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Altered Gene Expression and Function of Peripheral Blood Natural Killer Cells in Children with Autism

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Abstract

Immune related abnormalities have repeatedly been reported in autism spectrum disorders (ASD), including evidence of immune dysregulation and autoimmune phenomena. NK cells may play an important role in neurodevelopmental disorders such as ASD. Here we performed a gene expression screen and cellular functional analysis on peripheral blood obtained from 52 children with ASD and 27 typically developing control children enrolled in the case-control CHARGE study. RNA expression of NK cell receptors and effector molecules were significantly upregulated in ASD. Flow cytometric analysis of NK cells demonstrated increased production of perforin, granzyme B, and interferon gamma (IFN γ) under resting conditions in children with ASD ($p < 0.01$). Following NK cell stimulation in the presence of K562 target cells, the cytotoxicity of NK cells was significantly reduced in ASD compared with controls ($p < 0.02$). Furthermore, under similar stimulation conditions the presence of perforin, granzyme B, and IFN γ in NK cells from ASD children was significantly lower compared with controls ($p < 0.001$). These findings suggest possible dysfunction of NK cells in children with ASD. Abnormalities in NK cells may represent a susceptibility factor in ASD and

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may predispose to the development of autoimmunity and/or adverse neuroimmune interactions during critical periods of development.

Introduction

Autism spectrum disorders (ASD) are complex neurodevelopmental disorders which are typically diagnosed within the first three years of life. ASD are characterized by significant impairments in social interaction and communicative skills, as well as restricted and stereotyped behaviors and interests (Association, 2000). ASD includes both Asperger's syndrome and autism disorder, as well as pervasive developmental disorder not otherwise specified (PDD-NOS) (Association, 2000). Specific diagnosis is determined by the nature and severity of delays or deficits in communication and social interactions and the presence or absence of restricted and stereotyped behaviors/interests. Males are four times more likely to be diagnosed with ASD than females (Fombonne, 2005). Over the past decade, intense interest has focused on ASD, as the prevalence appears to be increasing (Fombonne, 2005). Recent estimates, including the recent CDC study, place overall prevalence of ASD at 1 per 150 children (Fombonne, 2005; Kuehn, 2007).

Despite expanding research in ASD, its etiologies remain poorly understood and the relative contribution from genetic, epigenetic, and environmental susceptibility factors remains widely debated (Ashwood et al., 2006). Twin studies indicate a strong heritability for ASD risk (Muhle et al., 2004), and whole genome scans have revealed potential ASD candidate genes on nearly every chromosome (Szatmari et al., 2007; Veenstra-VanderWeele and Cook, 2004). Several studies have demonstrated ASD associations with immune related genes, including: complement C4 null allele (Odell et al., 2005; Warren et al., 1991), HLA-DR β 1, and DR13 (6–9), and immune cell development genes, such as Reelin (RELN) and MET protooncogene (MET) (Muhle et al., 2004; Skaar et al., 2005). In addition, systemic abnormalities of the immune system have been one of the most common and long-standing reported findings in ASD (Money et al., 1971; Stubbs and Crawford, 1977). Extensive neuroimmune interactions, beginning as early as embryogenesis, offer one possible explanation for the involvement of the immune response in the development of ASD and the ongoing immune alterations demonstrated in affected individuals.

Immunological findings in ASD have been reported systemically and at the cellular level, including familial associations with autoimmune and/or immune disorders such as atopy and asthma (Ashwood and Van de Water, 2004; Comi et al., 1999; Money et al., 1971). Notably, altered production of proinflammatory signaling proteins, such as cytokines, have been identified in the plasma, peripheral immune cells, brain, and CSF of individuals with ASD (Ashwood et al., 2003; Ashwood et al., 2004; Ashwood and Wakefield, 2006; Croonenberghs et al., 2002; Jyonouchi et al., 2001; Molloy et al., 2006; Singh, 1996; Vargas et al., 2005; Zimmerman et al., 2005). Despite reported increases in inflammatory mediators in plasma and CNS tissue, immune cells isolated from individuals with ASD fail to respond appropriately to mitogen stimulation, such as phytohemagglutinin (Stubbs and Crawford, 1977; Warren et al., 1986). Moreover, there is a growing literature that demonstrates the increased presence of autoantibodies, especially to CNS proteins, in children with ASD and some mothers of children with ASD (Ashwood and Van de Water, 2004; Cohly and Panja, 2005; Wills et al., 2007; Zimmerman et al., 2007). In susceptible individuals, immune dysregulation may predispose to the generation of aberrant or inappropriate immune responses such as autoimmunity and/or adverse neuroimmune interactions which during critical developmental windows may ultimately lead to changes in neurodevelopment.

Despite the broad scope of immunological findings in ASD, no consistent and specific immunological dysfunction has emerged. Recently, a mRNA expression study of peripheral blood cells of young children with ASD found increased expression of natural killer (NK) cell-associated genes (Gregg et al., 2008). This is interesting as twenty years ago, Warren *et al.* reported decreased NK cell function in individuals with autism (Warren et al., 1987). NK cells have been implicated in the pathology of other neurological and behavioral disorders, including Tourette syndrome (Lit et al., 2007), schizophrenia (Yovel et al., 2000), and multiple sclerosis (Jie and Sarvetnick, 2004; Johansson et al., 2006). In addition, imbalances between inhibitory and activating NK signaling have been implicated in the development of several autoimmune diseases, including diabetes mellitus (van der Slik et al., 2007), rheumatoid arthritis (Villanueva et al., 2005), systemic lupus erythematosus and scleroderma (Pellett et al., 2007).

The findings of a possible association between NK cell dysfunction and ASD reported by Warren *et al.* (Warren et al., 1987) and RNA expression profiling implicating NK cells (Gregg et al., 2008) led us to directly address whether NK cell was altered in ASD. First, using the findings described by Gregg *et al.* (Gregg et al., 2008), we refined the profile of NK gene expression associated with autism. Secondly, we determined that the specific changes reflected in the RNA profiles translated to altered NK activity, and cytolytic protein production at the cellular level in a separate cohort of children diagnosed with ASD. Together, these RNA expression and cellular studies indicate a role for NK cells in the dysregulated immune responses reported in ASD.

Materials and Methods

Study Participants

Participants in the study were recruited in conjunction with the CHARGE (Childhood Autism Risk from Genetics and Environment) study conducted at the UC Davis M.I.N.D. Institute; Sacramento, CA. A full description of the study and the assessment criterion has been described (Hertz-Picciotto et al., 2006). This study was approved by the UC Davis institutional review board and complied with all requirements regarding human subjects. Informed consents were obtained from legal guardians of each participant.

All children, including those from the general population, were screened for autism traits using the Social Communication Questionnaire (SCQ) (Eaves et al., 2006). Children with a preliminary outside diagnosis of autism, as well as those scoring above the screening threshold on the SCQ were further tested to confirm diagnosis and completed the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1997) and the Autism Diagnostic Observation Schedule (ADOS) (Gotham et al., 2007) conducted at the M.I.N.D. institute. The ADI-R is a comprehensive clinical interview administered to parents or caregivers assessing language and communication, reciprocal social interaction, and repetitive, restricted, stereotyped behaviors or interests. Results from the interview are interpreted by using a diagnostic algorithm that correlates with the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and The International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) definitions of autism. The ADOS is an observational assessment of the children suspected of having autism in various structured and unstructured situations that provide a standardized set of conditions to observe behavior in the areas of communication, play, and other areas relevant to autism. Only children who scored above the cut-off for the ADOS modules 1 and 2 for ASD and met the criteria for autism in the ADI-R were included in the study as part of the ASD study group. To reduce confounding factors, children who were ill at the time of the study, or had a temperature above 98.9°F, or were prescribed anti-psychotics were excluded from the study. None of the children had any other known medical disorder or primary diagnosis (e.g. Fragile X or Rett syndrome).

Two separate cohorts totaling 79 children between 2 and 5 years of age participated in the study. Children were selected at random from the larger CHARGE study pool, which were matched for age, gender, and postal code (Hertz-Picciotto et al., 2006). Peripheral blood from 46 children was used for RNA analysis and included 35 children with autism spectrum disorders (ASD) and 11 age and gender matched typically developing general population non-sibling controls (GP). All of the ASD subjects met criteria for full autism, based on ADOS and ADI-R criteria. Eighteen of the ASD subjects met the requirements for regression based on questions 11 and 25 on the ADI-R assessment. An additional 33 children (17 ASD and 16 GP) from the CHARGE study were included in the NK cellular study. Of the 17 ASD children, 16 met criteria for full autism disorder; the remaining child was reclassified as having an ASD but not full autism after the start of the study following clinician review. Three ASD subjects in the cellular analysis group met the requirements for regression based on ADI-R measures. All subjects are described in Table 1.

Gene Expression Studies

RNA isolation and microarray processing—RNA isolation and microarray processing were performed as described in Gregg *et al.* (Gregg et al., 2008). Briefly, 15 ml blood was collected into 6 PAXgene vacutainer tubes (Qiagen; Valencia, CA). RNA quality was confirmed on an Agilent 2100 Bioanalyzer. Gene expression was assessed on the human U133 Plus 2.0 GeneChip (Affymetrix; Santa Clara, CA), consisting of over 54,000 oligonucleotide transcript probe sequences. Protocols from the Affymetrix *Expression Analysis Technical Manual* were used for labeling the RNA, hybridization to the chip, and scanning the array. Expression of select probes was validated by RT-PCR (Gregg et al., 2008). GENESPRING 7.2 software (Agilent Technologies Inc., Santa Clara, CA) was used for gene expression analysis. Background and data normalization were performed using GC-RMA followed by three-step normalization (data transformation, per chip normalization, and per gene normalization). The microarray data is available at the Gene Expression Ombudsmen (GEO) with the tracking series number GSE6575.

Principal Components Analysis—Initial findings identified in Gregg *et al.* (Gregg et al., 2008) suggested increased expression of a group of NK cell related genes in many of the children with ASD compared with typically developing GP children. To further investigate these findings, we performed a principal components analysis using the seven genes validated by RT-PCR (Table 2) to determine how much of the variation between groups was accounted for by these seven genes.

Expanded gene expression profile—Using results from the Principal Components Analysis, we conducted an unpaired t-test (fold change $\geq |1.5|$, $p \leq 0.05$, false discovery rate (FDR) $q \leq 0.05$) comparing gene expression in children with ASD having high expression of NK cell related genes with gene expression in children having low expression of NK cell related genes.

Functional annotation—Using the expanded gene expression profile, the Database for Annotation, Visualization, and Integrated Discovery (DAVID; <http://niaid.abcc.ncifcrf.gov/>), supplemented by a literature search, was used to assess pathway and functional integration for genes within the expanded gene expression profile. Each annotation cluster represents a group of annotation terms with similar biological meaning due to sharing similar gene members. Enrichment Scores are the geometric mean (in $-\log$ scale) of functional annotation p-values in the corresponding functional annotation cluster; higher enrichment scores indicate overall smaller p values within a cluster. For each annotation term, p values are Expression Analysis Systematic Explorer (EASE) scores, a modified Fisher Exact Test which uses a Gaussian hypergeometric probability distribution using sampling without replacement to identify

likelihood of observing identified genes by random chance (Hosack et al., 2003). False Discovery Rates (FDR) using a Benjamini-Hochberg multiple comparison correction are provided to adjust for multiple comparisons (Benjamini and Hochberg, 1995). Webgestalt (<http://bioinfo.vanderbilt.edu/webgestalt>) Gene Set Analysis Toolkit was used to further examine gene expression patterns.

Cellular Studies

NK Cell Isolation—Approximately 8–10 ml peripheral blood was collected in acid-citrate-dextrose Vacutainers (BD Biosciences; San Jose, CA). Precise volume of blood collected was determined followed by centrifugation at 2300 rpm for 10 min. Peripheral blood mononuclear cells (PBMC) were separated using Histopaque (Sigma; St. Louis, MO) density gradient centrifugation at 1700 rpm for 30 min. CD56⁺ NK cells were positively selected by magnetic bead separation following the manufacturer's protocol (Miltenyi Biotec; Auburn, CA). CD56⁺ cells were adjusted to 3×10^6 cells/ml in RPMI media (Sigma) containing 10% heat-inactivated FBS (Omega Biosciences Tarzana, CA). Purity of CD56⁺ lymphocytes for all samples exceeded 95% as assessed by flow cytometry.

NK cytotoxicity Assay—MHC-devoid K562 cells (ATCC; Manassas, VA) were used as targets to assess NK cell cytotoxicity. Sorted CD56⁺ PBMC were incubated for 20 hours at 37°C in round bottom 96-well culture plates with 100,000 calcein-AM labeled K562 target cells at 25:1, and 10:1 effector:target cell ratios, and 50:1 and 1:1 effector:target cell ratios when sufficient cell numbers were obtained. Cells were washed and stained with Texas-Red conjugated ethidium-homodimer-1 (Molecular Probes; Eugene, OR) and CD56-PE Cy-7 for 15 min. Cells were then washed and immediately analyzed on a LSR II flow cytometer (BD Biosciences). Positive and negative controls were included in every assay and run concurrently. Singly stained cells for each fluorophore used were run for compensation correction. Unstained negative controls included unlabeled K562 cells (with and without CD56⁺ cells) and unlabeled CD56⁺ cells. Positive controls for maximum lysis included calcein-AM labeled K562 cells fixed and permeabilized at the end of the incubation period, prior to staining. Controls for spontaneous lysis were calcein-AM cells not incubated with any target cells. Dead K562 cells were gated on as dual-positive for calcein-AM and ethidium homodimer and were easily distinguishable from effector cells labeled with CD56 and live K562 cells.

Flow Cytometry—All cells were analyzed on an LSR II flow cytometer (BD Biosciences). Variance was assessed prior to start of every assay. Additionally, compensation was calculated for each assay using FACS Diva Software (BD Biosciences). Singly stained K562 and CD56⁺ cells were used to calculate compensation for the cytotoxicity assay, and anti-mouse antibody coated compensation beads (BD Biosciences) were used for the NK stimulation assay. A minimum of 30,000 ungated events was counted for each tube. Cell gating was performed using FlowJo software (Treestar Inc; Ashland, OR). NK cells were discriminated from NKT cells by gating positive on CD56⁺ cells and negative for both CD3 and CD8. CD56⁺ NK cells were further divided into CD56^{Dim} and CD56^{Bright} NK cells based on intensity of CD56 staining. CD56^{Dim} NK cells are associated with high natural cytotoxicity, KIR surface expression, and low cytokine production (43). Singly stained compensation controls and isotype mAb controls (BD Biosciences) were run for each subject. Positive gating was determined by setting gates at isotype control maximums for each subject's cells. Additionally, cells were stained with CD3, CD8, and CD56 with isotype controls in place of PE and APC to appropriately set positive gates for perforin and IFN γ . NK cells have previously been separated into two categories: CD56^{Dim} cells, which are the principal producers of cytolytic proteins, and CD56^{Bright} cells, which are the principal cytokine producers (41). As such, CD56^{Dim}, and CD56^{Bright} NK cells were assessed separately in order to determine if there were differences

in perforin, granzyme B and IFN γ in ASD versus GP groups. Subset determination and examples of staining are shown in Figure 1.

Antibodies—For the analysis of cell surface markers we used the following mouse-anti-human antibodies: PerP-Cy5.5 labeled CD3, PE-Cy5 labeled CD8, and PE-Cy7 labeled CD56 (BD Biosciences). For intracellular molecule analysis (see below), we used PE labeled perforin, APC labeled IFN γ , and Alexa-700 labeled granzyme B (BD Biosciences).

NK Cell Stimulation—CD56⁺ PBMC were stimulated as above with unlabeled K562 cells at 25:1 and 10:1 effector:target cell ratios. Additional CD56⁺ PBMC were incubated alone as unstimulated controls. Brefeldin-A was added at a final concentration of 10 μ g/ml at the incubation start to trap newly synthesized proteins including cytokines (Sigma). 6 μ g/ml monensin was additionally added to prevent degradation of endocytic vesicles (Sigma). Cells were labeled with cell surface antibodies for 20 minutes at room temperature in PBS containing 0.5% BSA to block non-specific binding. Cells were washed and fixed at 4°C for 10 minutes with 4% paraformaldehyde containing saponin. Cells were washed with PBS containing saponin detergent and stained with intracellular antibodies for 30 minutes. Cells were washed again, resuspended in buffer containing saponin and analyzed by flow cytometry within 2 hours of collection. Singly stained compensation beads (BD Biosciences) were used to calculate compensation. Unlabeled cells were used as an additional control.

Statistics—Unpaired t-tests were used to examine the flow cytometry and cytotoxicity data ($p < 0.05$ considered significant). The calculations were conducted utilizing GraphPad Prism 5 Software (GraphPad Software; San Diego, CA).

Results

Gene Expression Study

Refined gene expression profile—Previously, we demonstrated that seven genes were expressed at higher levels in children with ASD compared with matched controls, using microarrays and validation by RT-PCR (Gregg et al., 2008). To further examine the differences in gene expression in ASD, we used these seven genes as input for a Principal Components Analysis (PCA) and compared gene expression profiles in 35 children with ASD with 11 typically developing general population control (GP) children (Table 2). There were significantly more children with ASD who had a high expression of these genes compared with GP controls (Chi-square=11.9, $p=0.0005$). Unpaired t-tests used to compare ASD subjects with high expression of these seven PCA genes with those GP that had low expression, revealed that a total of 626 probes showed differential expression between these groups. Of these 626 probes, expression of 82 were significantly higher and 544 were significantly lower in the ASD group that had increased expression of the seven PCA genes. Of interest, several of the upregulated transcripts were related to NK function (Table 3), whereas many of the probes with lower expression were related to cellular proliferation (Tables 3 and 4).

Functional annotation—The 82 upregulated probes in ASD corresponded to 59 known genes, the majority of which have been ascribed to leukocyte function, more specifically NK cell cytotoxic function (Table 3). Thirty of the 59 genes (42 of the 82 probes) were associated with leukocytes, and 22 of these 59 (37%) were associated with cytolytic cells, predominantly NK cells. There was increased expression for 11 probes for killer cell immunoglobulin receptors (KIR), and 9 of these were probes for inhibitory KIR (long cytoplasmic domains). Additionally, expression of CD94 (2 probes) and CD160 (1 probe), which are both involved in MHC-I recognition and cytolytic activity increased (Barakonyi et al., 2004; Terrazzano et al., 2002). The expression of KSP37, a gene restricted to NK cells and co-expressed with

perforin during viral infection (Ogawa et al., 2001), increased 2.5-fold. Similarly, the RNA levels for perforin (2 probes), a pore forming protein secreted by activated NK cells, also increased 2.5 fold. The expression of three probes specific for granzymes, which enter the pore formed by perforin to induce apoptosis, were also increased. Thus, a profile indicating paradoxically increased expression of both inhibitory (KIR long cytoplasmic domain) and cytolytic (CD94, CD160, granzymes, perforin) natural killer cell-related genes were identified. Using the up-regulated genes as inputs, functional annotation clusters from the DAVID database strongly supported these findings showing that pathways and functions associated with NK cell-mediated cytotoxicity and antigen presentation were altered in ASD (Table 4). Furthermore, functional annotation clusters using the down-regulated genes (544 probes) as inputs indicated that ribosomal processing, cellular biosynthesis, intracellular organelles, cellular metabolism and cellular proliferation were altered in ASD (Table 5).

Cellular Analyses

Altered NK cell frequency in ASD—To further expand upon these initial gene expression studies, we performed a series of functional and cellular analyses on isolated NK cells in ASD compared with matched control children. First we assessed the frequency of NK cells in peripheral blood in ASD. After cell isolation there were significantly more CD56⁺ NK cells, per ml blood drawn, from children with ASD ($21.24 \pm 3.40 \times 10^4$ CD56 cells/ml blood, mean \pm SEM, $p < 0.05$) as compared with typically developing GP children ($14.45 \pm 1.98 \times 10^4$ CD56 cells/ml).

Decreased NK cytotoxicity in ASD—We used MHC-devoid K562 cells as a target cell line to determine the ability of NK cells to recognize and lyse a target cell. NK-mediated cytotoxicity was significantly decreased in children with ASD compared with GP controls at a NK:K562 ratio of 25:1 ($p = 0.02$, Figure 2, values corrected for spontaneous K562 cell lysis).

Increased cytotoxic capacity in ASD—To determine the frequency of NK CD56^{Dim} and CD56^{Bright} cells that express cytolytic effector molecules in un-stimulated conditions, we assessed whether NK cells contained increased intracellular stores of the cytolytic granules, perforin and granzyme B. There were no differences in the ratio of CD56^{Dim/Bright} cells in ASD compared with GP controls (data not shown). The frequencies of CD56^{Dim}, and CD56^{Bright} NK cells subsets that stained positive for the functional proteins perforin, granzyme B and IFN γ are summarized in Table 6.

Under un-stimulated conditions, there was a significant increased frequency of CD56^{Dim} NK cells that had positive staining for perforin in the ASD group ($43.78 \pm 5.12\%$, mean \pm SEM) compared with GP controls ($24.52 \pm 7.12\%$, $p = 0.0001$) (Figure 3A). Similarly, there was a significant increase in the frequency of CD56^{Dim} NK cells that had positive staining for Granzyme B in the ASD group ($62.59 \pm 4.88\%$) compared with GP controls ($47.52 \pm 4.00\%$, $p = 0.02$, Figure 3B). These data are consistent with our genomic findings that showed a greater than two-fold increased RNA gene expression of perforin and granzyme B in ASD children compared with GP controls. We also assessed the frequency of CD56^{Bright} cells that had positive staining for perforin and granzyme B to determine if differences in the frequencies in CD56^{Bright} cells contributed to our findings. There were no statistically significant differences between the frequencies of CD56^{Bright} NK cells that were stained positive for perforin and granzyme B between the ASD and control groups (Table 6).

Cytolytic activity of stimulated NK cells—NK cells were cultured with MHC-devoid target cells at 10:1 and 25:1 effector:target cell ratios to activate the NK cells. The frequencies of perforin and granzyme B containing NK cells following stimulation was assessed by flow cytometry (Table 6). Following stimulation, there were significantly reduced frequencies of

CD56^{Dim} and CD56^{Bright} NK cells that stained positive for the cytolytic proteins perforin and granzyme B in children with ASD ($p < 0.005$). In contrast, in the GP control children, following stimulation with target cells there were an increased frequencies of CD56^{Dim} and CD56^{Bright} NK cells that stained positive for perforin and granzyme B ($p < 0.006$). As a result the frequencies of CD56^{Dim} cells that stained positive for perforin, following stimulation at the 10:1 NK:K562 cells ratio, were significantly decreased in ASD ($13.72 \pm 2.54\%$) compared with GP controls ($47.01 \pm 5.16\%$, $p < 0.0001$, Figure 4). It is interesting to note that there was very little overlap between ASD and GP controls for the frequency of CD56^{Dim} cells staining positive for perforin (Figure 4A, 10:1 stimulation shown). Similarly, the frequencies of CD56^{Dim} cells that stained positive for granzyme B, were significantly lower in ASD ($43.51 \pm 2.44\%$) compared with GP controls ($67.60 \pm 3.96\%$, $p < 0.0001$, Figure 4B), following stimulation at the 10:1 NK:K562 cells ratio. Results for the CD56^{Bright} NK cell subset followed a similar trend, indicating that the difference following stimulation was not restricted to the CD56^{Dim} NK population (Table 6).

NK cells producing IFN γ in ASD—We sought to determine the frequency of NK cells producing the immune-stimulatory cytokine, IFN γ . Previous reports have demonstrated that CD56^{Bright} NK cells are the principal cytokine producing NK cell subset (Cooper et al., 2001), and we found similar trends between NK subsets in both ASD and GP controls (Table 6). Under resting conditions, the frequency of IFN γ positive cells were increased in the CD56^{Bright} subset in children with ASD ($72.10 \pm 6.11\%$, mean \pm SEM) compared with GP controls ($53.43 \pm 6.10\%$, $p = 0.04$; Figure 5A, Table 6). This trend was also present in the CD56^{Dim} NK cell population with significantly higher frequencies of the CD56^{Dim} NK cells staining positive for IFN γ in ASD ($39.14 \pm 5.73\%$) compared with GP control ($19.11 \pm 3.90\%$, $p = 0.003$, Table 6). Following stimulation with K562 target cells, IFN γ staining in NK cells was increased in GP controls but not in ASD. Thus, within the CD56^{Bright} cell population, there were nearly 20% fewer NK cells staining positive for IFN γ in ASD children following stimulation, compared with an increase of similar magnitude (29%) in NK cells staining positive for IFN γ in GP controls after stimulation. Moreover, following stimulation at the effector:target ratio of 10:1 NK:K562 cells, the frequency of CD56^{Bright} cells staining positive for IFN γ was decreased in ASD ($52.13 \pm 5.60\%$) compared with GP controls ($82.02 \pm 4.77\%$, $p = 0.0006$, Figure 5B). The finding of reduced IFN γ production is consistent with the lower frequencies of NK cells staining for perforin and granzyme B proteins after stimulation that is observed in ASD. Collectively, the data suggest that following stimulation there is a down-regulation of already chronically activated NK cells in ASD.

Discussion

Findings from our current study demonstrate that there are clear and significant abnormalities in the gene expression, frequency and function of NK cells obtained from 2–5 year old children with ASD compared with GP controls. Previously, Gregg et al., examined gene expression changes in children with autism who were distinguished based on the pattern of onset of their disorder (i.e. early onset autism versus late onset/regressive autism) and compared these findings with typically developing controls (Gregg et al., 2008). Using microarray analysis, these studies revealed that 12 gene probes, representing 11 different genes, were differentially expressed in early onset and regressive types of autism compared with the controls. Seven of these genes, which were known to be expressed in NK cells and involved in NK cell-mediated pathways, were confirmed by RT-PCR. Transcriptomics does not always predict protein differences or functional deficiencies. Moreover, although the genes that were shown to be differentially expressed are predominantly associated with NK cells they can also be present in subsets of CD8⁺ T cells, some populations of CD4⁺ T cells including NKT cells, and in subsets of dendritic cells. The transcriptional data identified in Gregg et al. (2008), demonstrated changes in blood mRNA expression but did not investigate protein levels in the

NK cell population nor did it report any functional readouts from the NK cell population. In the current study we sought to identify and elucidate differences in the physiological responses of NK cells in autism and to further refine the transcriptional analysis with an emphasis on NK cell related genes and pathways. Direct cellular protein analyses on a single NK cell basis, as well as dynamic cellular functions following activation were performed. To further refine the gene expression analysis, a principal component analysis inputting the 7 genes previously identified as different in autism was performed. The present study adds new and important information which demonstrates that protein levels and functional responses are altered in NK cells in children with autism, and adds to the previous report showing NK cell related gene expression changes in autism. We demonstrate that there are: 1) increased levels of cytolytic proteins in resting NK cells; 2) altered NK response to stimulation; and 3) changes in gene expression consistent with altered cellular activation in ASD compared with controls. Similar gene changes to those reported here are also seen in gene expression profiling of isolated NK cells stimulated with IL-2 (Dybkaer et al., 2007), which suggests that circulating NK cells in ASD are persistently activated rather than quiescent.

Analysis of cell function on a single cell basis supports the gene expression findings. Under resting conditions, we observed increased frequencies of NK cells that were positive for the cytotoxic granule proteins, perforin, and granzyme B, and for the cytokine IFN γ in blood from children with ASD. Both CD56^{Dim} cytotoxic granule production of perforin and granzyme B, and CD56^{Bright} NK cells production of IFN γ were increased and suggested that abnormalities in NK cell function were not confined to one specific NK cell subset. Moreover, abnormalities in NK function were observed following stimulation, with ASD subjects demonstrating reduced NK cell mediated-target cell cytotoxicity, reduced frequencies of NK cells staining for cytolytic proteins, and reduced frequencies of NK cells staining for cytokine production compared with controls. One possible explanation for these findings is that the NK cells from the ASD group have attained their maximum activation potential *in vivo* and are not able to respond to further stimulation *in vitro*. This could explain why even though there was a high resting cytotoxic capacity (i.e. increased gene expression and protein levels of perforin and granzyme B) there was decreased lysis of K562 cells in the ASD group when they were assessed *in vitro*. It is interesting to note that in some autoimmune diseases, including those which affect neurological functions such as multiple sclerosis (MS), circulating lymphocytes are activated in the periphery and fail to respond to further mitogen stimulation *in vitro* (Gershwin et al., 1979; Vervliet and Schandene, 1985). Several previous studies support the potential role of autoimmune processes in autism, including increased familial histories of immune and autoimmune disorders (Ashwood and Van de Water, 2004; Croen et al., 2005; Mouridsen et al., 2007; Sweeten et al., 2003), as well as the frequent finding of brain-reactive autoantibodies in ASD (Cabanlit et al., 2007; Connolly et al., 2006; Connolly et al., 1999; Silva et al., 2004; Singh and Rivas, 2004; Singh et al., 1997; Todd et al., 1988; Wills et al., 2007; Zimmerman et al., 2007). Recently, two laboratories, one that used maternal samples obtained from the same CHARGE study population as utilized here (Braunschweig et al., 2008) and the second from a separate geographically distinct population based study (Early Markers in Autism) (Croen et al., 2008) both reported the presence of autoantibodies that were reactive to fetal brain proteins in plasma specimens from mothers with children with autism but not mothers with typically developing children or children with developmental delays. These data suggest that possible maternal immune responses could affect fetal brain development and may be involved in a significant number of cases of autism. The exact nature of the function of these putative autoantibodies, what they recognize and whether they influence immune development in the child still remains to be resolved.

Despite repeated confirmed associations with autoimmune disease, the contribution of NK cells in these conditions is unclear. NK cells may be protective in autoimmune processes by inhibiting or killing autoreactive T cells or by affecting self-antigen presentation by antigen

presenting cells (Segal, 2007). In MS for example, NK cells may be closely involved with remission of disease with mRNA expression of IL-5 and IL-2R by NK cells (Takahashi et al., 2001). Cytotoxic activity can also directly correlate with MS disease activity (Infante-Duarte et al., 2005; Kastrukoff et al., 1998). In addition, highly cytolytic CX3CR1-positive NK cells are reported in active MS disease (Infante-Duarte et al., 2005). Loss of CX3CR1 selectively decreased NK cell migration into the CNS and was associated with increased neurological symptoms and mortality in a murine EAE model of MS (37). The role of NK cells in CNS diseases and neurodevelopmental disorders are likely complex. However, it is possible that NK cells may be critical in regulating neuronal inflammation and that defects in NK cell activity could permit alterations in normal neuroimmune regulation and function.

As NK cells play an important role in the host defense against infections, it is possible that abnormal or altered NK cell-mediated responses may modify susceptibility to infections. Consistent with an infection risk factor are reports of cellular and humoral immune dysfunction in ASD, as well as associations with more restricted HLA phenotypes including HLA-DR4 [4]. Moreover, medical chart reviews have demonstrated that there is an increased risk of ear and genitourinary tract infections in infants and young children with ASD (Niehus and Lord, 2006; Rosen et al., 2007). This evidence suggests that a potential environmental factor that may lead to changes in early brain development is perinatal infection. Recently, Rastogi *et al.* reported humoral immune responses generated in fetuses following maternal influenza vaccination as early as 29 weeks gestation (Rastogi et al., 2007). This finding demonstrates that antigen can cross the placenta, be recognized, and elicit a competent response by the fetal immune system. A defect residing in immune cells of the fetus could explain the increased risk of infection observed in the first month of life in some children with ASD (Rosen et al., 2007). As fetal NK cells are one of the predominant immune cells early in fetal life (Thilaganathan et al., 1993), abnormalities in these cells may have a major effects on early neurodevelopment. TNF α and IFN γ , which are both produced by NK cells, can damage cells of the CNS (Czlonkowska et al., 2005; Lambertsen et al., 2004) and can disrupt neurodevelopment (Gilmore et al., 2005; Shi et al., 2003). In addition, the neuropoietic cytokine IL-6, also produced by NK cells, can have direct effects on neurons and glia, including changes in proliferation, survival, neurite outgrowth, and gene expression (Gadient and Patterson, 1999; Mehler and Kessler, 1998). However, reports that associate ASD with infectious agents only account for a small proportion of ASD cases and they have implicated more than one potentially causative pathogen (Chess, 1971; Deykin and MacMahon, 1979; Stubbs et al., 1984). As such, rather than a single specific causative agent it is more likely that broad immunological dysfunctions, such as abnormal NK cellular responses, could explain these findings.

This present study shows distinct and significant physiological differences in NK cell responses in children with ASD. We clearly demonstrate that there are profound abnormalities in NK cell function in young children recently diagnosed with ASD. An altered NK cell population may have several consequences which could impact upon immune function in ASD and could explain some of the immune findings previously observed in ASD. In addition, fetal/neonatal NK cells may have important roles in neuroimmune interactions during early brain development. Significant differences in NK cells may thus represent a susceptibility factor for ASD. Although NK cells are known for their rapid response to virally infected cells (Andoniou et al., 2006), the current study cannot determine a role for infection in ASD, rather that there is significant abnormality in the resting state and response of NK cells to stimulation. NK-related mechanisms for ASD development must be carefully determined in future studies. Such studies will need to examine gene expression and NK function in the same children in order to determine whether the dysregulated expression profile directly correlates with abnormal NK cell-mediated function. Nonetheless, these results indicate a clear and significant alteration in

the gene expression and NK cell function in young children with ASD, a finding that may provide critical insight into potential neuroimmune susceptibility factors in some cases of ASD.

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Nonstandard abbreviations used

ASD	autism spectrum disorder
PDD	pervasive developmental disorder

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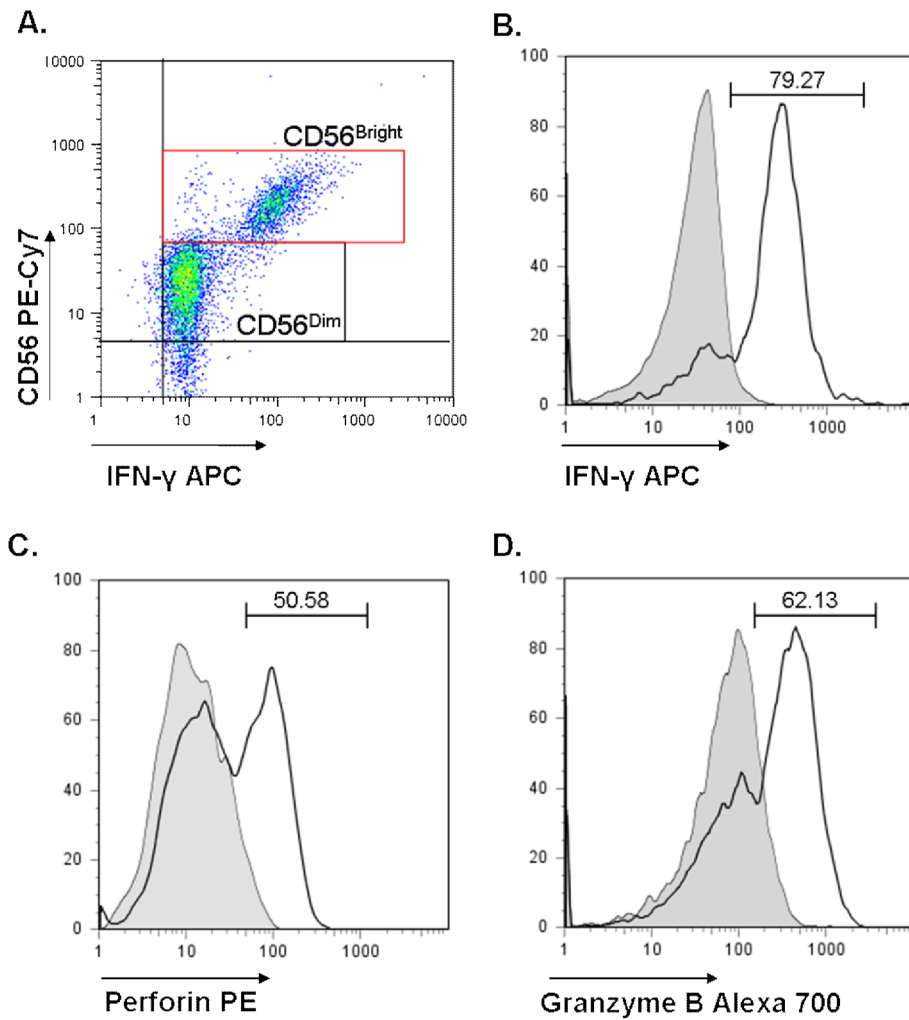


Figure 1. CD56^{Dim} and CD56^{Bright} subset determination

A representative dot plot showing markers for CD56⁺ NK cell subsets (A). Following selection of CD3⁻ CD8⁻ cells, CD56^{Dim} and CD56^{Bright} cells can be identified by density of CD56 staining. A representative example of an ASD subject is shown for determination of frequency of IFN γ positive CD56^{Bright} cells (B) and frequency of CD56^{Dim} cells positive for perforin (C) and granzyme B (D). The shaded line denotes isotype control. The percentage of positive cells is denoted above each histogram.

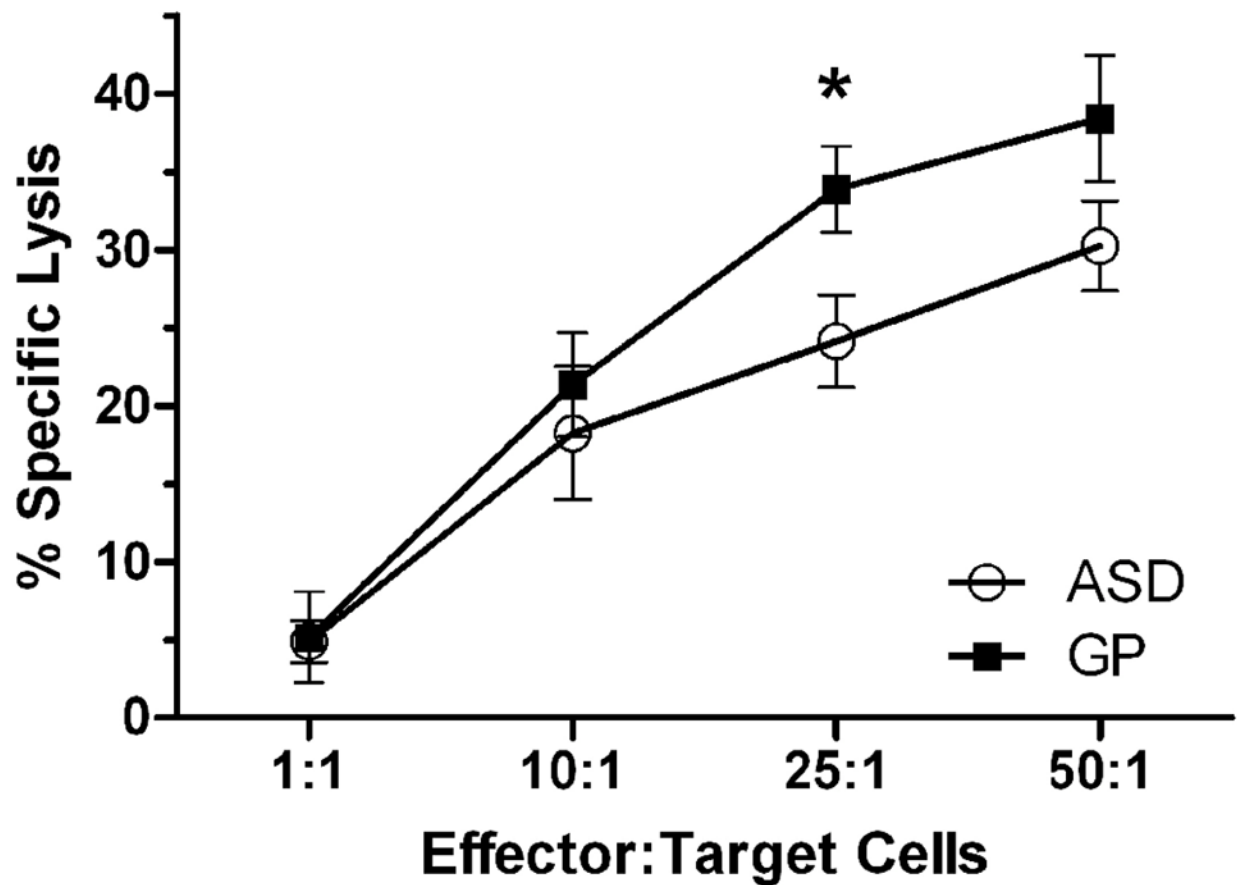


Figure 2. NK target cell lysis reduced in autism

Mean percent specific K562 lysis is shown for the indicated ratios of NK effector to K562 target cells. Bars represent standard error of the mean. A reduction in cytotoxic activity of cells obtained from ASD subjects was observed at cellular ratios above 1:1, which was significant at the 25:1 NK:K562 ratio ($p = 0.02$).

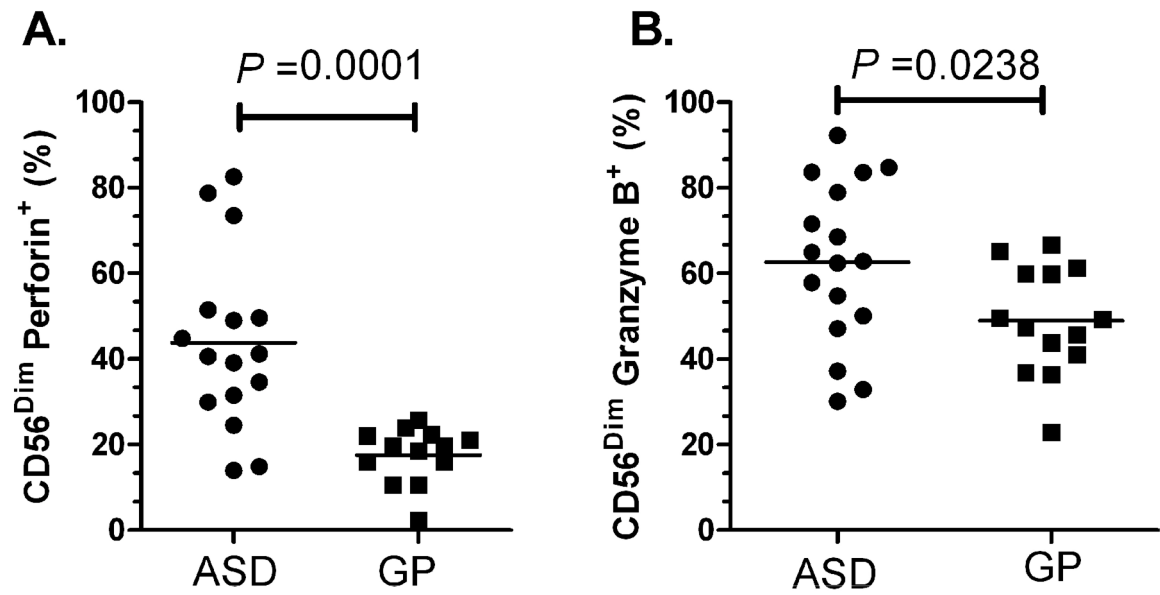


Figure 3. Increased resting cytolytic capacity in NK cells from ASD subjects
Under unstimulated conditions, a significantly higher frequency of CD56^{Dim} NK cells from children with ASD stained positive for cytolytic proteins A) perforin and B) granzyme B when compared with GP controls. Mean frequencies are shown.

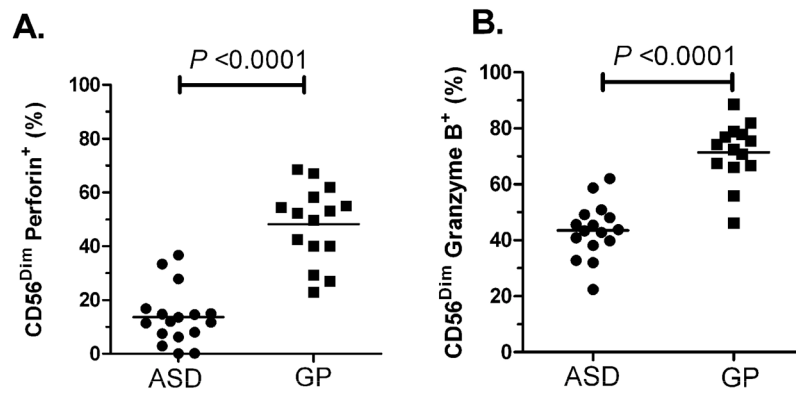


Figure 4. Reduced response to stimulation in NK cells from ASD subjects

Following stimulation with MHC-I deficient target cells at a 10:1 NK:K562 cell ratio, a significantly lower frequency of CD56^{Dim} NK cells stained positive for cytolytic proteins A) perforin and B) granzyme B in children with ASD compared with GP controls. Mean frequencies are shown.

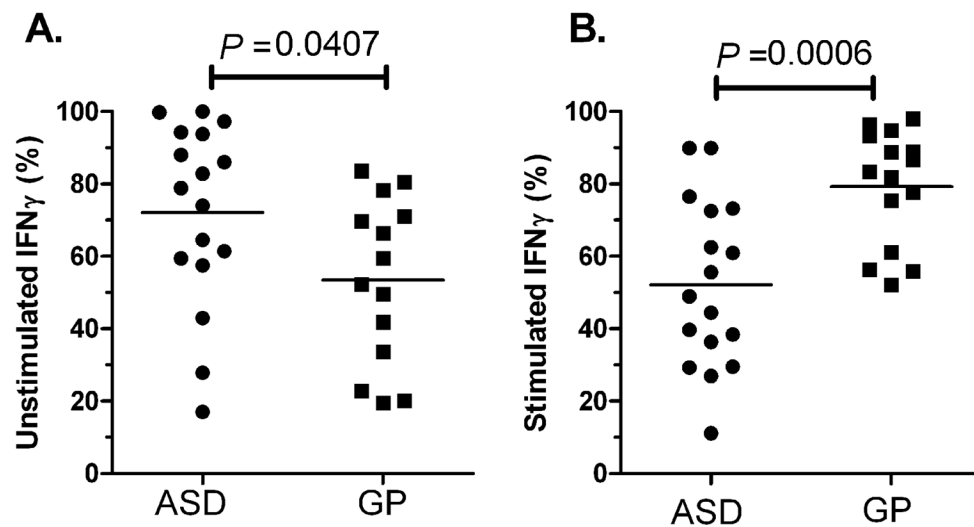


Figure 5. Frequency of IFN γ positive NK cells is altered in ASD

Frequencies of CD56^{Bright} NK cells which stain positively for IFN γ in ASD and GP children are shown for A) unstimulated and B) stimulated with 10:1 NK:K562 cells. In the absence of stimulation, the frequency of NK cells with positive staining for IFN γ was increased in children with ASD compared with GP controls. However, following stimulation decreased frequencies of IFN γ -positive NK cells were observed in the ASD group. Mean frequencies are shown.

Table 1

Descriptive statistics of the autism spectrum disorder (ASD) and typically developing general population (GP) study populations.

	Microarray Analysis		Cellular Studies	
	ASD	GP	ASD	GP
<i>n</i>	35	11	17	16
% Males	86%	82%	82%	81%
Average Age	3.6 years	3.5 years	3.9 years	3.3 years
Age Range	2.3–5.6 years	2.7–4.3 years	2.2–5.0 years	2.3–4.8 years

Table 2

Genes used as input to Principal Components Analysis. Differential gene expression in autism spectrum disorder (n=35) compared with typically developing children (n=11) (modified from Gregg *et al.*, 2008).

Gene symbol	Affymetrix GeneChip	TaqMan	
	Fold Change	Fold Change	<i>P</i> value
PAM	1.86	1.51	0.007
SPON2	1.87	1.86	0.005
IL2RB	1.56	1.35	0.046
PRF1	1.79	1.53	0.027
GZMB	2.01	1.72	0.014
CX3CR1	1.60	1.37	0.006
SH2D1B/EAT2	2.19	1.78	0.077

Table 3

Increased expression of NK cell related genes in peripheral blood from children with autism spectrum disorder compared with typically developing controls using microarray analysis.

Fold-change	Gene Name	Protein Name
2.2	CCL4	MIP-1 Beta
1.7	CCL5	RANTES
1.7	CD160	CD160
2.4	CTSW	Cathepsin W/Lymphopain
1.8	CX3CR1	Chemokine (CX3C motif) receptor 1
2.7	GZMB	Granzyme B
2.4	GZMH	Granzyme H
1.5	GZMM	Granzyme M
1.9	IL2RB	Interleukin 2 receptor beta
2.1	ITGB2	CD18/Beta 2 integrin
1.5	KIR2DL1	KIR, two domains, long cytoplasmic tail
1.7	KIR2DL2	
1.5	KIR2DL3	
1.6	KIR2DL5A	KIR, two domains, short cytoplasmic tail
2.2	KIR2DS2	
1.7	KIR2DS5	KIR, three domains, long cytoplasmic tail
1.7	KIR3DL1	
1.7	KIR3DL2	
1.7	KIR3DL3	CD94
1.8	KLRD1	
1.7	KLRF1	ITIM2
2.5	KSP37	KSP37 protein
1.5	NFATC2	Nuclear factor of activated T cells
2.5	NKG7	Natural killer cell group 7, sequence
1.9	PDGFD	Platelet derived growth factor D
2.6	PRF1	Perforin 1
2.2	PTGDR	prostaglandin D2 receptor
2.0	TBX21	T-box 21/TBET
2.0	TGFBR3	Transforming growth factor β receptor III
1.6	TRGC2	T cell receptor gamma constant 2

Table 4

The top two annotation clusters identified by DAVID for up regulated probes in ASD. Count defines the number of genes occurring within specified group. False discovery rate (FDR) denotes the number of genes expected to occur within a group by chance.

Enrichment Score: 5.96	Count	p Value	FDR
Defense response	22	1.20E-12	4.10E-09
Immune response	21	1.50E-12	2.50E-09
Response to biotic stimulus	22	3.00E-12	3.30E-09
Cellular signaling	28	8.20E-09	5.40E-06
Disulfide bond formation	27	9.10E-09	3.60E-05
Signal peptide	28	2.00E-07	4.00E-04
Response to pest, pathogen or parasite	13	4.90E-07	4.10E-04
Response to other organism	13	8.40E-07	5.70E-04
Enrichment Score: 5.74	Count	p Value	FDR
Natural killer cell mediated cytotoxicity	13	9.70E-13	2.10E-10
Antigen processing and presentation	9	5.40E-09	5.80E-07
Immunoglobulin subtype	13	2.90E-08	1.50E-04
Immunoglobulin-like	13	1.10E-07	2.70E-04
MHC class I receptor activity	6	2.00E-07	1.60E-04

Table 5

The top two annotation clusters identified by DAVID for down regulated probes in ASD. Count defines the number of genes occurring within specified group. False discovery rate (FDR) denotes the number of genes expected to occur within a group by chance.

Enrichment Score: 7.49	Count	p Value	FDR
Ribosome	31	2.00E-13	6.40E-11
Structural constituent of ribosome	31	4.90E-13	1.20E-09
Ribonucleoprotein complex	40	1.00E-12	2.10E-10
Protein biosynthesis	44	2.80E-10	4.80E-07
Macromolecule biosynthesis	47	4.00E-10	4.60E-07
Biosynthesis	62	2.70E-08	2.30E-05
Cellular biosynthesis	57	4.40E-08	3.00E-05
Large ribosomal subunit	11	1.70E-07	1.50E-05
Ribonucleoprotein	22	3.00E-07	4.00E-04
Ribosomal protein	20	3.10E-07	2.00E-04
Protein complex	76	1.40E-05	9.00E-04
Enrichment Score: 6.85	Count	p Value	FDR
Intracellular processing	250	2.10E-15	1.30E-12
Cellular physiological process	278	3.30E-12	1.10E-08
Intracellular organelle	215	5.30E-12	8.30E-10
Cellular metabolism	211	1.00E-07	5.90E-05
Intracellular membrane-bound organelle	179	3.90E-07	3.00E-05
Membrane-bound organelle	179	4.00E-07	2.80E-05
Metabolism	219	6.90E-07	3.30E-04
Physiological process	286	8.20E-07	3.50E-04

Table 6
Frequency of CD56^{Dim} and CD56^{Bright} NK cell subsets that stain positively for perforin, granzyme B and IFN γ before and after stimulation. Frequency of cells \pm standard error of mean (SEM) shown for each measure.

Assay	ASD	GP	P value
<i>Unstimulated, Perforin:</i>			
CD56 ^{Dim}	43.8 \pm 5.1 %	24.5 \pm 7.1 %	0.0001
CD56 ^{Bright}	14.9 \pm 2.3 %	11.3 \pm 2.3 %	NS
<i>Stimulated, Perforin:</i>			
CD56 ^{Dim} , 10:1 NK:K562	13.7 \pm 2.5 %	47.0 \pm 5.2 %	0.0001
CD56 ^{Dim} , 25:1 NK:K562	16.9 \pm 3.4 %	53.4 \pm 4.6 %	0.0001
CD56 ^{Bright} , 10:1 NK:K562	14.3 \pm 2.0 %	26.7 \pm 1.7 %	0.002
CD56 ^{Bright} , 25:1 NK:K562	12.6 \pm 1.7 %	23.8 \pm 3.6 %	0.005
<i>Unstimulated, Granzyme B:</i>			
CD56 ^{Dim}	62.6 \pm 4.9 %	47.5 \pm 4.0 %	0.02
CD56 ^{Bright}	19.6 \pm 1.8 %	17.1 \pm 2.2 %	NS
<i>Stimulated, Granzyme B:</i>			
CD56 ^{Dim} , 10:1 NK:K562	43.5 \pm 2.4 %	67.6 \pm 4.0 %	0.0001
CD56 ^{Dim} , 25:1 NK:K562	47.4 \pm 4.7 %	74.6 \pm 3.4 %	0.0001
CD56 ^{Bright} , 10:1 NK:K562	40.6 \pm 5.4 %	68.8 \pm 4.1 %	0.0004
CD56 ^{Bright} , 25:1 NK:K562	36.5 \pm 3.9 %	71.7 \pm 3.0 %	0.0001
<i>Unstimulated, IFNγ:</i>			
CD56 ^{Dim}	39.1 \pm 5.7 %	19.1 \pm 3.9 %	0.003
CD56 ^{Bright}	72.1 \pm 6.1 %	53.4 \pm 6.1 %	0.04
<i>Stimulated, IFNγ:</i>			
CD56 ^{Dim} , 0:1 NK:K562	21.2 \pm 3.4 %	43.2 \pm 4.1 %	0.0004
CD56 ^{Dim} , 25:1 NK:K562	30.6 \pm 8.2 %	46.6 \pm 4.4 %	NS
CD56 ^{Bright} , 10:1 NK:K562	52.1 \pm 5.6 %	82.1 \pm 4.8 %	0.0006
CD56 ^{Bright} , 25:1 NK:K562	56.4 \pm 7.9 %	85.9 \pm 5.0 %	0.009