

The Genome-Wide Expression Profile of Electroacupuncture in DNP-KLH Immunized Mice

Sung-Hwa Sohn · Sun Kwang Kim · Eunjung Ko · Youngseop Lee ·
Hwan-Suck Chung · Hyojung Lee · Hyunseong Kim ·
Deok-Sang Hwang · Sangsoo Nam · Hyunsu Bae

Received: 12 October 2009 / Accepted: 11 December 2009
© Springer Science+Business Media, LLC 2009

Abstract Previously, we demonstrated that electroacupuncture (EA) suppressed allergic reactions in DNP-KLH immunized mice. In this study, the mechanisms by which EA induces immunomodulation in the immunized mice were evaluated by genome-wide microarray analysis. The anti-allergic effects of EA in DNP-KLH immunized mice were confirmed by analyzing antigen specific IgE using ELISA. Microarray analysis, followed by real time RT-PCR validation, revealed that Th1 and Th17 cytokine-, opioid peptide-, and anti-apoptosis-related genes were up-regulated

upon treatment with EA. In addition, significant decreases in Th2 cytokine-, MAPK signaling pathway-, and apoptosis-related genes were observed following EA treatment.

Keywords Electroacupuncture · Gene expression profile · IgE · Microarray

Introduction

Several studies have demonstrated that electroacupuncture (EA) stimulation is effective for the treatment of allergic disorders caused by an imbalance of the Th1/Th2 cell response, such as chronic urticaria and asthma (Biernacki and Peake 1998; Chen and Yu 1998). Our previous study showed that EA reduces IgE in BALB/c mice immunized with 2,4-dinitrophenylated keyhole limpet protein (DNP-KLH) through the suppression of Th2 cytokines (Park et al. 2004). T cells differentiate into Th1, Th2, Th17, and T regulatory cells (Tregs) based on differences in cytokine production and the expression of transcription factors (de Jong et al. 2005; Steinman 2007; Szabo et al. 2003). Th1 cells express T-bet and secrete the cytokines interleukin 2 (IL-2), interferon γ (IFN- γ) and tumor necrosis factor β (TNF- β), which induce protective immunity against intracellular pathogens. Th2 cells express GATA3 and secrete the cytokines IL-4, IL-5, IL-10, and IL-13, which induces humoral immunity to expel parasites (Mosmann and Sad 1996; O'Garra 1998; Szabo et al. 2003; Zhu and Paul 2008). Th17 cells express ROR γ t and secrete the cytokines IL-17A and IL-17F, which induces protective immunity against extracellular pathogens (Steinman 2007).

Oligonucleotide chip microarray analysis is a molecular technique that enables the parallel analysis of gene expression by a very large number of genes encompassing a

Sung-Hwa Sohn and Sun Kwang Kim have contributed equally to this work.

S. K. Kim · E. Ko · Y. Lee · H.-S. Chung · H. Lee · H. Kim ·
H. Bae (✉)
Department of Physiology, College of Oriental Medicine,
Kyung Hee University, #1, Hoegi-dong, Dongdaemun-gu,
Seoul 130-701, Republic of Korea
e-mail: hbae@khu.ac.kr

D.-S. Hwang
Department of Oriental Gynecology, College of Oriental
Medicine, Kyung Hee University, Seoul 130-701,
Republic of Korea

S. Nam (✉)
Department of Acupuncture & Moxibustion, College of Oriental
Medicine, Kyung Hee University, #1, Hoegi-dong,
Dongdaemun-gu, Seoul 130-701, Republic of Korea
e-mail: dangun66@yahoo.co.kr

S.-H. Sohn
Brain Korea 21 Project for Medical Science, Yonsei University,
Seoul 130-701, Republic of Korea

Y. Lee
Department of Medical Research, Korea Institute of Oriental
Medicine, Daejeon 305-811, Republic of Korea

significant fraction of the experimental animal genome. This method is both qualitative and quantitative because it is able to detect changes in the levels of expression in experimental samples based on comparison with control samples (Aras et al. 2003; Huang et al. 2006). In this study, microarray analysis was conducted to elucidate the anti-allergic mechanisms of EA on DNP-KLH immunized mice.

Materials and Methods

Animals

Eight-week old female BLAB/c mice were purchased from Orient Bio Inc. (Sungnam, Korea). The animals were kept under pathogen-free climate controlled conditions and a 12 h light–dark cycle. Mice were allowed food and water ad libitum throughout the experiments. All animal experiments were performed in accordance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Kyung Hee University.

Experimental Groups and EA Stimulation

Mice were randomly divided into three groups ($n = 6$ –10/group): (1) Control group (NC; no treatments for 3 weeks, except for i.p. injection of saline on the 1st, 8th, and 15th day); (2) IMM group (immunization on the 1st, 8th, and 15th day + holder immobilization for 3 weeks); (3) EA group (immunization on the 1st, 8th and 15th day + holder immobilization + ST36 EA for 3 weeks). EA stimulation was performed as previously described (Park et al. 2004). Briefly, a pair of stainless steel needles (0.2 mm in diameter and 3 cm length) was inserted (3 mm in depth) into the ST36 acupoint, which is located at the anterior tibial muscle, 5 mm below and lateral to the anterior tubercle of the tibia, and into the point 5 mm distal from the first needle. EA stimulation at this point (ST36) is known to modulate immune responses in mice (Park et al. 2004; Yu et al. 1998). An electrical pulse of 3–5 V with a duration 0.25 ms and a frequency of 1 Hz was then delivered from an EA stimulator. EA stimulation was applied for 20 min.

DNP-KLH Immunization and Sample Preparation

Mice were immunized intraperitoneally with 4 µg of 2,4-dinitrophenylated keyhole limpet protein (DNP-KLH; Calbiochem, USA) and 4 mg of aluminum hydroxide (Sigma, USA). Acupuncture stimulations were started on the day of immunization and repeated each day for a period of 7, 14, and 21 days. Serum and spleen samples from all

groups of mice were obtained on the final experimental day and stored at -20°C until assayed.

Cytokine Assay

IgE concentration was measured with a quantitative sandwich enzyme-linked immunoassay kit (BD, USA). A 96-well microtiter plate (Coster, USA) was incubated overnight at 4°C with anti-mouse IgE monoclonal antibody in coating buffer, washed with PBS containing 0.05% tween 20 (Sigma, USA) and blocked with 5% FBS in PBS for 1 h at room temperature. Subsequently, 100 µl of sample were loaded and incubated for 2 h at room temperature. Then secondary peroxidase labeled biotinylated anti-mouse IgE monoclonal antibody in 5% FBS in PBS diluent for 1 h. Finally, the plates were treated with TMB substrated solution for 30 min and the reaction was stopped by the addition 2 N H_2SO_4 Stop solution 50 µl per well. Optical density was measured at 450 nm in microplate reader (TECAN, USA).

RNA Preparation

The RNA was then isolated from the spleen using an Rneasy[®] mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, after which the RNA was quantified using a NanoDrop (NanoDrop Technologies, Inc ND-1000; Wilmington, DE, USA).

Oligonucleotide Chip Microarray

Oligonucleotide chip microarray was performed using single round RNA amplification protocols, following the Affymetrix specifications (Affymetrix GeneChip Expression Analysis Technical Manual). Briefly, 3 µg of total RNA were used to synthesize first-strand complementary DNA (cDNA) using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primers (Proligo LLC, Boulder, CO) and the Superscript Choice System (Life Technologies, Invitrogen, Milan, Italy). After double-stranded cDNA synthesis, the products were purified by phenol–chloroform extraction, and then biotinylated antisense complementary RNA (cRNA) was generated through in vitro transcription using a BioArray RNA High-Yield Transcript Labeling Kit (ENZO Life Sciences Inc., Farmingdale, NY). The biotinylated labeled cRNA was then fragmented, and 10 µg of the total fragmented cRNA was hybridized to the Affymetrix Mouse 430 2.0 GeneChip array (P/N900470, Affymetrix Inc., USA). The Affymetrix Fluidics Station 400 was then used to wash and stain the chips, after which the nonhybridized target was removed. Next, the samples were incubated with a streptavidin–phycoerythrin conjugate to stain the biotinylated cRNA.

The staining was then amplified using goat IgG as blocking reagent and biotinylated antistreptavidin antibody (goat), followed by a second staining step using a streptavidin–phycoerythrin conjugate. The fluorescence was detected using the Genechip System Confocal Scanner (Hewlett-Packard), and analysis of the data contained on each GeneChip was conducted using the GeneChip 3.1 software produced by Affymetrix, using the standard default settings. To compare different chips, global scaling was used, with all probe sets being scaled to a user-defined target intensity of 150.

Data Analysis

The Beadstudio v3.0 was used to evaluate the expression signals generated by the Affymetrix Mouse 430 2.0 array. Global scaling normalization was then performed and the normalized data were log-transformed using base 2. Next, fold change and a Welch *t* test were applied to select the differentially expressed genes (DEGs) using a fold change threshold of 2.0-fold and a *P* < 0.05 to indicate significance. Each probe set used in the Affymetrix GeneChip produces a detection call, with P (present call) indicating good quality, M (marginal call) indicating intermediate quality, and A (absent call) indicating relatively low reliability. Therefore, probe sets that resulted in A calls in the compared groups were removed to filter false positives. A volcano plot was used to better visualize and compare the two DEG methods. The 2.0-fold DEGs were clustered using the GenPlex™ v3.0 software (ISTECH Inc., Korea) using hierarchical clustering with Pearson correlation as a similarity measure and complete linkage as the linkage method). In addition, gene ontology significance analysis was conducted to investigate the functional relationships among the 2.0-fold DEGs using high-throughput GoMiner. The 2.0-fold DEGs were then mapped to relevant pathways using GenPlex™ v3.0 software (ISTECH Inc., Korea). The pathway resources were provided by the KEGG database.

Real-Time PCR Analysis

Microarray verification was performed by real time RT-PCR analysis of selected genes using LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Seoul, Korea) and primers (Genotech Inc., Korea). Complementary DNA (cDNA) was synthesized using 2 µg of RNA in a reverse transcription reaction. Real time-PCR quantitative mRNA analyses were performed with a Roche LightCycler® 480 System (LC480) (Roche Diagnostics, Seoul, Korea) to quantify the amplicons. Cycling conditions on LC480 were as follows: 10 min at 95°C, followed by 45 cycles each consisting of 10 s at 95°C, 10 s at 65°C, 20 s at 72°C, and a cooling step of 30 s at 40°C. The sequences of

the mouse primers were as follows: Sod1 (forward primer 5'-tgg tgg tcc atg aga aac aa-3'; reverse primer 5'-gtt tac tgc gca atc cca at-3'), Il1f9 (forward primer 5'-gaa cag gac aaa ggg att gc-3'; reverse primer 5'-cag ggt ggt ggt aca aat cc-3'), Oprl1 (forward primer 5'-ctg gtt caa gga ctg ggt gt-3'; reverse primer 5'-ggc ctt gaa gtt ctc atc ca-3'), Oprk1 (forward primer 5'-gct gcc ctc tcc agc tat ta-3'; reverse primer 5'-gtc cct aaa aca ccg ctt ga-3'), Cyp2a4 (forward primer 5'-gac cga atg aag atg ccc ta-3'; reverse primer 5'-cga aac ttg gtg tcc ttg gt-3'), Itga4 (forward primer 5'-tca ctt gtg aac cct cag ca-3'; reverse primer 5'-cca gtg gtt tca acc ttc ct-3'), Foxf2 (forward primer 5'-atc cca gaa gag cga cag ac-3'; reverse primer 5'-cac aca cac ctc cct ttt ca-3'), and GAPDH (forward primer 5'-ttc acc acc atg gag aag gc-3'; reverse primer 5'-ggc atg gac tgt ggt cat ga-3'). PCR conditions for each target were optimized according to the primer concentration, the absence of primer dimer formation, and the efficiency of amplification of both the target genes and the housekeeping gene control. PCR reactions were carried out in a total volume of 20 µl in PCR master mix containing 10 µl 2X SYBR Green, 5 µM each of sense and antisense primer, and 2 µl of 1:2 diluted cDNA to a final volume of 20 µl with DEPC-treated H₂O. In order to normalize the cDNA content of the samples, we used the comparative threshold (*C_T*) cycle method, which consists of the normalization of the number of target gene copies versus the endogenous reference gene, GAPDH. The *C_T* is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold baseline when amplification of the PCR product is first detected.

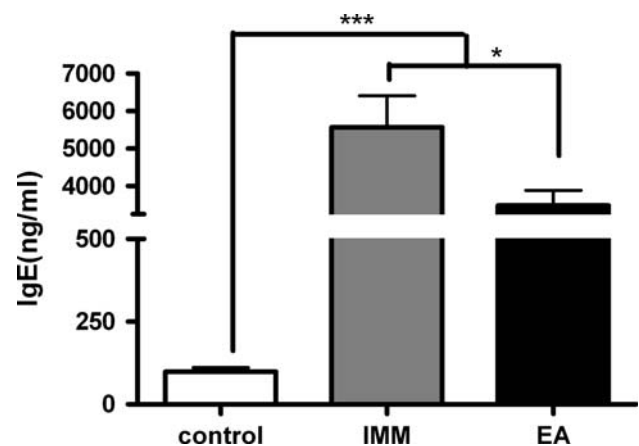


Fig. 1 Antigen-specific IgE production from serum in DNP-KLH immunized mice. Antigen-specific IgE levels were measured by ELISA. The data shown represent the means $\pm$ SEM. * *P* < 0.05 and *** *P* < 0.001 between the two indicated groups by Newman–Keuls multiple comparison test after one-way ANOVA. Control = no treatments, except for saline injection; IMM = DNP-KLH immunization + holder immobilization; EA = DNP-KLH immunization + holder immobilization + EA

Statistical Analysis

Statistical analysis of the data was conducted using the Prism 4.03 software (GraphicPad Software Inc., CA, USA). Data were analyzed by one-way ANOVA for multiple comparisons. One-way ANOVA was performed to compare values and post-test was done by Tukey's multiple comparison test. Results with a $P < 0.05$ were considered statistically significant.

Results

Anti-Allergic Effects in Response to EA

The secretion of antigen-specific IgE was measured in the experimental groups (IMM and EA) and the control group (Fig. 1). The antigen-specific IgE levels in the IMM group

were significantly higher than those in the control group. However, such increase in IgE levels was significantly attenuated by ST36 EA stimulation. These findings indicate that EA can down-regulate antigen-specific IgE secretion in DNP-KLH immunized mice.

Gene Expression Profiles in DNP-KLH immunized mice

Gene expression profiles were significantly up- or down-regulated in the IMM and EA groups when compared with the control group. Overall, 1,075 differentially expressed genes (4.2%) were detected in the experimental groups using approximately 45,100 oligonucleotide probes. Genes that showed highly altered expression levels were aligned according to the magnitude of the altered expression. Among these genes, annotation (function) indicated genes were selected. Most of the differentially expressed genes

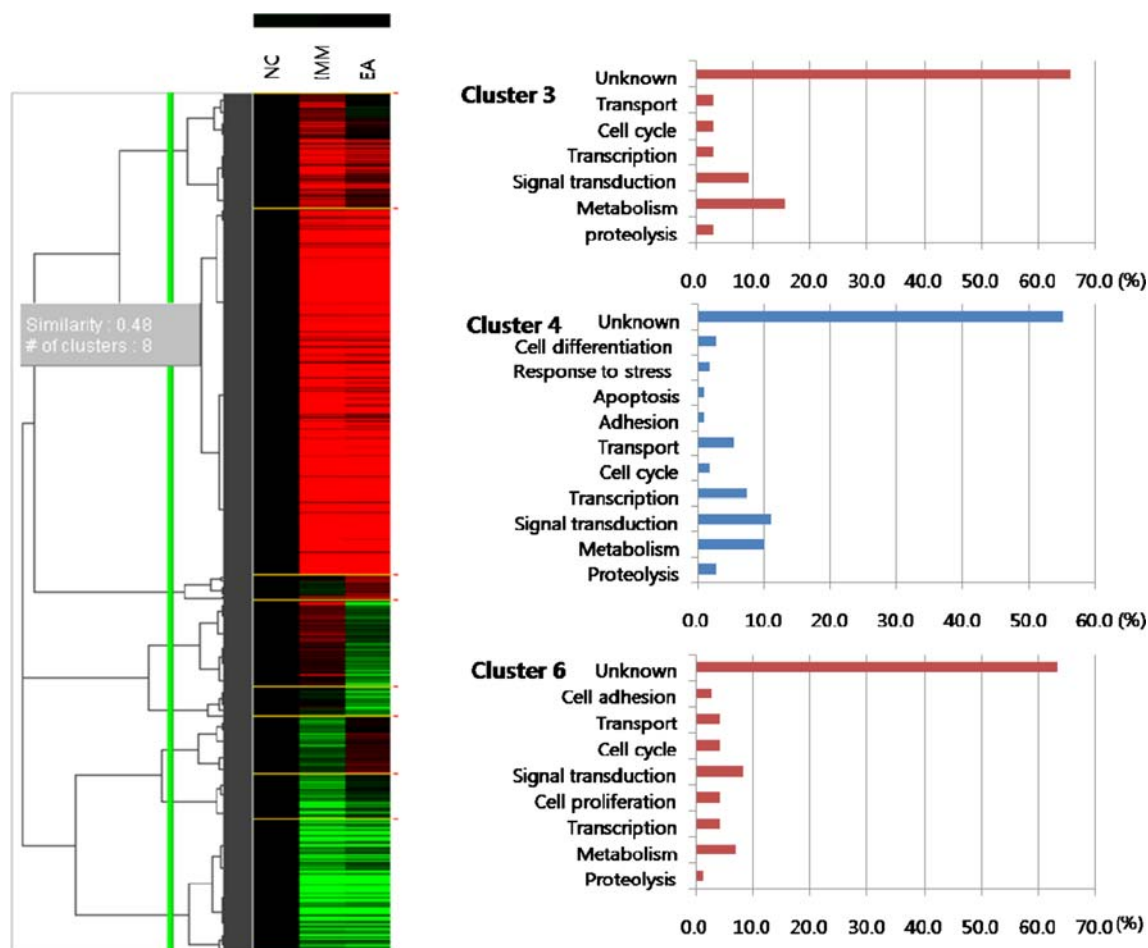


Fig. 2 Correlation matrix plot analysis for calculation of the reproducibility between triplicate samples. The R^2 value was calculated based on the intensity of signals from all probe sets on the Affymetrix Mouse 430 2.0 GeneChip array. Based on the R^2 values, the microarray hybridization patterns were highly consistent

among samples. A perfect relationship among samples would produce a slope of 1. NC = normal control (no treatments, except for saline injection); IMM = DNP-KLH immunization + holder immobilization; EA = DNP-KLH immunization + holder immobilization + EA

(61) are listed in Tables 2 and 3, which show a comparison of the expression levels of a variety of genes in the experimental groups and the control. All genes were grouped into functional categories and metabolic pathways based on the Kyoto Encyclopedia of Genes and Genomes (KEGG)

database. In order to assess the reliability of our microarray technique, we calculated the microarray reproducibility between triplicate RNA samples from three independent cell cultures. The correlation matrix plot comparing the control group to the IMM or EA group is shown in Fig. 2.

Fig. 3 Clustergram of up- and down-regulated genes in DNP-KLH immunized mice. Microarray data from control (NC) and experimental groups (IMM and EA groups) were combined and clustered. There were three independent samples evaluated for each treatment. Each gene is represented by a single row of clustered boxes and each experimental sample is represented by a single column. The entire clustered image is shown on the left. The cluster function categories are shown on the right

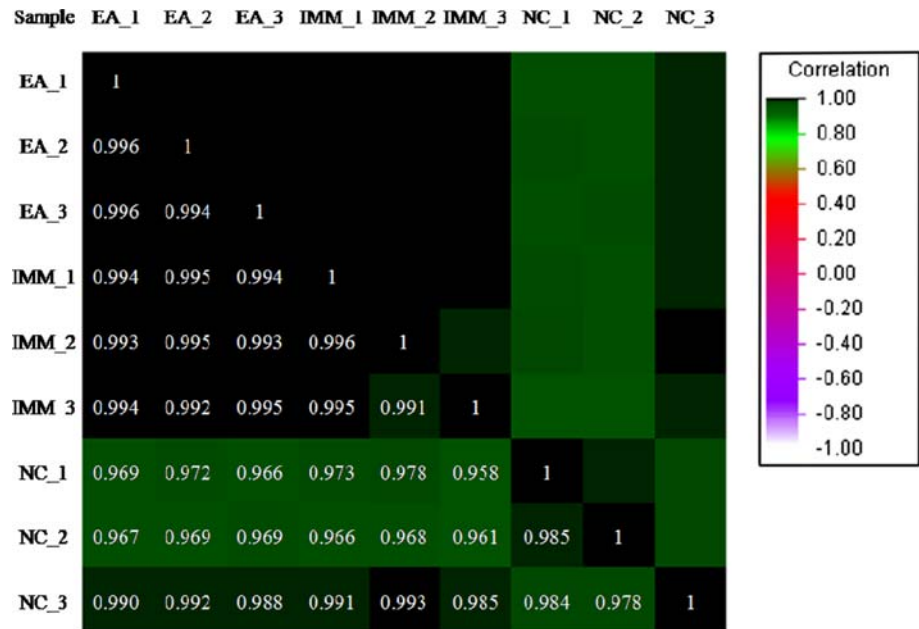


Table 1 Clusters of differentially expressed genes

Cluster	NC ^a	IMM	EA	Number of genes	Genes of interest ^b
1	n.d.	Up	Up	146	–
2	n.d.	Up	Up	456	–
3	n.d.	Down	Up	32	Phospholipase A2, group IVC (Pla2g4c) Protection of telomeres 1A (Pot1a)
4	n.d.	Up	Down	109	Phospholipase C, delta 1 (Plcd1) Cytochrome P450, family 3, subfamily a, polypeptide 11 (Cyp3a11) Cytochrome P450, family 2, subfamily j, polypeptide 5 (Cyp2j5) Transforming growth factor, beta receptor I (Tgfb1) Coagulation factor II (thrombin) receptor-like 3 (F2rl3) Opioid receptor, kappa 1 (Oprk1) Heat shock protein 1B (Hspa1b) Heat shock protein 1A (Hspa1a) Adrenergic receptor, alpha 1a (Adra1a)
5	n.d.	Down	Down	37	–
6	n.d.	Down	Up	71	Interleukin 12 receptor, beta 1 (Il12rb1) Tumor necrosis factor alpha induced protein 6 (Tnfaip6) Peroxiredoxin 6 (Prdx6) IL2-inducible T-cell kinase (Itk)
7	n.d.	Down	Down	58	–
8	n.d.	Down	Down	166	–

^a n.d. no statistically significant difference in expression levels

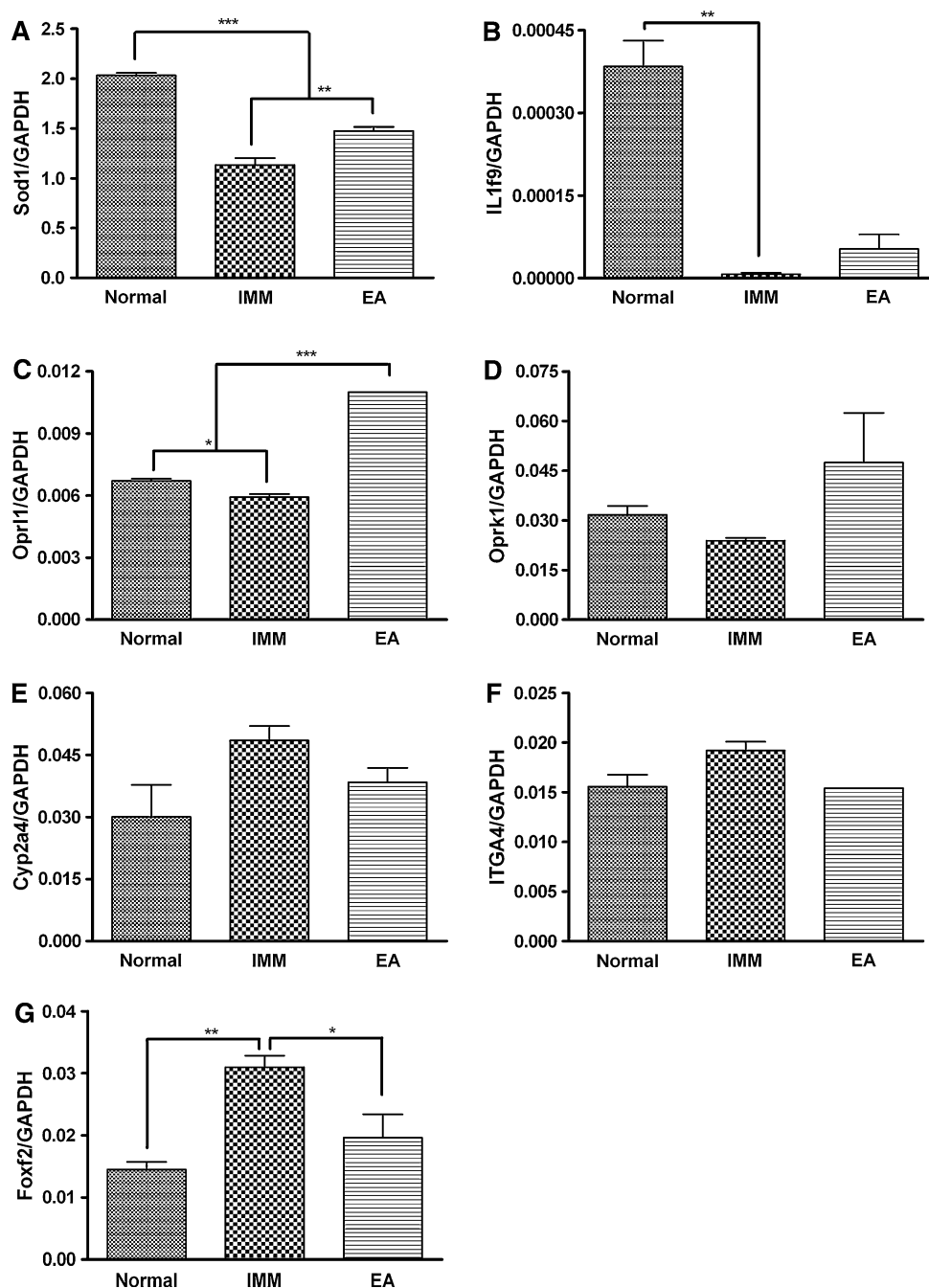
^b Symbol of genes are given in parenthesis

Clusters of Differentially Expressed Genes

We identified a total of 1,075 genes that were differentially expressed (≥ 0.5 log₂ unit difference) in the IMM or EA group when compared with the control group. To elucidate the molecular mechanism of EA in DNP-KLH immunized mice, a hierarchical clustering algorithm was used to group genes on the basis of similar expression patterns (Fig. 3; Table 1). These 1,075 differentially expressed genes were grouped into eight different expression clusters (Fig. 3; Table 1). Each row in Fig. 3 represents the hybridization

results for a single set of oligonucleotide probes in the array, and each column represents the expression levels of all genes in a single hybridization sample. Colors were used to compare the median expression level of each gene when compared with that of the control. Specifically, red represents an expression level greater than the control, green represents an expression level lower than the control and the color intensity denotes the degree of deviation from the control. The cells included in this map include samples from both the experimental groups and the control. Coordinately expressed genes are grouped into clusters, which

Fig. 4 Validation of gene expression by microarray analysis. The results were normalized as a ratio of each specific mRNA signal to the GAPDH gene signal within the same RNA sample and the values expressed. These real-time RT-PCR data were in agreement with the microarray data. **a** Sod1, **b** IL1f9, **c** Opr1, **d** Oprk1, **e** Cyp2a4, **f** ITGA4, and **g** Foxf2. Normal = normal control (no treatments, except for saline injection); IMM = DNP-KLH immunization + holder immobilization; EA = DNP-KLH immunization + holder immobilization + EA. Data shown are the means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ between the two indicated groups by Newman-Keuls multiple comparison test after one-way ANOVA



are named according to the cellular process in which they participate. The clustergram revealed that up- or down-regulated genes were grouped into functional categories (Fig. 3; Table 1).

Validation of Selected Genes via Real Time RT–PCR

A SYBR Green assay was used to confirm expression changes in seven selected genes, *Sod1*, *Il1f9*, *Oprl1*, *Oprk1*, *Cyp2a4*, *Itga4*, and *Foxf2*. The real-time RT–PCR assay yielded results that were in qualitative agreement with the microarray results (Fig. 4).

Discussion

Clinical and experimental studies have shown that sequential electrical stimulation applied to certain acupuncture loci on the human body, such as ST36, was effective in the treatment of stress induced immunodeficiency and physical disorders (Vasilenko et al. 1989). EA stimulation has effects on physiological systems that include induction of the secretion of cytokines, activation of NK cell activity, and the release of opioid peptides both in the periphery and the central nervous system (Bianchi et al. 1991; He 1987; Yuasa 2003). However, the studies that these results were based on evaluated only a single

Table 2 Up-regulation of genes based on comparison of gene expression between the control and experimental (IMM or EA group) groups

Genes	Probe ID	Symbol	Regulation profile and fold change		
			NC ^a	NC ^a vs. IMM	NC ^a vs. EA
Inflammatory and immune response-related genes					
CD209b antigen	1426157_a_at	Cd209b	1.00	0.03	0.13
CD163 antigen	1419144_at	Cd163	1.00	0.10	0.31
Dopamine beta hydroxylase	1459848_x_at	Dbh	1.00	0.18	0.51
Chemokine (C–C motif) ligand 3	1419561_at	Ccl3	1.00	0.19	0.91
Interleukin 28 receptor alpha	1446908_at	Il28ra	1.00	0.21	0.93
Interleukin 17D	1435714_x_at	Il17d	1.00	0.33	1.59
Interleukin 17 receptor C	1457471_at	Il17rc	1.00	0.33	3.04
Tumor necrosis factor receptor superfamily, member 9	1428034_a_at	Tnfrsf9	1.00	0.41	0.63
Interleukin 1 family, member 9	1425958_at	Il1f9	1.00	0.41	0.96
Interleukin 23, alpha subunit p19	1419529_at	Il23a	1.00	0.58	1.44
Transport-related genes					
Transient receptor potential cation channel, subfamily C, member 4 associated protein	1441184_at	Trpc4ap	1.00	0.10	0.48
Potassium inwardly rectifying channel, subfamily J, member 16	1435094_at	Kcnj16	1.00	0.11	0.23
Solute carrier family 30, member 10	1439934_at	Slc30a10	1.00	0.12	0.55
Regulating synaptic membrane exocytosis 2	1422809_at	Rims2	1.00	0.16	0.53
Signal transduction- related genes					
Sprouty protein with EVH-1 domain 1, related sequence	1442859_at	Spred1	1.00	0.13	1.35
Eph receptor A7	1445635_at	Epha7	1.00	0.14	0.73
Intersectin 1 (SH3 domain protein 1A)	1425899_a_at	Itsn1	1.00	0.25	0.72
Opioid receptor-like 1	1422099_a_at	Oprl1	1.00	0.43	0.93
Opioid receptor, kappa 1	1451813_at	Oprk1	1.00	0.44	1.65
Anti-apoptosis-related genes					
Bcl2-like 1	1426191_a_at	Bcl2l1	1.00	0.15	0.28
Cell cycle-related genes					
Cyclin B3	1427744_at	Ccnb3	1.00	0.25	1.04
Others					
Radical S-adenosyl methionine domain containing 2	1421009_at	Rsad2	1.00	0.16	0.33
Parkin	1449975_a_at	Park2	1.00	0.17	0.35
Superoxide dismutase 1, soluble	1447761_x_at	Sod1	1.00	0.21	0.76

^a NC control

Table 3 Down-regulation of genes based on comparison of gene expression between the control and experimental (IMM or EA group) groups

Genes	Probe ID	Symbol	Regulation profile and fold change		
			NC ^a	NC ^a vs. IMM	NC ^a vs. EA
MAPK signaling pathway-related genes					
Transformation related protein 53	1459781_x_at	Trp53	1.00	36.17	13.90
Similar to cytoplasmic nuclear factor of activated T-cells 4	1423379_at	Nfatc4	1.00	18.30	7.68
RAS-related C3 botulinum substrate 1	1437674_at	Rac1	1.00	17.20	5.36
Fibroblast growth factor 13	1418498_at	Fgf13	1.00	15.94	6.37
p21 (CDKN1A)-activated kinase 1	1459029_at	Pak1	1.00	8.82	1.80
Mitogen-activated protein kinase 8 interacting protein 2	1449225_a_at	Mapk8ip2	1.00	5.98	1.15
Fibroblast growth factor 9	1438718_at	Fgf9	1.00	5.71	2.90
Calcium channel, voltage-dependent, alpha 2/delta subunit 2	1426185_at	Cacna2d2	1.00	3.60	0.91
Protein kinase C, gamma	1455758_at	Prkcc	1.00	3.26	0.76
RAS protein-specific guanine nucleotide-releasing factor 1	1459056_at	Rasgrf1	1.00	3.00	1.50
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	1440426_at	Nfatc2	1.00	2.07	0.88
Cell adhesion-related genes					
Integrin alpha 4	1427615_at	Itga4	1.00	6.14	2.75
Apoptosis-related genes					
Paternally expressed 3	1447731_at	Peg3	1.00	15.70	0.85
Programmed cell death 10	1437530_at	Pdcd10	1.00	5.24	2.39
Ankyrin 2, brain	1440043_at	Ank2	1.00	4.53	2.04
Cytokine–cytokine receptor interaction-related genes					
Anti-Mullerian hormone	1450573_at	Amh	1.00	6.99	1.18
Growth differentiation factor 5	1419139_at	Gdf5	1.00	6.35	0.50
Interleukin 10-related T cell-derived inducible factor beta	1427624_s_at	Il1tfb	1.00	5.84	2.34
Transport-related genes					
ATPase, H+ transporting, lysosomal V1 subunit H	1447659_x_at	Atp6v1 h	1.00	46.14	11.85
Mitochondrial carrier homolog 2 (<i>C. elegans</i>)	1437806_x_at	Mtch2	1.00	25.71	11.22
Chloride channel calcium activated 3	1416306_at	Clca3	1.00	13.35	0.62
Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	1439385_x_at	Slc13a3	1.00	12.32	4.33
Centaurin, gamma 2	1459389_at	Centg2	1.00	12.18	4.89
Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	1457018_at	Kcnma1	1.00	9.45	1.36
Cytochrome P450, family 4, subfamily f, polypeptide 14	1419559_at	Cyp4f14	1.00	8.04	1.18
Cytochrome P450, family 4, subfamily f, polypeptide 15	1449316_at	Cyp4f15	1.00	7.76	2.73
Cytochrome P450, family 2, subfamily a, polypeptide 4	1422230_s_at	Cyp2a4	1.00	3.58	1.58
Gamma-aminobutyric acid (GABA-A) receptor, subunit beta 2	1450319_at	Gabrb2	1.00	3.07	1.51
ATPase, H+/K+ exchanging, beta polypeptide	1448911_at	Atp4b	1.00	2.95	0.75
Transcription-related genes					
Homeo box A11, opposite strand transcript	1457646_at	Hoxa11os	1.00	14.41	4.95
Forkhead box F2	1418220_at	Foxf2	1.00	1.99	0.98
Inflammatory and immune response-related genes					
Regenerating islet-derived 3 alpha	1449495_at	Reg3a	1.00	5.69	1.72
Sialic acid binding Ig-like lectin 1, sialoadhesin	1427706_a_at	Siglec1	1.00	4.92	1.53
Kallikrein B, plasma 1	1449034_at	Klkb1	1.00	2.43	0.90
Mannose binding lectin (C)	1418787_at	Mbl2	1.00	2.37	0.62
Complement component 1, q subcomponent, beta polypeptide	1457322_at	C1qb	1.00	2.21	0.68

factor. Microarray analysis enables the parallel analysis of expression of a very large number of genes encompassing a significant fraction of the entire genome. Therefore, we conducted an oligonucleotide microarray experiment to assay the effects of EA on DNP-KLH immunized mice. To the best of our knowledge, this study is the first to investigate the mechanism of acupuncture mediated anti-allergic effects by oligonucleotide microarray analysis. We found specific and significant alterations in the expression profile of EA stimulation in DNP-KLH immunized mice (Tables 1, 2, 3). The cellular processes represented by these genes include inflammatory and immune response, transport, signal transduction, apoptosis, cell cycle, cell adhesion, cytokine-cytokine receptor interaction, transcription, and MAPK signaling (Table 2, 3).

Th1 cells are involved in delayed type hypersensitivity and cytotoxic response, while Th2 cells regulate allergic disease by activating B cells and regulating IgG and IgE secretion (Packard and Khan 2003). In the present study, ELISA analysis revealed that ST36 EA stimulation suppressed the increased antigen-specific IgE production in DNP-KLH immunized mice (Fig. 1). The Th cytokine network is complex and the Th1, Th2, and Th17 immune responses are generally antagonistic. Th1 and Th17 immune responses dominate Th2 and are more important for T cell-mediated pathological processes (Colic et al. 2009). IL-23 belongs to the IL-12 cytokine family, shares its p40 subunit with IL-12 and has a unique p19 chain (Hunter 2005). IL-23 has been found to be important for IL-17 production and to increase the number of antigen-primed IL-17 expressing T cells (Park et al. 2005). In addition, IL-23 is an important factor involved in the regulation of Th17 cells in vivo (Bettelli et al. 2006). Furthermore, it has been shown that IL-1 signaling in T cells is required for early Th17 cell differentiation in vitro and in vivo (Chung et al. 2009). In the present study, the microarray analysis showed that EA stimulation led to an increase in genes related to Th1 cells such as *Il12rb1*, *Tnfrsf6*, *Tnfrsf9*, *Itk*, and *Il1f9* and in genes related to Th17 cells such as *Il17d*, *Il17rc*, and *Il23a*. Conversely, genes related to Th2 cells such as *Il1fb* expression were downregulated in response to EA stimulation when compared with IMM mice (Tables 1, 2, 3; Fig. 4b). In line with this result, our previous study showed that EA treatment suppressed the secretion of Th2-cytokines, including IL-4 and IL-13, from splenocytes of DNP-KLH immunized mice (Park et al. 2004).

Electoracupuncture enhancement of endogenous opioid peptides in the spleen, including endorphins and enkephalins, is well documented (Wu et al. 1995; Yuasa 2003). The results of the present study showed that EA stimulation increased the expression of opioid receptor-related genes, such as *Oprl1* and *Oprk1*, in DNP-KLH immunized mice (Table 2; Fig 4c, d). Dopamine β -hydroxylase (DBH)

catalyzes the conversion of dopamine to norepinephrine (Levi-Montalcini and Angeletti 1966). Norepinephrine and epinephrine play important roles in energy balance, thermoregulation, cardiovascular tone, behavior and stress response (Levi-Montalcini and Angeletti 1966; Thomas et al. 1998; Thomas and Palmiter 1997). In addition, *Dbh*^{-/-} mice do not have intrinsic developmental or functional immune defects. Indeed, *Dbh*^{-/-} mice have impaired T cell function that includes impaired Th1 cytokine (IFN- γ and TNF- α) production (Alaniz et al. 1999). The results of the present study demonstrated that *Dbh* expression increased in response to EA stimulation (Table 2). These findings support the evidence that the immunomodulatory effects of EA are mediated by not simply opioid but also non-opioid system (e.g., noradrenergic, serotonergic, and cholinergic) (Kasahara et al. 1993; Lee et al. 2007; Yu et al. 1998).

Spred1 overexpression is known to suppress IL-5-induced Ras-MAPK activity, which may regulate Th cell development (Myou et al. 2003; Yamashita et al. 1999). In addition, *Spred1* plays an important regulatory role in asthma by modulating the signaling of a limited repertoire of Th2 cytokines (Foster et al. 1996; Hamelmann et al. 1999; Inoue et al. 2005; Nakajima et al. 1992). The results of the present study indicated that EA stimulation led to increased *Spred1* expression, whereas the expression levels of Ras-MAPK pathway-related genes, such as *Trp53*, *Nfatc4*, *Rac1*, *Fgf13*, *Pak1*, *Mapk8ip2*, *Fgf9*, *Cacna2d2*, *Prkcc*, *Rasgrf1*, and *Nfatc2*, decreased in response to EA stimulation (Table 2, 3).

Electoracupuncture stimulation alone also affected many pathways. Specifically, EA stimulation was found to regulate genes involved in a variety of cytokine-cytokine receptor interactions, ECM-receptor interactions, complement and coagulation cascades, neuroactive ligand-receptor interactions, cell adhesion molecules (CAMs), hematopoietic cell lineage regulation, ABC transporters, leukocyte transendothelial migration, and adherens junctions regulation (Table 4).

Table 4 Regulation of pathway based on comparison of gene expression between the control and experimental (IMM or EA group) groups

KEGG pathway	Gene counts	P value
Cytokine-cytokine receptor interaction	27	0.00
ECM-receptor interaction	13	0.01
Complement and coagulation cascades	10	0.01
Neuroactive ligand-receptor interaction	23	0.02
Cell adhesion molecules (CAMs)	16	0.04
Hematopoietic cell lineage	10	0.04
ABC transporters—general	6	0.04
Leukocyte transendothelial migration	14	0.04
Adherens junction	13	0.04

Taken together, the identification of EA stimulation-related genes will help elucidate the mechanism of acupuncture mediated anti-allergic effects, which will open a new route for therapy and provide new potential treatments of allergic disorder.

Acknowledgments This work was supported by a grant from Kyung Hee University in 2009 (KHU-20080600).

References

- Alaniz RC, Thomas SA, Perez-Melgosa M, Mueller K, Farr AG, Palmiter RD, Wilson CB (1999) Dopamine beta-hydroxylase deficiency impairs cellular immunity. *Proc Natl Acad Sci USA* 96:2274–2278
- Aras RA, Lee Y, Kim SK, Israel D, Peek RM Jr, Blaser MJ (2003) Natural variation in populations of persistently colonizing bacteria affect human host cell phenotype. *J Infect Dis* 188:486–496
- Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235–238
- Bianchi M, Jotti E, Sacerdote P, Panerai AE (1991) Traditional acupuncture increases the content of beta-endorphin in immune cells and influences mitogen induced proliferation. *Am J Chin Med* 19:101–104
- Biernacki W, Peake MD (1998) Acupuncture in treatment of stable asthma. *Respir Med* 92:1143–1145
- Chen CJ, Yu HS (1998) Acupuncture treatment of urticaria. *Arch Dermatol* 134:1397–1399
- Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, Watowich SS, Jetten AM, Tian Q, Dong C (2009) Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 30:576–587
- Colic M, Gazivoda D, Vucevic D, Vasiljic S, Rudolf R, Lukic A (2009) Proinflammatory and immunoregulatory mechanisms in periapical lesions. *Mol Immunol* 47:101–113
- de Jong EC, Smits HH, Kapsenberg ML (2005) Dendritic cell-mediated T cell polarization. *Springer Semin Immunopathol* 26:289–307
- Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG (1996) Interleukin 5 deficiency abolishes eosinophilia, airways hyper-reactivity, and lung damage in a mouse asthma model. *J Exp Med* 183:195–201
- Hamelmann E, Cieslewicz G, Schwarze J, Ishizuka T, Joetham A, Heusser C, Gelfand EW (1999) Anti-interleukin 5 but not anti-IgE prevents airway inflammation and airway hyperresponsiveness. *Am J Respir Crit Care Med* 160:934–941
- He LF (1987) Involvement of endogenous opioid peptides in acupuncture analgesia. *Pain* 31:99–121
- Huang S, He J, Zhang X, Bian Y, Yang L, Xie G, Zhang K, Tang W, Stelter AA, Wang Q, Zhang H, Xie J (2006) Activation of the hedgehog pathway in human hepatocellular carcinomas. *Carcinogenesis* 27:1334–1340
- Hunter CA (2005) New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 5:521–531
- Inoue H, Kato R, Fukuyama S, Nonami A, Taniguchi K, Matsumoto K, Nakano T, Tsuda M, Matsumura M, Kubo M, Ishikawa F, Moon BG, Takatsu K, Nakanishi Y, Yoshimura A (2005) Spred-1 negatively regulates allergen-induced airway eosinophilia and hyperresponsiveness. *J Exp Med* 201:73–82
- Kasahara T, Amemiya M, Wu Y, Oguchi K (1993) Involvement of central opiodergic and nonopiodergic neuroendocrine systems in the suppressive effect of acupuncture on delayed type hypersensitivity in mice. *Int J Immunopharmacol* 15:501–508
- Lee Y, Kim SK, Kim Y, Lee H, Shin MK, Hong MC, Min BI, Bae H (2007) The α -adrenoceptor mediation of the immunomodulatory effects of electroacupuncture in DNP-KLH immunized mice. *Neurosci Lett* 423:149–152
- Levi-Montalcini R, Angeletti PU (1966) Second symposium on catecholamines. Modification of sympathetic function. *Immunosympathectomy. Pharmacol Rev* 18:619–628
- Mosmann TR, Sad S (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 17:138–146
- Myou S, Zhu X, Myo S, Boetticher E, Meliton AY, Liu J, Munoz NM, Leff AR (2003) Blockade of airway inflammation and hyperresponsiveness by HIV-TAT-dominant negative Ras. *J Immunol* 171:4379–4384
- Nakajima H, Iwamoto I, Tomoe S, Matsumura R, Tomioka H, Takatsu K, Yoshida S (1992) CD4⁺ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am Rev Respir Dis* 146:374–377
- O'Garra A (1998) Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8:275–283
- Packard KA, Khan MM (2003) Effects of histamine on Th1/Th2 cytokine balance. *Int Immunopharmacol* 3:909–920
- Park MB, Ko E, Ahn C, Choi H, Rho S, Shin MK, Hong MC, Min BI, Bae H (2004) Suppression of IgE production and modulation of Th1/Th2 cell response by electroacupuncture in DNP-KLH immunized mice. *J Neuroimmunol* 151:40–44
- Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133–1141
- Steinman L (2007) A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* 13:139–145
- Szabo SJ, Sullivan BM, Peng SL, Glimcher LH (2003) Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol* 21:713–758
- Thomas SA, Palmiter RD (1997) Thermoregulatory and metabolic phenotypes of mice lacking noradrenaline and adrenaline. *Nature* 387:94–97
- Thomas SA, Marck BT, Palmiter RD, Matsumoto AM (1998) Restoration of norepinephrine and reversal of phenotypes in mice lacking dopamine beta-hydroxylase. *J Neurochem* 70:2468–2476
- Vasilenko AM, Kirilina EA, Sarybaeva DV (1989) Prevention and correction of stress-induced immunodeficiency by atrial electroacupuncture. *Patol Fiziol Eksp Ter* 3:21–24
- Wu GC, Zhu J, Cao X (1995) Involvement of opioid peptides of the preoptic area during electroacupuncture analgesia. *Acupunct Electrother Res* 20:1–6
- Yamashita M, Kimura M, Kubo M, Shimizu C, Tada T, Perlmutter RM, Nakayama T (1999) T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. *Proc Natl Acad Sci USA* 96:1024–1029
- Yu Y, Kasahara T, Sato T, Asano K, Yu G, Fang J, Guo S, Sahara M, Hisamitsu T (1998) Role of endogenous interferon-gamma on the enhancement of splenic NK cell activity by electroacupuncture stimulation in mice. *J Neuroimmunol* 90:176–186
- Yuasa Y (2003) Control of gut differentiation and intestinal-type gastric carcinogenesis. *Nat Rev Cancer* 3:592–600
- Zhu J, Paul WE (2008) CD4 T cells: fates, functions, and faults. *Blood* 112:1557–1569