytic step necessary for the activation of alternative complement pathway and activates factor B that leads to subsequent cleavage of C3 into C3a (anaphylatoxin) and C3b (Xu et al., 2001). Anaphylatoxins trigger the release of histamine from basophils and mast cells, play a role in immune and allergic

response, and contribute to polarising T-cells towards a Th2 phenotype (Esterbauer, Krempler, Oberkofler, & Patsch, 1999; Hawlisch, Wills-Karp, Karp, & Kohl, 2004; Taylor et al., 1999; Xu et al., 2001). Recent studies suggest its possible involvement in allergic rhinitis and glomerulonephritis, a type 3 hypersensitivity reaction (Abrera-Abeleda, Xu, Pickering, Smith, & Sethi, 2007; Ciprandi, De Amici, & Marseglia, 2009); however, its role in food allergy is still unknown. In our study, we observed an up-regulation of *Adn* in both microarray and qPCR experiments that strongly suggested its involvement in the OVM-induced food allergy.

Carbonic anhydrase 3 (*Car3*) is a well known cytosolic protein that is abundant in skeletal muscle, adipocytes and liver. The mammalian carbonic anhydrases reversibly hydrates carbon dioxide, generating both bicarbonate and hydrogen ions to maintain pH homeostasis (Sly & Hu, 1995). Carbonic anhydrase 3 is rapidly glutathionylated *in-vivo* and *in-vitro* when cells are exposed to oxidative stress; this role suggests that *Car3* may function as a member of the defence system against oxidative stress and reactive oxygen species (Kim et al., 2004; Zimmerman, Wang, Zhang, Bogdanovich, & Forster, 2004). Hypoxia is a condition that is induced during anaphylaxis, and available reports indicate that the generation of high levels of oxygen free radicals during anaphylaxis, bronchial asthma and nocturnal asthma exacerbates these conditions (Jarjour, Busse, & Calhoun, 1992; Weiss & Bellino, 1986). Given this, up-regulation of *Car3* in both microarray and real-time RT-PCR experiments suggested that it may play a protective role against hypoxia that might have induced in mice after OVM challenge.

Conclusion

Results from this study illustrate that the genes differentially expressed in response to OVM sensitisation and challenge in the spleen of BALB/c mice may play role in the process of egg allergy. This study results further suggest that the genes *Adn* and *Car3* that were previously not known to be involved in food allergy may play roles in the process of OVM-mediated food allergy along with other pro-inflammatory or pro-allergy genes including *Serpinh1*, *Cxcl10*, *Igh4*, *Il6* and *Ptgs2*. Results from this study may provide a valuable insight into the molecular events that take place in egg allergy conditions. In future these results may contribute to the generation of a reference database of biomarkers of allergic response that may help the identification of potential allergenicity of other food proteins.

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