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Increased IgG4 levels in children with autism disorder

Amanda Enstrom [a,f], Paula Krakowiak [b,f], Charity Onore [a,f], Isaac N. Pessah [c,f], Irva Hertz-Picciotto [b,f], Robin L. Hansen [d,f], Judy A. Van de Water [f,e], Paul Ashwood [a,f,*]

[a] Department of Medical Microbiology and Immunology, 2805, 50th Street, Wet lab building, Sacramento, CA 95817, USA
[b] Department of Public Health Sciences, University of California at Davis, CA, USA
[c] Department of Veterinary Molecular Biosciences, University of California at Davis, CA, USA
[d] Department of Pediatrics, University of California at Davis, CA, USA
[e] Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis, CA, USA
[f] The Medical Investigation of Neurodevelopmental Disorders (M.I.N.D.) Institute, University of California at Davis, CA, USA

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ABSTRACT

Accumulating evidence indicates that immune dysfunction is associated with autism disorders in a significant subset of children. Previous reports have shown abnormal immunoglobulin (Ig) levels, including an increased presence of autoreactive antibodies in the circulation of individuals with autism. As IgG is the predominant antibody isotype in circulation, we expected that an altered immune response could result in an abnormal IgG subclass profile in children with autism. We examined circulating plasma levels of IgG1, IgG2, IgG3, and IgG4 in 241 children from the CHARGE (Childhood Autism Risks from Genetics and the Environment) study, a large epidemiologic case-control investigation, including 114 children who meet full criteria for autism disorder (AU), 96 typically developing control children (TD) from a randomly selected sample of the general population, and 31 children with developmental delays (DD). We report significantly increased levels of the IgG4 subclass in children with AU compared with TD control children ($p = 0.016$) and compared with DD controls ($p = 0.041$). These results may suggest an underlying immunological abnormality in AU subjects resulting in elevated IgG4 production. Further investigation is necessary to elucidate the relationship between immunological findings and behavioral impairments in autism.

1. Introduction

Autism spectrum disorders are a group of relatively common pervasive neurodevelopmental disorders affecting between 2 and 6 children per 1000 (Chakrabarti and Fombonne, 2005; Fombonne, 2005; Yeargin-Allsopp et al., 2003). Autism presents early in childhood and is characterized by deficits in verbal and non-verbal communication, restricted and repetitive stereotypical patterns of behavior, and impairments in social interactions. The etiology of autism is unknown with both environmental and genetic factors considered to play key roles. Multiple genetic studies of children and adults with autism have indicated various candidate genes, many of them related to immune system function, including C4 null allele and MHC haplotypes B44-SC30-DR4, HLA-DR β1, DR4, and DR13 (Daniels et al., 1995; Ferrante et al., 2003; Lee et al., 2006; Torres et al., 2006; Warren et al., 1995). Other genes that have important roles in regulating the developing immune system, such as MET, PTEN and RELN have also been implicated in autism (Beilmann et al., 1997; Butler et al., 2005; Garbett et al., 2008; Pardo and Eberhart, 2007; Skaar et al., 2005). Environmental triggers, such as thalidomide, valproic acid, and prenatal and early postnatal infections have also been associated with autism (Ashwood et al., 2006; Fortier et al., 2007; Meyer et al., 2006; Miyazaki et al., 2005; Narita et al., 2002; Niehus and Lord, 2006; Rodier et al., 1997; Rosen et al., 2007; Shi et al., 2003; Vojdani et al., 2003; Williams et al., 2001). Evidence includes increased rates of infection in neonates who were later diagnosed with autism, (Rosen et al., 2007), and links with congenital rubella, cytomegalovirus and other viral infections (Chess, 1971; Stubbs et al., 1984). In addition, several researchers have described various immune abnormalities, including increased levels of inflammatory mediators and the presence of autoimmune phenomena (Ashwood et al., 2006; Cohly and Panja, 2005; Korvatska et al., 2002). Taken together, these findings suggest that immunological imbalances or alterations during critical neurodevelopmental junctures could be important in a significant subset of children with autism.

Descriptions of aberrant immune function in individuals with autism have been reported for nearly 40 years. Among these findings there has been a long standing focus on autoimmune processes in individuals with autism and/or their primary and secondary family members compared with the general population

* Corresponding author. Address: Department of Microbiology and Immunology, and The M.I.N.D. Institute, 2805, 50th Street, Wet Lab Building, Sacramento, CA 95817, USA. Fax: +916 703 0367.
E-mail address: pashwood@ucdavis.edu (P. Ashwood).

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(Ashwood and Van de Water, 2004; Cohly and Panja, 2005; Comi et al., 1999; Money et al., 1971). Reports of self-reactive antibodies, especially to brain and CNS proteins (Cabanlit et al., 2007; Connolly et al., 2006, 1999; Kozlovskaia et al., 2000; Silva et al., 2004; Singh and Rivas, 2004; Singh et al., 1993; Todd et al., 1988; Vojdani et al., 2002, 2004; Wills et al., 2007), have been one of the most robust and consistently demonstrated immunological findings in autism. Furthermore, depending on the study, an estimated 25–70% of children with autism have antibodies that are reactive to neuronal proteins (Connolly et al., 1999; Singh et al., 1997). Their presence may indicate that a potential breakdown in the regulatory network which controls the immune response has occurred.

Immunoglobulin (Ig) G is the most prevalent antibody isotype in human circulation and consists of four subclasses. Each subclass of IgG has different biological properties. IgG1 and IgG3 are predominantly responsible for protection against re-infection based on their ability to activate complement, which induces platelet binding and clearance of infectious agents from the body. In contrast, IgG2 and IgG4 do not bind complement. IgG4 is univalent and like IgE has been implicated in allergy development. IgG4 is also thought to act as a blocking antibody, produced in response to chronic exposure as a means of immune regulation (Aalberse and Schuurman, 2002).

The clinical implications of skewed antibody subclasses are unclear. Croonenberghs et al. demonstrated that increased serum IgG2 and IgG4 concentrations were present in children with autism and were associated with certain behavioral outcomes (Croonenberghs et al., 2002). We compared IgG subclass levels among three groups of children; those who met the cut-offs for autistic disorder (AU), those with typical development and those with developmental delay. We also evaluated whether IgG subclass levels were associated with several clinical standardized measures of behavior and child neurodevelopment in both AU and control children.

2. Methods

2.1. Study design

Children in this project were participants in the Childhood Autism Risk from Genetics and the Environment (CHARGE) study, a large ongoing population-based case-control investigation being conducted at the University of California, Davis (Hertz-Picciotto et al., 2006). To be eligible for the CHARGE study, children must be between the ages of 24 and 60 months, born in California, living with their biological parents who speak English or Spanish, and residing within a defined catchment area. Children are recruited from three groups; children diagnosed with autism, diagnosed with developmental delay, or children sampled from the general population. Cases from the first two groups are identified from the California Department of Developmental Services Regional Center system that co-ordinates services for persons with developmental disabilities. The third group is sampled from birth certificate files with frequency matching by child's age, gender, and broad geographic area to the projected distribution of these factors in the autism case group. Children are evaluated to confirm or preclude developmental diagnoses by trained staff at the Medical Investigations of Neurodevelopmental Disorders (M.I.N.D.) Institute at the University of California, Davis. Standardized instruments considered to be gold-standard for diagnosing autism were administered, and along with standard assessments of cognitive and adaptive function, are described below. Further details on study design, recruitment, and data and specimen collection protocols are described in greater detail elsewhere (Hertz-Picciotto et al., 2006).

Participants in this project were 241 children recruited between March 2003 and August 2006, including 114 children diagnosed with autism (AU) based on gold-standard diagnostic assessments, 96 typically developing healthy (TD) controls from the general population and 31 children with developmental delays but not an autism spectrum disorder (DD). Samples were selected based on available volumes of plasma from consecutively recruited participants. Demographic data are detailed in Table 1. Informed consent was obtained from a parent for each study participant. This study was conducted with the approval of the institutional review boards of the UC Davis and the State of California.

2.2. Diagnosis and behavioral assessments

An autism disorder diagnosis was confirmed by qualified practitioners who have achieved research-reliability using the two gold-standard instruments: the Autism Diagnostic Observation Schedules (ADOS) (Lord et al., 2000, 2003) and the Autism Diagnostic Interview-Revised (ADI-R) (Le Couteur et al., 2003; Lord et al., 1997). Diagnostic algorithms have been published for both tests for autism and for autism spectrum disorders. The ADOS utilizes

Table 1
Participant demographic and diagnostic information. Data shown as median (interquartile range).

	Autism (n = 114)	TD (n = 96)	DD (n = 31)
Age, yrs*	3.47 (3.01–4.31)	3.21 (2.77–3.84)	3.57 (2.97–4.52)
Gender, n (%) male*	114 (92.1%)	72 (75.0%)	19 (61.3%)
Race/ethnicity			
White	60 (52.6%)	47 (49.0%)	16 (51.6%)
Hispanic	32 (28.1%)	34 (35.4%)	8 (25.8%)
Mixed or other**	22 (19.3%)	15 (15.6%)	7 (22.6%)
*Mother's education level**			
High school or less	15 (13.3%)	21 (21.9%)	4 (12.9%)
Some college/vocational	55 (48.7%)	26 (27.1%)	16 (51.6%)
Bachelor degree or higher	43 (38.0%)	49 (51.0%)	11 (35.5%)
Mother's birthplace			
US	89 (78.1%)	78 (81.3%)	26 (83.9%)
Mexico	6 (5.3%)	6 (6.2%)	2 (6.4%)
Other country	19 (16.7%)	12 (12.5%)	3 (9.7%)
Delivery payer			
Government program	20 (17.7%)	11 (11.5%)	6 (19.3%)
Private insurance	93 (82.3%)	85 (88.5%)	25 (80.7%)

* $P < 0.05$ likelihood ratio Chi-square test (child's gender, mother's education); $P < 0.05$ one-way ANOVA (child's age).
** Other race categories include Black, Asian, and Pacific Islander or Native Hawaiian.

clinician-directed semi-structured play to assess social, communication, and imaginative play areas, which include stereotypic behaviors and restricted interests that are typical of autism. Four different modules are available depending on the expressive language skills of the child. Based on age and skill level, 92 AU participants were administered ADOS module 1, designed for the least verbal children. Only results from ADOS module 1 were used for correlative analysis of IgG isotypes with behavior as there was insufficient power for analysis using ADOS module 2. The ADI-R is an interview-based assessment administered to the primary caregiver of the child and measures qualitative impairments in reciprocal social interaction, communication, and repetitive and stereotyped behaviors. The ADOS and ADI-R administered together provide a reliable assessment of AU in young children, including those with mental retardation (de Bildt et al., 2004). To satisfy the definition of AU used in the CHARGE study, participants needed to score at or above levels meeting established criteria for autism diagnosis on the social interaction, communication, and stereotyped behaviors domains of the ADI-R prior to 36 months and meet the social + communication cut-off for AU on the ADOS module 1 or 2 (Hertz-Picciotto et al., 2006; Lord et al., 2003).

Parents of all participants, regardless of the child's diagnosis, completed the self-administered Aberrant Behavior Checklist (ABC), which is designed to assess inappropriate and maladaptive behaviors in children (Aman and Singh, 1994). This instrument assesses the frequency of behaviors related to the following domains: irritability, lethargy, stereotypic behaviors, hyperactivity, and inappropriate speech. Elevated scores on the ABC indicate increasing severity of impairment in the behavioral domains assessed. The ABC was completed for the majority of the participants in the three groups with only 19 uncompleted assessments. To confirm non-ASD status, the primary caregivers of children entering the study from the DD or TD group were additionally administered the Social Communication Questionnaire (SCQ). The SCQ, which is an adaptation of the Autism Diagnostic Interview-Revised (ADI-R) (Rutter et al., 2003) is a brief caregiver-completed screen for autism spectrum disorders that focuses on communication and social skill development across the child's lifetime, as well as in the most recent three months. Children with an ASD will score above 14 on the SCQ, with good agreement with the ADI-R, whereas children without an ASD will score less than 15 (Rutter et al., 2003). In the CHARGE study children with scores 15 or greater are assessed for autism with ADI-R and ADOS and are removed from the TD or DD control comparison groups (Hertz-Picciotto et al., 2006).

During a clinic visit each child's cognitive function was assessed using the Mullen Scales of Early Learning (MSEL) (Mullen, 1995) and adaptive function using the Vineland Adaptive Behavior Scales (VABS) (Sparrow and Cicchetti, 1985). The MSEL is a standardized test administered to children between 3 and 60 months of age and requires the child to complete requested activities in the presence of the examiner (e.g. block sorting). The MSEL addresses the areas of visual reception, fine motor skills, receptive language, and expressive language. The VABS is a standardized parental interview that can be utilized across age ranges. Areas of VABS assessment include communication (receptive, expressive, written), daily living skills (personal, domestic, community), socialization (interpersonal, coping, play), and motor skills (fine and gross). The composite scores from MSEL and VABS were used to designate delayed (DD) and typically developing (TD) groups. Delayed development was defined as having a score of 70 or less on MSEL and/or a score of 69 or less on VABS. Children with a score of 71 or above on MSEL and a score of 70 or above on VABS (i.e. within 2 standard deviations from the mean) were considered as typically developing. Only typically developing children who were unrelated to any other group were included in the TD group. Only one AU subject did not complete the MSEL during the clinic visit. Decreasing standardized scores on the MSEL and VABS assessments indicate a lower level of functional ability.

2.3. IgG isotyping

Peripheral blood was collected in acid-citrate-dextrose Vacutainer tubes (BD Biosciences; San Jose, CA) and centrifuged for 10 min at 2300 rpm. Plasma was harvested and stored at −80 °C until samples were assayed. Plasma was analyzed by Luminex using Beadlyte human IgG subclass beads (Upstate; Lake Placid, NY). The assay kit allowed for threshold of detection limits of as little as 3 ng/ml of IgG1, 16 ng/ml IgG2, 0.2 ng/ml IgG3, and 0.1 ng/ml IgG4. Analyte-specific antibody conjugated beads were incubated with 25 μl of 1× biotinylated detection antibody solution and 50 μl of either standard, or 50 μl of plasma diluted to 1:8000 in human isotyping serum diluent (supplied in kit) in the dark for 1 h at room temperature on an orbital shaker (500–600 rpm). After wells were washed and aspirated by vacuum manifold aspiration, 25 μl of strepavidin-conjugated R-phycoerythrin was added to each well and incubated in the dark for 1 h at room temperature on an orbital shaker (500–600 rpm). R-phycoerythrin fluorescence was measured on a Bio-Plex 200 System (Bio-Rad Laboratories Inc.; Hercules, CA) to determine concentration.

2.4. Statistical analysis

Descriptive statistics were computed for selected sociodemographic variables across diagnostic groups. *p*-Values were calculated using likelihood ratio Chi-square test or one-way ANOVAs. Covariates of interest as possible confounders, included the following demographic and socioeconomic factors: child's age at blood draw, child's gender, child's race/ethnicity, maternal education level, mother's place of birth (US vs. elsewhere), and delivery payer (private insurance vs. government program). In analyses involving questionnaire and assessment score data, diagnostic group was also evaluated as a covariate. Bivariate linear regression analyses involving each covariate (i.e. child's age, sex, race/ethnicity, maternal education, birthplace, delivery payer, and diagnostic group) in relation to the outcome of interest (i.e. IgG isotypes, ABC scores, assessment scores) were performed to determine associations with each outcome and to assess potential confounding by these variables. Covariates were considered to be associated with an outcome if the *p*-value was $\leqslant 0.20$ and were fitted into weighted multiple linear regression models for further evaluation.

2.5. Analysis weights

Analysis weights were constructed to correct for under-represented subjects in the CHARGE study with regard to key demographic and socioeconomic status (SES) factors including mother's education, birthplace, child's race and ethnicity, and delivery payer. First, to calculate these weights, the probability of participation in the study was determined. Pools of potential CHARGE Study participants used during recruitment were identified for each of the three study groups (AU, DD, and TD) using Regional Center data and birth files. Next, these pools were combined and probabilities for participation, contingent on subject group and a set of sociodemographic variables from the birth files, were obtained. A variable was created to distinguish participants from non-participants. To evaluate the sociodemographic factors, each variable was individually fitted into a logistic regression model adjusted for subject group, where the outcome was participant (in study) versus non-participant (not in study). Finally, a logistic regression model with SES, demographic, and subject group variables, as well as interaction terms (involving subject group and selected sociodemographic variables), was fitted using forward

selection to calculate predicted probabilities of participation. The analysis weight for each subject was defined as the inverse of the probability of participation for that subject based on the sociodemographic characteristics and subject group.

2.6. Data analyses

Tolerance and eigenvalues were used to evaluate collinearity in all multiple linear regression models. In primary analyses, natural log-transformed IgG isotypes (outcome) were compared by group (predictor) in weighted multiple linear regression models adjusted for child's age and gender. Secondary analyses examined the association between IgG isotype levels (predictor) and behavioral assessment scores using weighted multiple linear regression models adjusted for diagnostic group, as well as child's age, gender, mother's birthplace, and/or delivery payer. Analyses were not adjusted for multiple comparisons. Findings with p-value <0.05 were considered significant. All analyses were carried out using SAS version 9.1 (SAS Inc.; Cary, NC) and Prism 5 Software (GraphPad Software; San Diego, CA).

3. Results

3.1. Comparison of study groups

The median ages of AU participants was 3.5 years (interquartile range [IQR]: 3.0–4.3), for TD participants 3.2 years (IQR: 2.8–3.8) and for DD participants 3.6 years (IQR: 3.0–4.5) ($p = 0.01$, AU vs. TD; Table 1). In the AU group there were 92% males compared with 75% males in the TD group (AU vs. TD; $p = 0.001$) and 61% males in the DD group ($p = 0.0001$, AU vs. DD). Approximately, one-half of participants in each group were Caucasian. Over 50% of mothers of TD participants attained a maximum education level of at least a Bachelor's degree compared with 38% mothers of AU participants ($p = 0.005$). Although more mothers of TD participants used private insurance to pay for delivery compared with mothers of AU participants (89% versus 82%) this was not statistically significant.

3.2. Increased IgG4 subclass is associated with AU diagnosis

There were significantly increased levels of the IgG4 subclass in children with AU (3.2 (1.0–8.0) mg/dl median (interquartile range)) compared with TD controls (1.7 (0.5–3.5) mg/dl, $p = 0.016$, Fig. 1, Table 2). Furthermore, there was a statistical difference in IgG4 levels in AU children compared with DD controls (1.8 (0.7–5.0) mg/dl, $p = 0.041$, Fig. 1, Table 2). No statistical difference in IgG4 levels was observed between DD and TD children. IgG2 subclass levels were marginally raised in children with AU (8.3 (6.6–12.2) mg/dl compared with TD controls (7.4 (5.6–10.8) mg/dl), but did not reach statistical significance ($p = 0.051$, Table 2). IgG2 levels were not different between DD (9.5 (7.2–14.6) mg/dl) compared with TD. IgG1 and IgG3 subclasses were not statistically different between any of the study groups (Table 2).

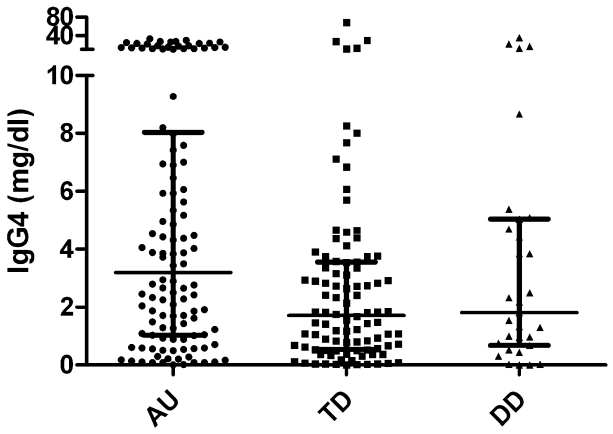

Fig. 1. Plasma IgG4 levels (ng/ml) in autism. IgG4 levels were significantly increased in children with autism (AU) compared with typically developing (TD) controls ($p = 0.016$) and children with other developmental disabilities (DD) ($p = 0.041$). The bars represents the median plus interquartile range for IgG4 levels in each group.

Table 2
IgG isotype values (mg/dl) shown as median (interquartile ranges). IgG4 is significantly increased in autism (AU) subjects compared with typically developing (TD) and developmental delayed (DD) controls.

	AU ($n = 114$)	TD ($n = 96$)	DD ($n = 31$)
IgG1	101.1 (82.1–128.0)	104.8 (85.5–129.4)	112.4 (96.6–135.4)
IgG2	8.3 (6.6–12.2)	7.4 (5.6–10.8)	9.5 (7.2–14.6)
IgG3	2.8 (2.0–4.1)	2.9 (2.3–3.9)	3.1 (2.0–5.4)
IgG4	3.2 (1.0–8.0)[a,b]	1.7 (0.5–3.5)	1.8 (0.7–5.0)

[a] $P < 0.05$, AU vs. TD.
[b] $P < 0.05$, AU vs. DD.

To address whether IgG subclass levels were associated with developmental and behavioral measures as previously suggested (Croonenberghs et al., 2002), we fitted weighted multiple linear regression models to examine the association between IgG4 subclass levels with data from the following behavioral, cognitive and adaptive assessments: ABC, MSEL, and VABS (Table 3) for all participants. Although IgG4 was positively associated with stereotypical behavior ($t = 2.65$, $p = 0.01$) and with lethargy ($t = 4.21$, $p = 0.001$) using the ABC behavioral assessment in weighted analysis that was unadjusted for any covariates, when each regression model was adjusted for diagnostic group, child's age, gender, mother's birthplace and delivery payer the associations between IgG4 and stereotypical behavior ($t = 2.26$, $p = 0.13$) and with lethargy ($t = 2.55$, $p = 0.11$) approached but failed to reach statistical significance. No significant associations were observed between any IgG isotypes levels and other ABC subscales, or in measures using MSEL or VABS assessments in unadjusted or adjusted analyses.

Further analyses were conducted within the group of children with AU only to determine whether IgG subclasses were associated with severity of autistic symptoms using autism-specific assessments. Among AU children assessed using the ADOS module 1, higher IgG4 was associated with increased severity of social interaction impairments as measured by the ADOS module 1 ($t = 2.13$, $p = 0.036$) in models that were weighted and adjusted for child's age and gender. Increased severity of communication impairments in AU participants as measured by ADOS module 1 were not statistically significantly associated with IgG4 levels in weighted linear regression models adjusted for child's age and gender ($t = 1.64$, $p = 0.093$). There were no associations between IgG1, IgG2 or IgG3 levels and ADOS measurements. In AU participants there were no significant associations noted for any IgG subclasses and reciprocal social interaction, verbal communication, non-verbal communication and stereotyped behaviors using ADI-R assessments following weighted analysis and adjustment for age and gender. There were no associations of any IgG subclass levels with assessments scores on the MSEL, with VABS or ABC in the AU only participants.

4. Discussion

The major finding of our study was that IgG4 levels were increased in children with autistic disorder compared with typically developing control children and compared with developmentally

Table 3
Participant behavioral and assessment scores. Data shown as median (interquartile range).

	AU (n = 114)	TD (n = 96)	DD (n = 31)
Aberrant Behavior Checklist			
Irritability	13.0 (9.0–19.0)	1.0 (0.0–4.0)	7.5 (3.0–18.0)
Lethargy	12.0 (8.0–18.0)	0.0 (0.0–0.0)	3.5 (1.0–9.0)
Stereotypy	6.0 (4.0– 9.0)	0.0 (0.0–0.0)	0.0 (0.0–4.0)
Hyperactivity	18.0 (14.0–26.0)	1.5 (0.0–5.0)	10.0 (4.0–23.0)
Inappropriate speech	2.0 (0.0– 4.0)	0.0 (0.0–1.0)	0.0 (0.0–2.0)
Total sore	56.0 (39.0–73.0)	3.5 (0.0–10.0)	30.0 (13.0–46.0)
Mullen Scales of Early Learning			
Visual reception	20.0 (20.0–29.0)	58.0 (49.5–64.5)	20.0 (20.0–30.0)
Fine motor	20.0 (20.0–27.5)	52.0 (45.0–59.0)	20.0 (20.0–21.0)
Receptive language	20.0 (20.0–25.0)	51.0 (46.0–61.0)	20.0 (20.0–26.0)
Expressive language	20.0 (20.0–23.0)	53.0 (43.0–62.0)	20.0 (20.0–27.0)
Composite score	50.0 (49.0–58.0)	106.0 (95.0–121.5)	49.0 (49.0–56.0)
Vineland Adaptive Behavior Scales			
Communication	60.0 (54.0–66.0)	107.5 (98.0–116.0)	62.0 (56.0–70.0)
Socialization	63.0 (58.0–69.0)	102.0 (93.0–113.0)	64.0 (57.0–76.0)
Daily living	63.0 (57.0–72.0)	105.5 (99.0–114.5)	68.0 (59.0–79.0)
Motor skills	72.0 (62.0–86.0)	111.0 (103.5–120.0)	58.0 (54.0–68.0)
Composite score	60.0 (54.0–68.0)	111.0 (99.0–120.0)	61.0 (52.0–66.0)

delayed children unaffected by autism. Our data supports a previous report that showed IgG4 levels were increased in autism (Croonenberghs et al., 2002). This previous study also hinted that increased IgG4 levels were associated with increasing severity of aberrant behaviors in autism. In the current investigation there was some evidence to suggest that IgG4 levels may be associated with autism-specific social interaction impairments among the children with autism based on measurements using the gold-standard ADOS assessment. However, as no other behavioral measures obtained from separate behavioral assessments, including reciprocal interactions on the ADI-R, found any statistical associations between IgG4 levels and behaviors, these data are difficult to interpret and should be treated with caution until further future investigations are performed. In addition, although our statistical analyses addressed potential confounding factors from socioeconomic status, by weighting and adjusting the analysis, these factors are only proxy measures for socioeconomic status and further large-scale studies need to be designed that fully account for socioeconomic and demographic factors.

There are several limitations to this current study. First, while we did not include any participant who showed visible signs of illness or who had a fever, our use of a single cross-sectionally obtained blood sample would not capture temporal fluctuations in the IgG isotype levels based on health status or environmental factors such as vaccinations. Secondly, limiting the ADOS analysis to module 1 was necessary as the algorithm scores between modules 1 and 2 are not interchangeable, but this clearly limited the sample to the more severely language impaired children, who, within the age group studied are usually the more cognitively impaired. Other measures of communication impairments should be examined in further studies that have sufficient power for analysis within groups of children comparable with regard to level of cognitive development, to determine whether the associations between IgG4 and communication skills are replicated. These studies must be conducted within case groups, as diagnostic group may be a confounding factor when correlating IgG levels with behavioral outcomes. Thirdly, in the autism group used for this study, there was a slightly higher than expected prevalence in boys relative to girls, as compared with the literature, although epidemiologic reviews have shown that there is considerable variability in the male:female ratio (reviewed by Fombonne, 2005) which range from 1.3 to 15.7. In addition, the recent CDC survey that obtained much larger case series in six states showed a two-fold variation for sex ratio (Rice et al., 2007). However, within any subsample of CHARGE, there will be some slight variations in the gender ratio due to random variability. Since gender was not related to IgG4 the variations in gender ratio would not have influenced the principal results of this study.

The salient finding of this study is the demonstration of an increase in IgG4 levels among children with autism compared with TD and DD controls. This result is present after adjusting for socioeconomic and demographic factors and adds to the growing body of research suggesting immune alterations accompany the behaviorally defined disorder of autism. Evidence related to specific behavioral domains is weak, but in any case, all associations reported here need to be replicated in larger future studies and especially in prospective studies that examine children prior to the development of autism's core features.

Although not statistically significant, levels of IgG1 and IgG3 appear lower in AU compared with the TD controls. We recently found a statistically significant decrease in total IgG levels in children with AU compared with controls as assessed by ELISA (Heuer et al., 2008). These studies and the current study differ in the analytes that were measured. For example, here we used a Luminex based technique to measure IgG subclasses and do not directly measure total IgG, whereas the other study measured total IgG but not individual IgG subclasses. The different buffering systems, specificity of antibodies, antibody sensitivities, different assay platforms and different product manufacturers used make it hard to directly compare between these two studies; however, the slight decrease in IgG1 (the major IgG subclass in plasma, especially in pediatric cases) in this study would suggest that the same trend would be observed i.e. that total IgG levels are reduced in AU compared with controls. Moreover, the results from these two papers would suggest that in AU there is a possible shift in IgG constituents, such that there is an increased level of IgG4 on a reduced background of total IgG. This finding is consistent with a previous report of increased IgG4 determined by nephlometry in children with autism compared to unaffected siblings (Trajkovski et al., 2004). Of interest, associations between behavior and IgG levels were noted in both this study and the investigation of total IgG reported by Heuer and colleagues (Heuer et al., 2008). Future studies in a larger cohort in which total IgG and its individual subclasses are measured using the same technique would clarify these issues.

IgG4 is a relatively unique subclass of IgG. IgG4 unlike IgG1, IgG2, and IgG3 does not bind strongly to any of the antibody recep-

tors (CD16, CD32) found on human leukocytes (Aalberse and Schuurman, 2002; van der Zee et al., 1986). IgG4 binds to the receptor CD64 (FcγRI) on monocytes and macrophages with ten times less affinity than either IgG1 or IgG3. Moreover, the circulating structure of IgG4 is functionally monovalent and differs from IgG1, IgG2, or IgG3, which contain two binding sites (Aalberse et al., 1983; van der Zee et al., 1986). These features drastically alter the biological function of the IgG4 antibody, shifting its function to that of a blocking or inhibiting antibody rather than one of protection through the more conventional routes, such as complement fixation (van der Neut Kolfschoten et al., 2007). Increased IgG4 is found in patients with atopy as well as prolonged chronic antigen stimulation (Aalberse et al., 1983; Shakib, 1986). Under prolonged antigen exposure, IgG4 allergen-specific antibodies increase eventually overtaking IgG1-specific titers (Aalberse et al., 1983). These IgG4 antibodies directly interfere with IgG1 antibody function (Aalberse and Vermeulen, 2001; van der Zee et al., 1986), possibly serving a protective role by decreasing IgG1-mediated inflammation and tissue damage.

If one considers IgG4 as a blocking antibody induced in the presence of chronic antigen exposure, including potential autoantigens or food allergens, this could relate to several of the phenomena described in autism, including increased food allergies and the presence of autoantibodies (Ashwood and Van de Water, 2004; Jyonouchi et al., 2002). The increased IgG4 in ASD children may be indicative of a genetic tendency towards either (1) chronic self-antigen exposure or (2) poor clearance of microbial antigens due to some other immune abnormality. Either situation is equally likely, both increased self-reactive (primarily neuroreactive) antibodies (Cabanlit et al., 2007; Connolly et al., 2006, 1999; Kozlovskaia et al., 2000; Singh and Rivas, 2004; Singh et al., 1997, 1993; Todd et al., 1988; Wills et al., 2007), and other altered immunological findings (Ashwood and Van de Water, 2004) have been reported in autism.. Indeed, the association of increased IgG4 with severity of behavioral and cognitive impairment may be related to another primary factor, such as tissue damage releasing sequestered neuronal antigens or underlying chronic inflammation. An area for further research is to determine if neuronal antibodies reported in other studies (Cabanlit et al., 2007; Connolly et al., 2006, 1999; Kozlovskaia et al., 2000; Singh and Rivas, 2004; Singh et al., 1997, 1993; Todd et al., 1988; Wills et al., 2007) are of the IgG4 subclass, suggesting they may be blocking antibodies.

The implications of the association between higher IgG4 levels and AU are unclear, however, one could speculate that a dysregulation in immune function may skew the immune response towards an elevated IgG4 antibody titer. Furthermore, potential gene-environment interactions, in which chronic antigen exposure in individuals who have a genetic predisposition towards immune dysregulation could thus, lead to increased IgG4 concentrations. The link between such immune dysregulation and changes in neurodevelopment and, subsequently behavior, are still unknown. This study provides further specificity to the growing evidence for a role of the immune system in the development and exacerbation of behavioral defined autism disorders, and for potentially critical interactions of the nervous and immune systems in such disorders.

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