

A Proteomic Investigation of B Lymphocytes in an Autistic Family: A Pilot Study of Exposure to Natural Rubber Latex (NRL) May Lead to Autism

Chen Shen · Xin-liang Zhao · Weina Ju ·
Xiao-bing Zou · Li-rong Huo · Wu Yan · Jun-hua Zou ·
Guo-di Yan · Edmund C. Jenkins · W. Ted Brown ·
Nanbert Zhong

Received: 24 August 2010 / Accepted: 5 October 2010
© Springer Science+Business Media, LLC 2010

Abstract Autism is a multi-factorial neurodevelopmental disorder. We have investigated the molecular mechanism involved in a Chinese family with autism by a proteomic approach. Antibody chips containing 500 spots of human protein antibodies were used to screen for differentially

expressed proteins in the peripheral B lymphocytes between autistic and non-autistic siblings in this family. Four proteins relevant to immuno-pathway, including IKK α that was up-regulated and Tyk2, EIF4G1 and PRKCI that were down-regulated, were identified differentially expressed in autistic versus non-autistic siblings. Western blot analysis and reverse transcription quantitative polymerase chain reaction validated the differential expression of these four proteins. Based on the function of these differentially expressed proteins, relevant studies on immunoglobulin E (IgE) level, nuclear factor kappa B signaling activation and cell cycle were conducted in both autistic and non-autistic children of this family. Considering the fact that the family members were in close contact with natural rubber latex (NRL) and that IgE-mediated cross-reactions could be triggered by *Hevea brasiliensis* (Hev-b) proteins in NRL, we hypothesize that immune reactions triggered by close contact with NRL might influence the functions of B lymphocytes by altering expression of certain proteins identified in our experiments thus contributing to the occurrence of autism.

C. Shen · X.-l. Zhao · L.-r. Huo · W. Yan · J.-h. Zou · G.-d. Yan ·
N. Zhong
Peking University Center of Medical Genetics,
Beijing 100191, China

C. Shen · X.-l. Zhao · W. Yan · J.-h. Zou · G.-d. Yan · N. Zhong
Department of Medical Genetics,
Peking University Health Science Center,
Beijing 100191, China

W. Ju · E. C. Jenkins · W. T. Brown · N. Zhong (✉)
New York State Institute for Basic Research in Developmental
Disabilities,
1050 Forest Hill Road,
Staten Island, NY 10314, USA
e-mail: nanbert.zhong@omr.state.ny.us

X.-b. Zou
Department of Pediatrics,
The Third Affiliated Hospital of Sun Yat-Sen University,
Guangzhou 510630, China

L.-r. Huo
Department of Microbiology and Immunology,
Shanxi Medical University,
Taiyuan 030001, China

Present Address:
C. Shen
Beijing Children's Hospital Affiliated to Capital Medical
University,
Beijing 100045, China

Keywords Autism · Neurodevelopmental disorder · IKK α ·
Tyk2 · NFkB · NRL

Abbreviations

IgE	Immunoglobulin E
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
NRL	Natural rubber latex
Hev-b	<i>Hevea brasiliensis</i>
EBV	Epstein–Barr Virus

Introduction

Autism is a neurodevelopmental disorder characterized by impairments in social interaction and communication as well as by stereotypic or repetitive behaviors. These features usually appear before 3 years of age and are accompanied by mental retardation, epilepsy, or hyperactivity (Bailey et al. 1996; Risch et al. 1999). The etiology of autism is not clear; however, the pathogenesis involves both genetic and environmental factors. Chromosomal and/or genetic abnormalities can affect gene transcription and translation; however, the ultimate changes occur in specific protein expression, distribution, and activity. Mutation of specific genes, including neurologins or Shank3 (Chih et al. 2004; Moessner et al. 2007), has been determined to result in autistic spectrum disorders. Immunological factors such as the increased activities of immunoglobins (Ig), in addition to the function of B lymphocytes that produce Ig, were also considered as a trigger for autism. For example, vaccination or related to immune disease may associate with autism (Parker et al. 2005; Ashwood and Van de Water 2004). Exposure to allergens can result in allergic reactions and in allergic and/or autoimmune disease (Astrakhan et al. 2007; Valenta et al. 2009; Lindelöf et al. 2009). It has been reported that the mean probability of autoimmune disorders is greater in families with autism (Comi et al. 1999; Mouridsen et al. 2007; Atladóttir et al. 2009). Autistic children could show abnormal immune responses and have elevated serum immunoglobulin E (IgE) and/or other Ig abnormalities (Magalhães et al. 2009; Bakkaloglu et al. 2008; Cohly and Panja 2005; Molloy et al. 2006; Singer et al. 2006; Enstrom et al. 2009), which may provide a clue for treatment (Careaga et al. 2010). *Hevea brasiliensis* (Hev-b) proteins in natural rubber latex (NRL), the key material used in natural rubber production and processing, are considered allergenic according to studies of a variety of autoimmune diseases and lead to elevated IgE secretion. Dochniak (Dochniak 2007) hypothesized that exposure to Hev-b proteins in NRL can lead to autism, depending on the timing, frequency, intensity, and type of exposure. Hev-b proteins may trigger IgE-mediated reactions and influence immune responses to other exogenous/endogenous proteins, which may lead to autism (Dochniak 2007).

Recently we have identified an autistic family (Fig. 1a) that has three autistic children, in addition to three normal children, with DSM-IV criteria. Three autistic children had serious language difficulties, social interaction impairments, obvious attention disorder, and moderate intellectual disabilities. We noticed that the family members were in close contact with NRL, due to the fact that the family owned a natural rubber production company, especially the mother

during her pre-pregnancy, pregnancy, and postnatal periods. Therefore, this pilot study was designed, with a proteomic approach, to investigate whether close contact with NRL might contribute to the development of autism in this family.

Materials and Methods

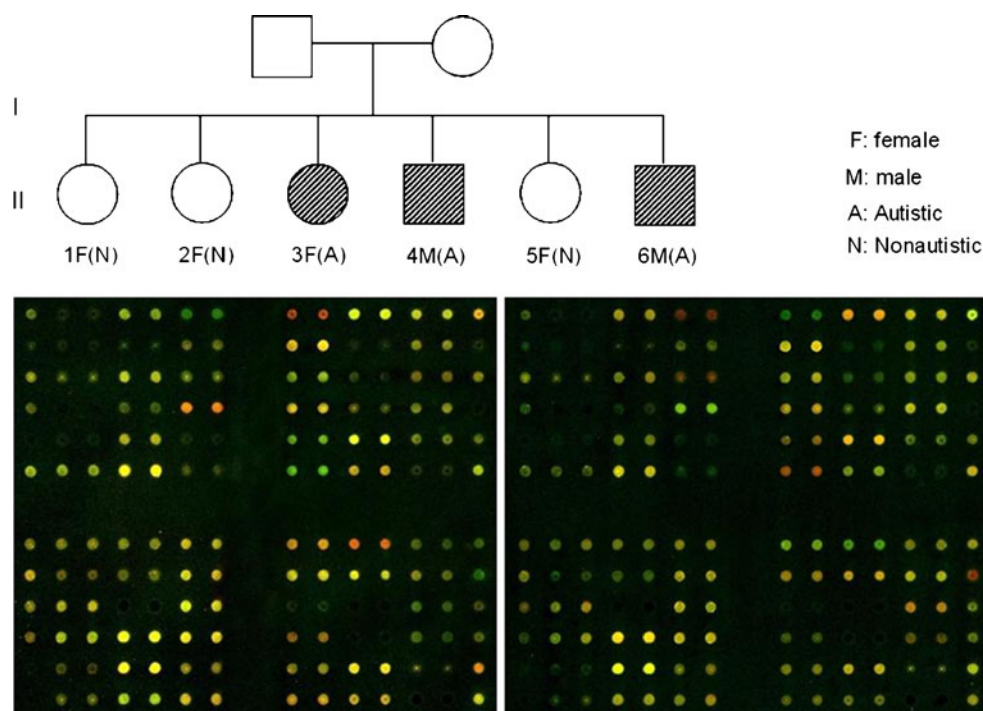
Autistic Family and Establishment of B Lymphoblastoid Cell Lines

A family (Fig. 1a) with three autistic children and three non-autistic siblings was clinically identified by DSM-IV in Guang-Zhou city, Guang-Dong province of South China, with a protocol approved by the Institutional Review Board for clinical investigations including this study. All three autistic children showed no eye contact, non-communication, language delay, and distractibility. B lymphoblastoid cell lines were established with peripheral blood obtained from all children in the family, by following a standard procedure published elsewhere (Neitzel 1986; Mobyuddin et al. 2004). In brief, Epstein–Barr Virus (EBV) supernatant was prepared by culturing the EBV-producing marmoset B cell line B95.8. We then obtained samples of peripheral blood from all family members and separated the mononuclear cells by using Ficoll PLUS-Paque (GE Healthcare Bio-Sciences). The EBV supernatant, along with cyclosporin A (Sigma–Aldrich, St. Louis, MO, USA) was used to transform B lymphocytes. Cells were incubated in RPMI-1640 medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) at 37°C in a 5% CO₂ atmosphere.

Antibody-Chip Detection

Antibody Microarray Express 500 Kits (Clontech, Mountain View, CA) were used following the manufacturer's instructions. In the antibody chip screening, chip pairs of 3F versus 2F (“3F vs. 2F”) and 4M versus 5F (“4M vs. 5F”) were designed to limited the affect of age and sex on the outcome of the experiment. Proteins were extracted from EB-established cell cultures, quantified by the bicinchoninic acid method, labeled with fluorescent dyes (Cy5 and Cy3; GE Healthcare Bio-Sciences), and purified with a Microspin desalting column (Pierce Biotechnology, Rockford, IL). In antibody-chip detection, each sample to be analyzed for protein expression was labeled with two fluorescent dyes, Cy5 and Cy3. Dye-swap chip-pair screenings were used to minimize the effects of inherent variations in dye labeling (Fig. 1b). After antibody array incubation, we scanned the slides with the Genepix 4000B system (Axon, CA) at the following settings: 635 nm (Cy5

Fig. 1 a A family with autism which was in close contact with NRL. Children in this family are shown from the oldest (1F) to the youngest (6M): 1F(N), 12 years old; 2F(N), 9 years old; 3F(A), 8 years old; 4M(A), 6 years old; 5F(N), 5 years old; 6M(A), 4 years old. "A" indicates autistic and "N" for non-autistic. During the antibody chip screening, chip pairs of 3F versus 2F ("3F vs. 2F") and 4M versus 5F ("4M vs. 5F") were designed to limit the affect of age and sex to the outcome of the experiment. **b** Dye-swap antibody chip-pair screening of scanned microarrays. The *left panel* shows a screening image of "A" labeled with Cy3 and "N" labeled with Cy5. The *right panel* shows a screening image of "A" labeled with Cy5 and "N" labeled with Cy3



channel): Photomultiplier tube (PMT)=670 V, power=33%; 652 nm (Cy3 channel): PMT=550 V, power=33%. The altered proteins were sorted with the threshold setting at 1.3-fold for up-regulation or 0.77-fold for down-regulation.

Western Blot Analysis

Western blot analysis was performed according to standard procedures. The primary antibodies, including mouse anti-IKK α (I Kappa K alpha), mouse anti-Tyk2 (tyrosine kinase 2), goat anti-EIF4G1 (eukaryotic translation initiation factor 4 gamma 1), and goat anti-PRKCI (protein kinase C iota), were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted at 1:100 for Western blot. Rabbit antibody of NF- κ B (nucleus factor-kappa B) p100/p52 (phospho-ser866) was from Signalway Antibody (Signalway Antibody Co., Ltd., USA) and diluted at 1:1,000. Rabbit antibody of I κ B α (inhibitor alpha of NF- κ B) was obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China) and diluted at 1:1,000. The secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted at 1:2,000. These are horseradish peroxidase (HRP)-conjugated donkey anti-goat, HRP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit.

RT-qPCR Analysis

Each RNA sample (2 μ g) was subjected to cDNA synthesis with MMLV (Moloney murine leukemia virus) reverse

transcriptase (Promega Corp., Madison, WI, USA). We used SYBR Premix Ex TaqTM II kit (Takara Bio Inc., Shiga, Japan) and ABI PRISM 7,300 Real-Time polymerase chain reaction System (Applied Biosystems, Foster City, CA, USA) to carry out quantitative PCR (polymerase chain reaction) experiments. Primers used to amplify cDNAs and the lengths of the PCR products are listed in Table 1.

IgE Concentration Detecting in Total Cell Lysis

Levels of IgE in total B lymphocyte lysates were quantified with an SX05003 system (Senxiong Biotech, Shanghai, China). To establish a standard curve, eight dots containing 0, 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 ng/ml, respectively, were applied. A standard curve for IgE was drawn with Y-axis as 490-nm absorbance (optical density, OD) and X-axis as concentration of standard IgE in nanograms per milliliter. Total protein extracts (3.5 ng/ μ l) from B lymphocytes of autistic or non-autistic children were used for IgE level determination. IgE concentrations of autistic and non-autistic children were calculated according to their absorption and the standard curve above.

Cell Cycle Analysis

For each sample, 1×10^6 cells were harvested, washed by $1 \times$ PBS (Phosphate-buffered saline) and prepared for single cell suspension. Next, these cells were fixed by 70% alcohol at 4°C overnight. The next day, after being washed by $1 \times$ PBS, cells were resuspended in 500 μ L $1 \times$ PBS with

Table 1 Dye-swap antibody chip-pair screening

3F vs. 2F	Swiss-Prot	4M vs.5F	Swiss-Prot
Up-regulated differentially expressed proteins			
ANXA2	P07355	AP3M1	Q9Y2T2
CDC27	P30260	BLNK	O75498
IKK α	O15111	CAV2	P51636
CTBP2	P56545	CCNB1	P14635
FDFT1	P37268	CDC37	Q16543
FKBP10	Q96AY3	CDC40	O75471
ITGB3	P05106	IKK α	O15111
LCK	P06239	CSPG5	O95196
MAP2K5	Q92961	CYCS	P00001
MCM5	P33992	DLG1	Q12959
MSH2	P43246	DYNLL1	Q15701
NRP2	O60462	F11R	Q9Y624
NUP88	Q99567	FKBP1A	P20071
PER2	O15055	FN1	P02751
RASGRF2	Q9UK56	FUBP1	Q12828
TIAL1	Q01085	GLUL	P15104
TJP1	Q07157	GOLGA2	Q9NYF9
TP53	P04637	HSPD1	P10809
		MAP4	P27816
		MNAT1	P51948
		NDP52	Q13137
		NONO	Q15233
		NOS2	P35228
		PARP1	P09874
		PLCB1	Q9NQ66
		PLK3	Q9H4B4
		PSIP1	O95368
		RASA2	Q15283
		STAT3	P40763
		TRAX	Q99598
		UBE2I	P50550
		VASP	P50552
		XRCC5	P13010
Down-regulated differentially expressed proteins			
CDC25C	P30307	AMPH	P49418
CDC37	Q16543	ANXA2	P07355
DAP3	P51398	AP2B1	P21851
DLG1	Q12959	APPBP1	Q13564
EIF4G1	Q04637	CALB2	P22676
EPS8	Q12929	CANX	P27824
FKBP1A	P20071	CBX2	P30658
GLUL	P15104	CEP250	O60588
HAX1	O00165	CHD3	Q12873
KIF3B	O15066	CMYA1	Q8TCG7
LCP2	Q13094	CPE	P16870
NCOR2	Q9Y618	CSNK1E	P49674
PRKCH	P24723	DDX1	Q92499
PRKCI	P41743	DOK2	O60496

Table 1 (continued)

3F vs. 2F	Swiss-Prot	4M vs.5F	Swiss-Prot
PRKCQ	Q04759	DUSP3	P51452
PPP1R2	P41236	E2F1	Q01094
SQSTM1	Q13501	EIF4G1	Q04637
STAT3	P40763	EIF5	P55010
SYT1	P21579	FDFT1	P37268
TEAD1	P28347	HMOX1	P09601
TYK2	P29597	HSPH1	Q92598
		ITGA3	P26006
		LCK	P06239
		MAPT	Q1RMF6
		MCM4	P33991
		MCM5	P33992
		MKI67	P46013
		MSH2	P43246
		NEDD4	P46934
		NRP2	O60462
		NUMA1	Q14980
		NUP88	Q99567
		ORC5L	O43913
		P4HB	P07237
		PCNT	P48725
		PER2	O15055
		PIK3R1	P27986
		PKN1	Q16512
		PPP2CA	P67775
		PPP5C	P53041
		PRKAR1A	P10644
		PRKAR2B	P31323
		PRKCI	P41743
		PRKCD	Q05655
		PTGS2	P35354
		PTPRF	P10586
		RAB11A	P24410
		RASGRF2	Q9UK56
		SCARB1	Q14016
		SERPINB5	P36952
		SHC3	Q92529
		SNX2	O60749
		STRN	O43815
		SYCP3	P70281
		TJP1	Q07157
		TOP2B	Q02880
		TYK2	P29597
		Vti1b	Q9UEU0
		XPA	P23025

Fig. 2 **a** Western blot of IKK α , TYK2, EIF4G1, and PRKCI proteins among six children of the family studied, with β -actin as a loading control. **b** Gray values of the bands were measured by Band Scan 5.0. The *X-axis* shows the four candidate proteins, and the *Y-axis* shows the ratio of autistic group compared with non-autistic group. Data are shown as mean $\pm$ SEM. By unpaired *t* test, two-tailed *p* value: “A”, *p*=0.0023; “B”, *p*=0.0035; “C”, *p*=0.0372; “D”, *p*=0.0095. **c** Quantitative RT-PCR results of IKK α , TYK2, EIF4G1, and PRKCI in autistic compared to non-autistic siblings

50 μ g/mL propidium iodide and 100 μ g/mL RNase A, and then incubated for half an hour at 4°C. After that, flow cytometry (FCM) was used for cell cycle analysis

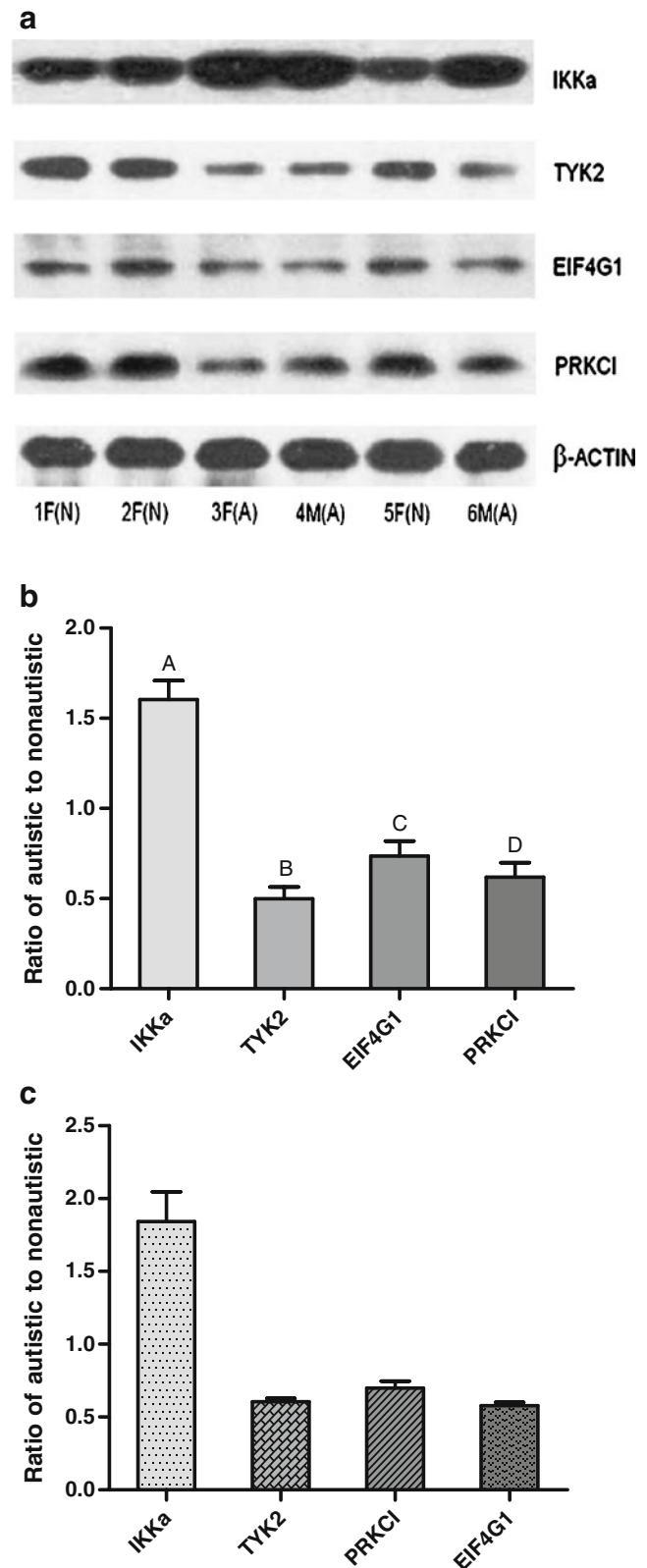
Results

Identification of Differentially Expressed Proteins

With dye-swap antibody chip-pair screening (Fig. 1b), 18 proteins were identified up-regulated in the pair of 3F(A) vs. 2F(N) and 33 were up-regulated in the pair of 4M(A) vs. 5F(N). Within these two groups of up-regulated proteins, IKK α was observed in both pairs. In addition, several proteins, such as CDC27 vs. CDC37 (and CDC40), FKBP10 vs. FKBP1A, and MAP2K5 vs. MAP4 showed that their different isoforms may have up-regulated in both pairs. Similarly, three differentially expressed proteins EIF4G1, PRKCI, and TYK2, along with protein isoforms PPP1R2 vs. PPP5C, PRKCQ vs. PRKCD were identified down-regulated in both pairs (Table 1). Further validation of differentially up-regulated IKK α and down-regulated EIF4G1, PRKCI, and TYK2 was performed by Western blot and reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Fig. 2). The analysis on the gray scale of the Western blot bands indicated that IKK α was up-regulated to 1.60-fold of control protein level, while RT-qPCR analysis showed an up-regulation of IKK α to 1.84-fold of control RNA level. Results also indicated that the translational level of Tyk2 in autistic children was down-regulated to 49% of control protein level compared with non-autistic children, while RT-qPCR showed a down-regulation of Tyk2 to 60.4% of control RNA level. PRKCI was down-regulated to 62.0% of control level by Western blot. RT-qPCR data showed PRKCI was down-regulated to 57.9% of normal level. Western blot analysis and RT-qPCR data showed that EIF4G1 was down-regulated to 73.5% of control protein level and to 69.9% of control RNA level.

Detection of Total Lymphocyte Protein IgE

We found the average IgE level in total cell lysates of cultured B lymphocytes from autistic children was 766.0238 ng/ml $\pm$ 155.6379 (mean $\pm$ SD) and that from non-autistic siblings was



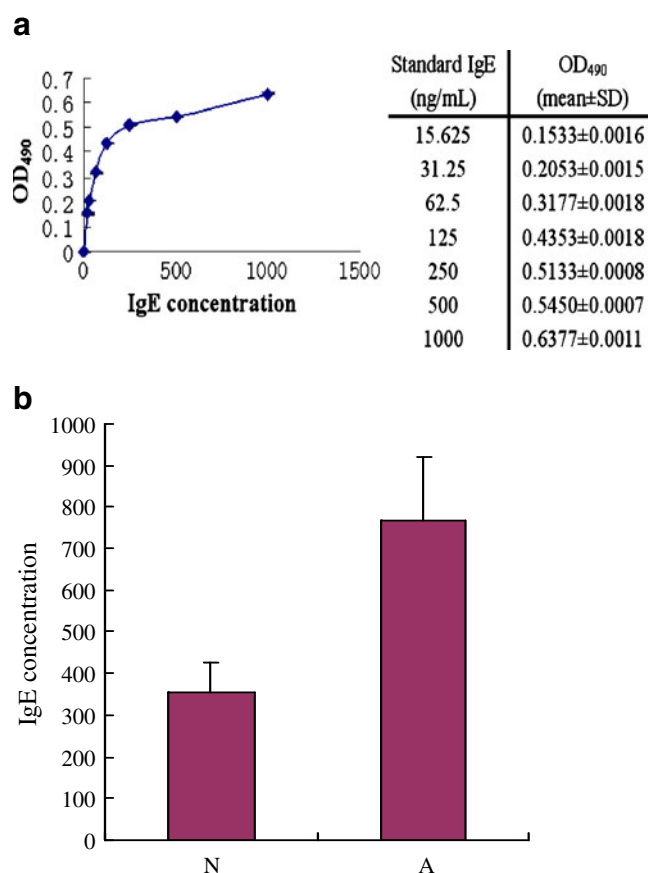


Fig. 3 Detection of IgE. **a** Standard curve of IgE was drawn with *Y*-axis as 490-nm absorbance (optical density, *OD*) and *X*-axis as concentration of standard IgE. **b** One hundred microliters of total protein extracts (3.5 ng/ μ l) from B lymphocytes of autistic or non-autistic siblings were used for IgE assay. IgE concentrations of autistic and non-autistic children were calculated according to their absorption and the standard curve above. The average IgE concentration of autistic children was 766.0238 ng/ml $\pm$ 155.6379 (mean $\pm$ SD) and that of non-autistic children was 355.3102 ng/ml $\pm$ 71.00042. By unpaired *t* test, two-tailed *p* value is 0.0274

355.3102 ng/ml $\pm$ 71.00042, which showed autistic children had a significant higher IgE level (*p*<0.05) than non-autistic siblings in this Chinese family (Fig. 3).

Activation of NF-Kb Signaling

In autistic children compared with non-autistic children, the protein level of I κ B α did not change. (Fig. 4a), but nuclear P52 was increased (Fig. 4b). This means that, in autistic children, the NF- κ B signaling was activated not in the canonical way but in the non-canonical way.

Cell Cycle Analysis

FCM analysis showed that the percentage of G0/G1 phase cells decreased but that of S phase cells increased in autistic children compared with non-autistic siblings. The cell

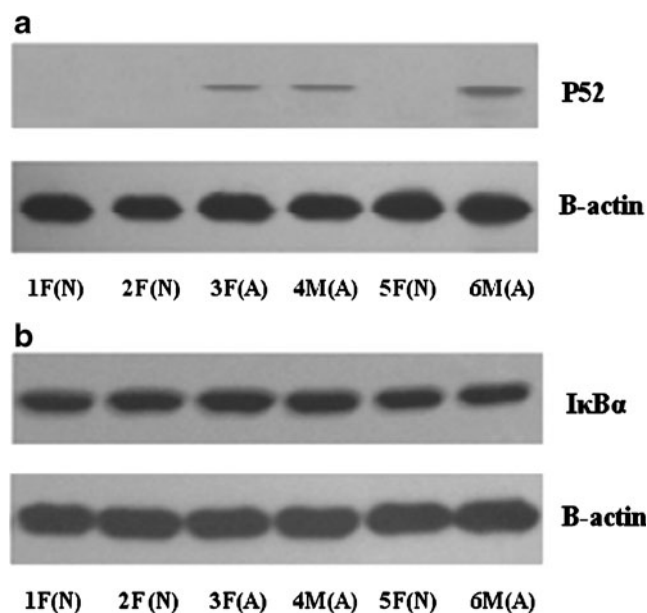


Fig. 4 NF- κ B signaling activation. **a** Western blot of P52 (phosphorylated) using nuclear protein extracts. A protein band probed by anti-P52 antibody can be detected at lanes of 3F(A), 4M(A), and 6M(A) but not at lanes of non-autistic (N) siblings. **b** Western blot of I κ B α using total protein extracts. B-actin was used as a control in Western blotting of both **a** and **b**

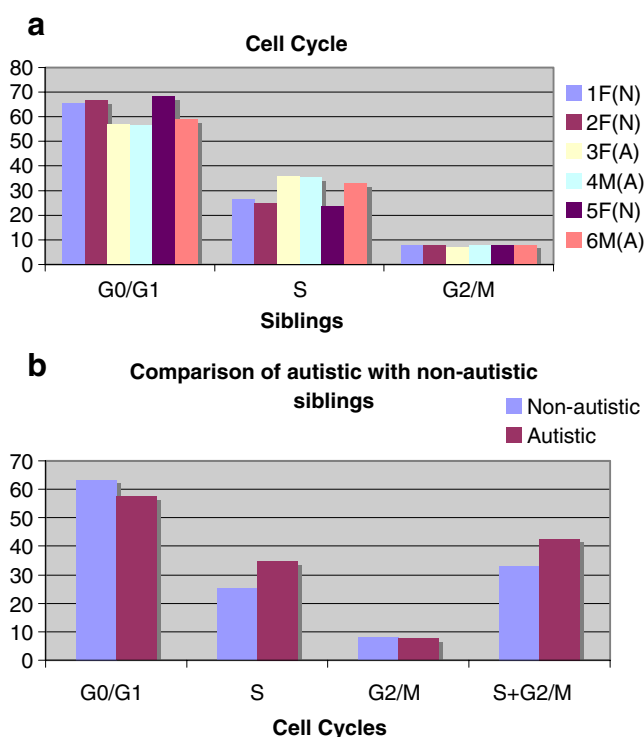


Fig. 5 Cell cycle: **a** Cell percentage of each phase in cell cycle by FCM. **b** Analysis of cell cycle in autistic children and non-autistic children. By unpaired *t* test, two-tailed *p* values: ^I*p*<0.01 for G0/G1, ^{II}*p*<0.01 for S, ^{III}*p*>0.05 for G2/M, and ^{IV}*p*<0.01 for S+G2/M

percentage of “S+G2/M” phase which represents cell proliferation also increased significantly in autistic children ($p < 0.005$; Fig. 5a, b)

Discussion

Quite a lot studies have been reported on the relation between autism and immune-mediated disorders such as allergies and autoimmune diseases (Astrakhan et al. 2007; Valenta et al. 2009; Lindelöf et al. 2009). Parents of autistic children have higher rates of abnormal immune response (Comi et al. 1999; Mouridsen et al. 2007; Atladóttir et al. 2009). Some research groups have argued that autism is an autoimmune disease (Ashwood and Van de Water 2004). Dochniak hypothesized that exposure to Hev-b proteins in NRL can lead to autism, depending on the timing, frequency, intensity, and type of exposure; Hev-b proteins can trigger IgE-mediated reactions and influence immune responses to other exogenous/endogenous proteins, which may lead to autism (Dochniak 2007). In our current study, three autistic children were identified from a Chinese family in south China, in which the parents run a natural rubber production business and have been in close contact with NRL. Hypothesizing that close contact with NRL may be the underlying mechanism for this family having three children affected by autism, we undertook a proteomic approach to study the differentially expressed proteins in hope of identifying any protein that may associate with autistic phenotypes. Not to our surprise, the altered proteins detected by antibody chips were mostly immuno-relevant ones including elevated IKK α and decreased Tyk2, EIF4G1, and PRKCI. Previous studies have reported the presence of autism susceptibility gene loci at bands 3q26.31-q27.3 and 19p13 (Allen-Brady et al. 2009; Ylisaukko-oja et al. 2006; McCauley et al. 2005), which embedded genes of PRKCI, EIF4G1, and Tyk2 that we identified in this study (Firmbach-Kraft et al. 1990; Fields and Regala 2007; Prévôt et al. 2003). Combining our findings of higher IgE level in B lymphocytes from autistic children indicates a relationship of abnormal immune reactions and autism. The underlying mechanism can be that possibly Tyk2 acts to phosphorylate the IFN- α /beta receptor alpha chain, which plays an obligatory role in human immunity. Mutations in Tyk2 can cause type II hyper-IgE syndrome (Minegishi et al. 2006; Minegishi and Karasuyama 2007). Usually, B lymphocytes produce IgE in response to certain endogenous lymphokines. It has been reported that IgE synthesis can occur without lymphokines in patients with hyper-IgE syndrome, suggesting that IgE synthesis occurs in a terminally differentiated B lymphocyte population; terminally differentiated B lymphocytes that specifically produce IgE are no longer sensitive to

regulatory signals (Vercelli et al. 1990). In our present experiments, the IgE level was higher in cell lysates from autistic children than that of non-autistic siblings, which is consistent with previous results (Stern et al. 2005) and coupled with the down-regulation of Tyk2. It looks like the terminally differentiated B lymphocytes have a larger population in autistic children than non-autistic siblings of the present family. Tyk2 may also move the Th1/Th2 balance toward Th1. In Tyk $^{-/-}$ mice, IL-12-mediated Th1 cell differentiation is decreased while IL-4-mediated Th2 cell differentiation is increased (Seto et al. 2003). In autism, similar alterations occur in immune regulation involving pro-inflammatory cytokine autoimmunity. Cell-mediated immunity is impaired, as evidenced by low numbers of CD4 cells along with a concomitant T cell polarity and an imbalance of the Th1/Th2 subset toward Th2 (Molloy et al. 2006), and levels of all Th2 cytokines, including IL-4, are significantly increased (Singer et al. 2006). Shifts of the T cell response from Th2 to Th1 or a reduction of Th2-type cytokine production proved to be of therapeutic benefit in patients with autism spectrum disorder (Boris et al. 2007).

IKK α is essential for B lymphocyte development and function (Kaisho et al. 2001). IKK α /IKK β heterodimer phosphorylates I κ B α (inhibitor alpha of Nuclear factor kappa B) on both serine 32 and serine 36, which are required for targeted degradation of I κ B α , thereby activating the essential transcription factor NF- κ B (Nuclear factor kappa B) in canonical way (Régner et al. 1997). IKK α /IKK α homodimer activates NF- κ B in a non-canonical (alternative) way by phosphorylating the C terminal region of p100 followed by degradation of the p100 to generate activated p52 in the nucleus (Hayden and Ghosh 2004). IKK α -activated p52/p100 (NF- κ B2)-dependent pathway plays a role in the regulation of the cell cycle and cell proliferation (Schneider et al. 2006; Barré and Perkins 2007; Ishikawa et al. 1997). In our results, when autistic children were compared to non-autistic siblings, the protein level of I κ B α did not show significant alteration (Fig. 4b). Rather, p52 appeared in nucleus in autistic children (Fig. 4a). These indicated that, in autistic children, the NF- κ B signaling was activated in a non-canonical way. In autistic children compared with non-autistic siblings, cell counts in G0/G1 phase decreased in number but increased in S phase. Cell percentage of “S+G2/M” phase which represents cell proliferation increased significantly in autistic children. In addition, IKK α may serve as a novel target for the regulation of monocyte function in allergy, in which IgE plus trinitrophenyl induced prominent synthesis and rapid phosphorylation of IKK α (Peng et al. 2005).

PRKCI is over-expressed in numerous tumors (Regala et al. 2005; Zhang et al. 2006). It may be involved in cell polarization and the formation of epithelial tight junctions; it plays roles in the secretory response to nutrients. Besides,

PRKCI is implicated in the activation of NF- κ B signaling pathways through recruitment to IKK complex and phosphorylating IKK α and IKK β (Win and Acevedo-Duncan 2008). EIF4G1 has been identified as a candidate citrullinated auto-antigen in patients with rheumatoid arthritis (Okazaki et al. 2006). Rheumatoid arthritis is a common systemic autoimmune disease of unknown etiology, which is thought to be associated with autism (Comi et al. 1999).

As an antibody producer, B lymphocytes contribute to the humoral immune system. NRL may influence the function of B lymphocytes such as causing differences in IgE production and alteration in protein expression. In this study, we compared the protein expression pattern of B lymphocytes in autistic children with that in non-autistic children. By analyzing the functions of altered proteins, we further investigated the IgE level, the activation of NF- κ B, and the cell cycle. With our knowledge, results generated from this pilot study is the first time illustrating the exposure to NRL may lead to IKK α differentially up-regulated and EIF4G1, PRKCI, and TYK2 down-regulated in autism persons. Based upon the discussion above, we hypothesized that during neurodevelopment, in this family, NRL triggered cross-reaction down-regulated Tyk2 in B lymphocytes, which developed terminally differentiated B lymphocytes to produce IgE in the absence of stimulation; elevated IgE could also up-regulate IKK α and activate it by phosphorylation; and then down-regulation of PRKCI occurred, which appeared to be an inverse feedback to up-regulated IKK α for PRKCI, that also activates IKK α . IKK α activates NF- κ B signaling in a non-canonical way, which alters the cell cycle. The abnormal status of B lymphocytes found in autistic children of this family may support the hypothesis that exposure to NRL is associated with autism. Furthermore, close contact with NRL may have contributed to the occurrence of autism in this family by altering the expression of certain immuno-relevant proteins, by triggering abnormal immune reaction and influencing the cell cycle. To further test our hypothesis, detailed investigations should be pursued with a population-based larger size of samples to explore (1) if there is an increase in autism in people who work or live near rubber processing plants, (2) if there is a spike in the prevalence of autism around those types of plants, and (3) are there any differences in autistic prevalence in the areas of rubber plants versus others without similar exposures.

The complex and precise signal transmission in the central nervous system occurs as a result of the generation and maturation of functional synapses. There is evidence that abnormalities in immunity affect nervous system development. For example, immune deficiency or severe inflammatory disease can lead to impaired hippocampal neurogenesis, which can result in impaired hippocampus-dependent cognitive abilities (Ziv and Schwartz 2008). But how immune

factors affect development, behavior and changes within the CNS to cause autism is still unclear (McAllister and van de Water 2009; Libbey and Fujinami 2010).

Finally, we would like to point out that all results of this study were generated from B lymphocytes that came from the peripheral bloods of autistic and non-autistic siblings. Because autism is a neurodevelopmental disorder and the ideal material for conducting biological study is brain tissue, using peripheral blood-derived lymphocytes may have a potential bias. However, due to the lack of brain tissue for studying the relationship, at a translational level, between contacts of NRL and autism in neurons, the only possible materials that could be obtained from autism children would be skin cultures and lymphocyte cultures. Compared to lymphoblasts, cultured fibroblasts transformed with SV-40 would be closer to brain. Unfortunately, we could not obtain the skin biopsy due to the difficulty of cooperation from the autistic children, therefore, lymphocytes became the only available material we could use for this current study, although we were aware of the potential bias. A similar report using peripheral bloods studying plasma β -endorphin and pain reactivity in autistic children was reported previously (Tordjman et al. 2009) and recently, lymphoblastoid cell lines were studied that revealed at least a subset of brain-related microRNAs implicated in autism (Talebzadeh et al. 2008).

Conclusion

Our present results indicate that immune responses caused by specific contact with Hev-b proteins during prenatal and/or neonatal development may alter the immune system balance, influence functions of B lymphocytes by altering expression of certain proteins identified in our experiments, and finally, contribute to the occurrence of autism in this family.

Acknowledgements The authors wish to thank the family that participated in this study. This work was supported in part by the “985” program (985-2-035-39) of the Chinese Ministry of Education, the “973” project (2007CB511902) from the Ministry of Science and Technology, the National Nature Science Foundation (30671157), and the New York State Office for People with Developmental Disabilities (NYS OPWDD).

References

- Allen-Brady K, Miller J, Matsunami N, Stevens J, Block H, Farley M, Krasny L, Pingree C, Lainhart J, Leppert M, McMahon WM, Coon H (2009) A high-density SNP genome-wide linkage scan in a large autism extended pedigree. *Mol Psychiatry* 14:590–600
- Ashwood P, Van de Water J (2004) Is autism an autoimmune disease? *Autoimmun Rev* 3:557–562

- Astrakhan A, Omori M, Nguyen T, Becker-Herman S, Iseki M, Aye T, Hudkins K, Dooley J, Farr A, Alpers CE, Ziegler SF, Rawlings DJ (2007) Local increase in thymic stromal lymphopoietin induces systemic alterations in B cell development. *Nat Immunol* 8:522–531
- Atladóttir HO, Pedersen MG, Thorsen P, Mortensen PB, Deleuran B, Eaton WW, Parner ET (2009) Association of family history of autoimmune diseases and autism spectrum disorders. *Pediatrics* 124:687–694
- Bailey A, Phillips W, Rutter M (1996) Towards an integration of clinical, genetic, neuropsychological, and neurobiological perspectives. *J Child Psychol Psychiatry* 37:89–126
- Bakkaloglu B, Anlar B, Anlar FY, Oktem F, Pehlivanlı B, Unal F, Ozbesler C, Gökler B (2008) Atopic features in early childhood autism. *Eur J Paediatr Neurol* 12(6):476–479
- Barré B, Perkins ND (2007) A cell cycle regulatory network controlling NF-kappaB subunit activity and function. *EMBO J* 26:4841–4855
- Boris M, Kaiser CC, Goldblatt A, Elice MW, Edelson SM, Adams JB, Feinstein DL (2007) Effect of pioglitazone treatment on behavioral symptoms in autistic children. *J Euroinflammation* 4:3
- Careaga M, Van de Water J, Ashwood P (2010) Immune dysfunction in autism: a pathway to treatment. *Neurotherapeutics* 7:283–292
- Chih B, Afridi SK, Clark L, Scheiffele P (2004) Disorder-associated mutations lead to functional inactivation of neuroligins. *Hum Mol Genet* 13:1471–1477
- Cohly HH, Panja A (2005) Immunological findings in autism. *Int Rev Neurobiol* 71:317–341
- Comi AM, Zimmerman AW, Frye VH, Law PA, Peeden JN (1999) Familial clustering of autoimmune disorders and evaluation of medical risk factors in autism. *J Child Neurol* 14:388–394
- Dochniak MJ (2007) Autism spectrum disorders—exogenous protein insult. *Med Hypotheses* 69:545–549
- Enstrom A, Krakowiak P, Onore C, Pessah IN, Hertz-Picciotto I, Hansen RL, Van de Water JA, Ashwood P (2009) Increased IgG4 levels in children with autism disorder. *Brain Behav Immun* 23:389–395
- Fields AP, Regala RP (2007) Protein kinase C iota: human oncogene, prognostic marker and therapeutic target. *Pharmacol Res* 55:487–497
- Firmbach-Kraft I, Byers M, Shows T, Dalla-Favera R, Krolewski JJ (1990) Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. *Oncogene* 5:1329–1336
- Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. *Genes Dev* 18:2195–2224
- Ishikawa H, Carrasco D, Claudio E, Ryseck RP, Bravo R (1997) Gastric hyperplasia and increased proliferative responses of lymphocytes in mice lacking the COOH-terminal ankyrin domain of NF-kappaB2. *J Exp Med* 186:999–1014
- Kaisho T, Takeda K, Tsujimura T, Kawai T, Nomura F, Terada N et al (2001) I kappa B kinase alpha is essential for mature B cell development and function. *J Exp Med* 193:417–426
- Libbey JE, Fujinami RS (2010) Role for antibodies in altering behavior and movement. *Autism Res* 3:147–152. doi:10.1002/aur.144
- Lindelöf B, Granath F, Tengvall-Linder M, Lindelöf H, Ekblom A (2009) Allergy and autoimmune disease: a registry-based study. *Clin Exp Allergy* 9:110–115
- Magalhães ES, Pinto-Mariz F, Bastos-Pinto S, Pontes AT, Prado EA, de Azevedo LC (2009) Immune allergic response in Asperger syndrome. *J Neuroimmunol* 216:108–112
- McAllister AK, van de Water J (2009) Breaking boundaries in neural-immune interactions. *Neuron* 64:9–12
- McCauley JL, Li C, Jiang L, Olson LM, Crockett G, Gainer K, Folstein SE, Haines JL, Sutcliffe JS (2005) Genome-wide and ordered-subset linkage analyses provide support for autism loci on 17q and 19p with evidence of phenotypic and interlocus genetic correlates. *BMC Med Genet* 6:1
- Minegishi Y, Karasuyama H (2007) Hyperimmunoglobulin E syndrome and tyrosine kinase two deficiency. *Curr Opin Allergy Clin Immunol* 7:506–509
- Minegishi Y, Saito M, Morio T, Watanabe K, Agematsu K, Tsuchiya S, Takada H, Hara T, Kawamura N, Ariga T, Kaneko H, Kondo N, Tsuge I, Yachie A, Sakiyama Y, Iwata T, Bessho F, Ohishi T, Joh K, Imai K, Kogawa K, Shinohara M, Fujieda M, Wakiguchi H, Pasic S, Abinun M, Ochs HD, Renner ED, Jansson A, Belohradsky BH, Metin A, Shimizu N, Mizutani S, Miyawaki T, Nonoyama S, Karasuyama H (2006) Human tyrosine kinase two deficiency reveals its requisite roles in multiple cytokine signals involved in innate and acquired immunity. *Immunity* 25:745–755
- Mobyuddin A, Ayub Q, Sidiqi S, Carvalho-Silva DR, Mazhar K, Rehman S, Firasat S, Dar A, Tyler-Smith C, Mehdi SQ (2004) Genetic instability in EBV transformed lymphoblastoid cell line. *Biochem Biophys Acta* 1670:2356–2360
- Moessner R, Marshall CR, Sutcliffe JS, Skaug J, Pinto D, Vincent J, Zwaigenbaum L, Fernandez B, Roberts W, Szatmari P, Scherer SW (2007) Contribution of SHANK3 mutations to autism spectrum disorder. *Am J Hum Genet* 81:1289–1297
- Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, Dienger K, Manning-Courtney P, Altaye M, Wills-Karp M (2006) Elevated cytokine levels in children with autism spectrum disorder. *J Neuroimmunol* 172:198–205
- Mouridsen SE, Rich B, Isager T, Nedergaard NJ (2007) Autoimmune diseases in parents of children with infantile autism: a case-control study. *Dev Med Child Neurol* 49:429–432
- Neitzel H (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum Genet* 73:320–326
- Okazaki Y, Suzuki A, Sawada T, Ohtake-Yamanaka M, Inoue T, Hasebe T, Yamada R, Yamamoto K (2006) Identification of citrullinated eukaryotic translation initiation factor 4 G1 as novel autoantigen in rheumatoid arthritis. *Biochem Biophys Res Commun* 341:94–100
- Parker S, Todd J, Schwartz B, Pickering L (2005) Thimerosal-containing vaccines and autistic spectrum disorder: a critical review of published original data. *Pediatrics* 115:200
- Peng Y, Power MR, Li B, Lin TJ (2005) Inhibition of IKK down-regulates antigen+IgE-induced TNF production by mast cells: a role for the IKK-IkappaB-NF-kappaB pathway in IgE-dependent mast cell activation. *J Leukoc Biol* 77:975–983
- Prévôt D, Darlix JL, Ohlmann T (2003) Conducting the initiation of protein synthesis: the role of eIF4G. *Biol Cell* 95:141–156
- Regala RP, Weems C, Jamieson L, Copland JA, Thompson EA, Fields AP (2005) Atypical protein kinase C iota plays a critical role in human lung cancer cell growth and tumorigenicity. *J Biol Chem* 280:31109–31115
- Régner CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M (1997) Identification and characterization of an Ikappa B kinase. *Cell* 90:373–383
- Risch N, Spiker D, Lotspeich L, Nouri N, Hinds D, Hallmayer J, Kalaydjieva L, McCague P, Dimiceli S, Pitts T, Nguyen L, Yang J et al (1999) A genomic screen of autism: evidence for a multilocus etiology. *Am J Hum Genet* 65:493–507
- Schneider G, Saur D, Siveke JT, Fritsch R, Greten FR, Schmid RM (2006) IKKalpha controls p52/RelB at the skp2 gene promoter to regulate G1- to S-phase progression. *EMBO J* 25:3801–3812
- Seto Y, Nakajima H, Suto A, Shimoda K, Saito Y, Nakayama KI, Iwamoto I (2003) Enhanced Th2 cell-mediated allergic inflammation in Tyk2-deficient mice. *J Immunol* 170:1077–1083
- Singer HS, Morris CM, Williams PN, Yoon DY, Hong JJ, Zimmerman AW (2006) Antibrain antibodies in children with autism and their unaffected siblings. *J Neuroimmunol* 178:149–155

- Stern L, Francoeur MJ, Primeau MN, Sommerville W, Fombonne E, Mazer BD (2005) Immune function in autistic children. *Ann Allergy Asthma Immunol* 95:558–565
- Talebizadeh Z, Butler MG, Theodoro MF (2008) Feasibility and relevance of examining lymphoblastoid cell lines to study role of microRNAs in autism. *Autism Res* 1:240–250
- Tordjman S, Anderson GM, Botbol M, Brailly-Tabard S, Perez-Diaz F, Graignic R, Carlier M, Schmit G, Rolland A-C, Bonnot O, Trabado S, Roubertoux P, Bronsard G (2009) Pain reactivity and plasma β -endorphin in children and adolescents with autistic disorder. *PLoS ONE* 4:5289. doi:[10.1371/journal.pone.0005289](https://doi.org/10.1371/journal.pone.0005289)
- Valenta R, Mittermann I, Werfel T, Garn H, Renz H (2009) Linking allergy to autoimmune disease. *Trends Immunol* 30:109–116
- Vercelli D, Jabara HH, Cunningham-Rundles C, Abrams JS, Lewis DB, Meyer J, Schneider LC, Leung DY, Geha RS (1990) Regulation of immunoglobulin (Ig)E synthesis in the hyper-IgE syndrome. *J Clin Invest* 85:1666–1671
- Win HY, Acevedo-Duncan M (2008) Atypical protein kinase C phosphorylates IKK α in transformed non-malignant and malignant prostate cell survival. *Cancer Lett* 270:302–311
- Ylisaukko-oja T, Alarcón M, Cantor RM, Auranen M, Vanhala R, Kempas E, von Wendt L, Järvelä I, Geschwind DH, Peltonen L (2006) Search for autism loci by combined analysis of autism genetic resource exchange and finnish families. *Ann Neurol* 59:145–155
- Zhang L, Huang J, Yang N, Liang S, Barchetti A, Giannakakis A, Cadungog MG, O'Brien-Jenkins A, Massobrio M, Roby KF, Katsaros D, Gimotty P, Butzow R, Weber BL, Coukos G (2006) Integrative genomic analysis of protein kinase C (PKC) family identifies PKC δ as a biomarker and potential oncogene in ovarian carcinoma. *Cancer Res* 66:4627–4635
- Ziv Y, Schwartz M (2008) Immune-based regulation of adult neurogenesis: implications for learning and memory. *Brain Behav Immun* 22:167–176