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Decreased Numbers of CD57+CD3− Cells Identify Potential Innate Immune Differences in Patients with Autism Spectrum Disorder

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Abstract. *Background/Aim: Autism spectrum disorders (ASD) are complex, and severe heterogeneous neurodevelopmental pathologies with accepted but complex immune system abnormalities. Additional knowledge regarding potential immune dysfunctions may provide a greater understanding of this malady. The aim of this study was to evaluate the CD57+CD3− mature lymphocyte subpopulation of natural killer cells as a marker of immune dysfunction in ASD. Materials and Methods: Three-color flow cytometry-based analysis of fresh peripheral blood samples from children with autism was utilized to measure CD57+CD3− lymphocytes. Results. A reduction of CD57+CD3− lymphocyte count was recorded in a significant number of patients with autism. Discussion and conclusion: We demonstrated that the number of peripheral CD57+CD3− cells in children with autism often falls below the clinically accepted normal range. This implies that a defect in the counter-regulatory functions necessary for balancing pro-inflammatory cytokines exists, thus opening the way to chronic inflammatory conditions associated with ASD.*

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Autism and autism spectrum disorders (ASD) are complex and severe, heterogeneous neurodevelopmental pathologies. Their multifactorial nature suggests they originate from the interactions of several genes with environmental, lifestyle and immunological factors (1). ASD diagnostic criteria substantially changed in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (2). The revised diagnosis represents a new and hopefully more accurate depiction of these disorders.

Despite significant progress in our understanding of the associated immunobiology of autism, the pathogenesis, as well as defined molecular mechanisms, remain unclear (3). The dramatic increase in the prevalence of autism (one in 42 boys and one in 189 girls) (4) underscores the urgent need for a broader understanding of the immunological underpinnings of this disease (3, 5). The DSM-5 criteria of autism diagnosis continue to limit this disorder to the evaluation of social, communication skills, and behavioral criteria. However, others are proposing to sub-type of ASD based on a combination of socio-behavioral and biomedical criteria (6-8). Our long-lasting clinical experience points towards a multifactorial disorder combining disorders from four major sub-groups, namely immune disorders, infection, intestinal dysfunction and environmental or maternal toxicant exposure (*i.e.* valproic acid, endocrine disrupting plasticizers, ethanol, air pollution, organophosphates and heavy metals). Sufficient immunological evidence presently exists to encourage the identification of more specific biological criteria (9), thus enabling better diagnostic categorization, and in turn, leading to better management of the complex clinical picture associated with ASDs.

Various potential biomarkers of autism are emerging and several studies have tried to support an immunology-based foundation of autism pathophysiology (10). However, to date, neither a biochemical nor immunological foundation has been identified that can be efficiently translated into the clinic (7). The availability of such knowledge would provide valuable information for physicians to treat their patients and to follow therapy (11).

Recently, Shen *et al*. demonstrated increased extra-axial fluid persistence, as measured by serial magnetic resonance imaging, as a predictive tool in early risk assessment (12). Following this, Bradstreet *et al*. proposed a new methodology of imaging the extra-axial fluid spaces with transcranial ultrasonography and observed significantly increased extra-axial fluid in an autistic cohort when compared to age-matched controls (13). Both Shen *et al*. and Bradstreet *et al*. noted the potential for immunological dysregulation within the arachnoid membranes in the formation of increased extra-axial fluid in the ASD population.

An understanding of the precise mechanisms of immunological dysregulation is incomplete; however, recent progress is allowing more insight into the complex imbalances associated with ASD (14, 15). Autism is now accepted to be commonly associated with significant immunological abnormalities ranging from the gastrointestinal associated immune system to the microglial innate immune cells of the central nervous system (16-20). Recently, endocannabinoid system dysregulation of autism-associated macrophages has been reported (21). Others see the endocannabinoid system, a vital regulator of immune function and pyramidal cell activity, as a central mechanism in autism pathophysiology (22).

While the function of natural killer (NK) cells ($CD56^+CD16^+CD3^-$) is well characterized, the function of the $CD57^+$ subset of NK cells is less well understood. These cells are known to have killing capability, and are believed to be a terminally differentiated subset of classic NK cells that are less responsive to cytokines (23, 24). Cells with NK activity, and in particular, the $CD57^+$ subset, have been consistently characterized as key regulators of the innate immune system and are tied to both immune deficiency and autoimmunity (25).

Previous studies suggest that altered NK cell activity may play a role in autism pathology (26). Low NK cell cytotoxic activity was identified in autistic children, and this lower activity correlated with decreased intracellular levels of glutathione, as well as the interleukins IL2 and IL15 (27). These interleukins are among the key regulators of homeostasis and function of NK cells (28). The work of Enstrom *et al*. further identified a possible NK cell dysfunction in children with autism (29), indicating that the abnormalities in NK cells may represent a susceptibility factor in ASD. Up-regulation of gene transcription in NK cells, including those for perforin and granzyme, was also found in children with ASD, when compared to typical developing controls (29). Alterations in NK cell function during critical periods of development may represent a predisposition to autoimmunity and to adverse neuroimmune interactions. Indeed, NK cell-related gene expression changes found in children with autism are consistent with altered cellular activation. Differential alternative mRNA splicing in NK cell-mediated cytotoxicity pathways has also been reported in blood of individuals with autism (30). Here we further explore the $CD57^+CD3^-$ NK cell population as potential evidence of immune dysfunction in autism, particularly in an ASD subgroup mainly affected by chronic infections.

Materials and Methods

Participants. In this study, 104 children (aged 3 to 22 years; mean age= 6.7±3.6; males: 78, females: 26) with autism and 31 age-matched healthy controls (aged 1 to 12 years; mean age= 5.2±3.4 years; males: 19, females: 12) were investigated. In addition, 119 healthy adult controls (mean age= 45.1±16.6 years; males: 65, females: 54) were utilized in order to validate previously published methods. All participants were recruited into the study from the outpatient Biomedical Center for Autism Research and Treatment, Bari, Italy, and R.E.D. Laboratories, Zellik, Belgium. Prior to enrollment, participants were administered the Autism Diagnostic Interview-Revised version, the Childhood Autism Rating Scales (CARS) (subject children were required to score at least 30 points on the CARS scale) and the Autism Diagnostic Observation Schedule-Generic to document the diagnosis of autism (21, 31, 32). The Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) autism spectrum disorder criteria were satisfied for all cases (2). Potential participants were excluded if they had any of the following: a neurological or comorbid psychiatric disorder, epilepsy, history of liver, renal or endocrine disorders, and current infection of any origin. Mental retardation or behavioral disorders, including pervasive developmental disorder–not otherwise specified, and inclusion criteria for attention deficit-hyperactivity disorder, were all considered exclusion criteria. Children diagnosed with Asperger's syndrome (although included in the DSM-5), fragile X syndrome and tuberous sclerosis, were also excluded from the study. An IQ test was not performed and children with autism did not receive any pharmacological interventions. Other exclusion criteria were celiac disease or other major diseases of the intestinal tract, such as inflammatory bowel disease or hepatic disorders. Informed consent for blood drawing was obtained from the parents of all children enrolled in the study in compliance with Italian/European legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki).

Quantification of CD57 antigen-expressing cells. Fresh peripheral blood samples from all participants were drawn for routine blood testing using sterile EDTA-containing tubes. Anticoagulated blood was stained using the whole-blood lysis method and analyzed using a three-color flow cytometry-based assay. All antibodies and flow cytometry-related reagents were obtained from BD Biosciences (San Jose, CA USA). Whole blood was stained with antibodies directed against the surface antigens CD45 (marker for human leucocytes; BD 345809), CD3 (marker for human T-lymphocytes; BD 555333),

and CD57 (BD 333169). Cells were enumerated using BD Trucount™ Absolute Counting Tubes, as per the manufacturer's instructions. The absolute numbers of CD57+/CD3− lymphocytes (cells per μl of whole blood) were determined by flow cytometry using FACS Canto II™ platform.

Quantification of NK CD3/CD56/CD16 antigen-expressing cells. Fresh peripheral blood samples from all participants were drawn for routine blood testing in sterile EDTA-containing tubes. Anticoagulated blood was stained using the whole-blood lysis method. Whole blood was stained with antibodies directed against the surface antigens CD3 (marker for human T-lymphocytes; BD 555339), CD16 (BD 561304) and the CD56 (BD 555516). All antibodies and flow cytometry-related reagents were obtained from BD Biosciences (San Jose, CA USA). The assay was conducting using BD Trucount™ Absolute Counting Tubes, as per the manufacturer's instructions. The absolute numbers of CD3/CD56/CD16-positive lymphocytes (cells per μl of whole blood) were determined by flow cytometry using FACS Canto II™ platform.

Statistical analysis. All data are expressed as the mean with standard deviation. Student's *t*-test was used to determine statistical significance of the results and *p*-values of less than 0.001 were considered statistically significant. In addition, the Kruskal–Wallis one-way analysis of variance test was used to determine more stringent statistical significance. This test was performed using the analytical software offered by Vassarstats.net(Vassar College, NY, USA). The statistical results were: H=108.38; df=2; $p<0.0001$, where H is the Kruskal–Wallis statistic, and df degrees of freedom.

Results

Flow cytometry-based assays for cell counting are widely used and well-established methods. The normal range for the $CD57^+CD3^-$ lymphocyte subset was set to 60-360 cells/μl, as previously established (33). However, to further validate our in-house method, we also evaluated $CD57^+CD3^-$ lymphocytes for adult controls. All the controls had $CD57^+CD3^-$ counts within the normal range (129.2±52.4 cells/μl).

$CD57^+CD3^-$ expression in children with ASD and age-matched healthy controls was assessed using the same method. $CD57^+CD3^-$ counts for age-matched healthy controls were within the normal range (136.00±78.70 cells/μl). In sharp contrast, when compared both to expected laboratory controls (60-360 cells/μl) (33), and age-matched healthy controls, we observed a significant reduction of $CD57^+CD3^-$ lymphocytes counts in patients with autism (Figure 1).

Specifically, of the 104 enrolled patients with autism, 73 (70.19%) had counts lower than 100 cells/μl (49.12±26.68 cells/μl, $p<0.001$) of whole blood. Forty-seven (45.19%) patients had $CD57^+CD3^-$ counts below the lower limit of normal range (<60 cells/μl of whole blood) (32.00±15.10 cells/μl, $p<0.001$). Of significant concern, 20 out of the 104 (19.23%) demonstrated very low $CD57^+CD3^-$ counts of fewer than 30 cells/μl of whole blood (18.64±8.02, $p<0.001$). Only 31 (29.81%) patients with autism had $CD57^+CD3^-$

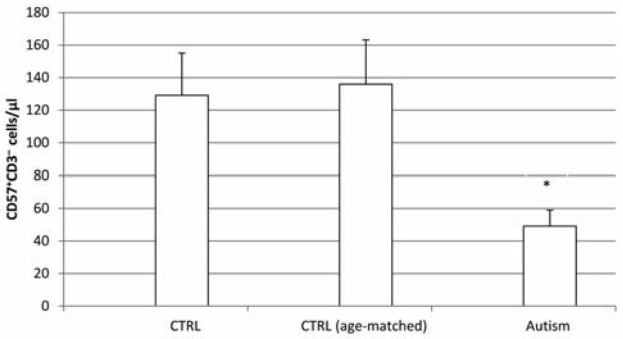

Figure 1. *$CD57^+/CD3^-$ lymphocyte counts. The graph shows the $CD57^+CD3^-$ lymphocyte counts for: children with autism, adult controls (CTRL) and age-matched child controls. *$p<0.001$.*

counts of more than 100 cells/μl of whole blood (161.03±56.14 cells/μl), which were within the normal range. In the autistic group, four participants may be considered as young adults (mean age: 18.75±2.36 years, range: 17 to 22 years); they also had $CD57^+CD3^-$ counts below the lower limit of the normal range (40.75±10.34 cells/μl).

In summary, 70% of the analyzed patients with autism had $CD57^+CD3^-$ counts below the normal range.

In the initial study linking decreased $CD57^+$ counts to chronic infection (33), the authors showed that the classic NK cell population (namely $CD56^+$ cells) remained unaffected, *i.e.* most of the chronic patients with low $CD57^+$ had normal $CD56^+$ counts. As already emphasized, having seen the previous work on CD56 NK cells in ASD, our goal was not to re-assess this population in our study. However, CD56 lymphocyte subset analysis was also performed. We counted in-house $CD56^+CD3^-$ lymphocytes of controls. All controls had $CD56^+CD3^-$ counts within the normal range (31.10±9.83 cells/μl). When compared to age-matched healthy controls, we did not observe a significant reduction of $CD56^+CD3^-$ lymphocytes counts in patients with autism (34.25±5.44 cells/μl), however, this value falls in the expected range for laboratory controls (3-95 cells/μl).

Discussion

To the best of our knowledge, this study demonstrated for the first time a potential involvement of $CD57^+$ NK cells in autism immunopathology. The CD57 (B3GAT1, HNK-1; Leu7) antigen is a terminally sulfated glycan carbohydrate epitope detected on human NK cells and is generally believed to reflect immunosenescence (33). The expression of CD57 glyco-epitopes are not limited to NK cells but appears on many other immune cells, as well as neurons. Decreased expression of the CD57 epitope in the brain is associated with learning and memory deficits in mice (34).

Increased $CD56^+/CD3^-$ profiles are observed in patients with rheumatoid arthritis and autoimmune diseases (25, 35). Transient increases in $CD56^+CD3^-$ cells are observed in many infections (36, 37). While in distinction, low $CD56^+CD3^-$ counts are described during the acute and convalescent phases of wild-type measles infections, while vaccine administration of measles virus was not associated with alterations of circulating $CD57^+$ NK cells (38, 39).

It is well established that systemic juvenile rheumatoid arthritis is associated with macrophage activation syndrome (40). One suggested explanation for unregulated macrophage activity is the absence of circulating $CD56^{bright}$ NK cells, which in turn down-regulates the pro-inflammatory function of macrophages (41, 42). Abnormal macrophage activity has been associated with autism based on the observed clinical responses to the administration of vitamin D-binding protein, Gc macrophage-activating factor (GcMAF) (43). While GcMAF is considered to be an activator of macrophages based on its effects on human cancer cells and observed *in vitro* effects (44), recent observations indicate its apparent effect is mediated, at least in part, *via* the regulation of transcription of endocannabinoid receptors (45).

The role of vitamin D3 deficiency in numerous inflammatory states and autoimmune states, including autism, was recently reviewed (46, 47). Very interestingly, it was recently proposed that vitamin D3 could be a potential therapeutic tool for dysregulated NK-cell immunity (48, 49). Indeed, vitamin D_3 has immuno-regulatory effects on NK-cell cytotoxicity, cytokine secretion and degranulation activity (48).

Some interesting observations seem to link atopy and increased risk of autism (50-53). Furthering the understanding of the source of the NK-cell deficiency in atopic dermatitis, Katsuta *et al*. observed that NK cells are selectively targeted for apoptosis in the presence of activated monocytes (54). These monocytic cells are strongly dysregulated in autism (16). It also appears that the role of mast-cell activation will overlap with these NK-atopic observations and explain why some atopic-presenting individuals with ASD have normal IgE levels and negative antigen-specific skin tests. Theoharides and co-workers have proposed that mast-cell activation reactions occur in ASD in the absence of IgE mediation (55). Mast cell granules appear to present cross-reactive epitopes with CD57, and it is possible that mast-cell activation further marks those (*i.e*. CD57) cells for selective apoptosis (56). Importantly, Stricker *et al*. reported that the numbers of $CD57^+CD3^-$ cells were decreased in chronic (not acute) Lyme disease (33).

It is now apparent that autism is unequivocally associated with abnormalities in both innate and adaptive immune responses (16, 57). Maternal immune system activation in animal models results in autism-related behaviors in offspring (58). Children with autism have increased plasma levels of several interleukins, such as IL1, IL6, IL8 and IL12, interferon γ, and macrophage migration-inhibitory factor, and decreased levels of transforming growth factor-β (59-61). Increased number of B-cells in children with autism has also been reported (13).

It is important to distinguish NK cell function from the numbers of circulating NK cells, since either or both may be altered in autism. Furthermore, children with autism demonstrated NK cell dysfunction and these abnormalities in NK cells could represent a susceptibility factor in autism (29).

One study found an increased absolute number of NK cells in patients with autism (29). However, only CD56 and CD3 markers were used to select and count NK cells. CD56 and CD57 can be used to define multiple distinct NK cell subsets; transcriptional signature, phenotypic properties, and the functional capacity of human $CD57^+$ NK cells is that of different from $CD57^-$ NK cells (62, 63). The fact that we found a decrease in $CD57^+CD3^-$ counts in our autistic population, in contrast to $CD56^+CD3^-$ counts which did not change but showed values in the normal range, may indicate that a specific subset of non-T-lymphocytes with NK activity could be strictly involved in autism. $CD57^+CD3^-$ cells are known to have NK capability, although they appear to be distinct from the $CD56^+CD57^-CD3^-$ NK population. CD57 is considered a marker for NK cells that are highly mature and terminally differentiated (62). Indeed, CD56 and CD57 could be independently correlated as their expression patterns define different stages of NK-cell differentiation and maturation (64). In addition, not all NK-cell markers (*i.e*. CD57 and killer cell immunoglobulin-like receptor (KIR)) are expressed in the same way, but seem to indicate different NK-cell subsets in a particular cell state (65-67). In a previous study, low NK cell activity was found in 45% of a subgroup of children with autism (68). Lower levels of NK cells were also reported in individuals with Rett syndrome (27, 69), a disease that has several similarities to autism. Interestingly, we recently published a case showing increase $CD57^+CD3^-$ counts after specific therapy in a boy with autism together with significant ASD criteria improvement (70).

Conclusion

In summary, our data suggest that CD57+CD3– NK cells may play a role in the pathophysiology of autism. Lower levels of $CD57^+$ NK cells may reflect an immunopathological foundation for the creation and/or maintenance of autism, with a possible link to the ASD subgroup characterized by chronic infection. While preliminary in nature, our observations are consistent with a growing body of evidence supporting immunopathology in ASD. The limitation of our study was the restricted sample set. In addition, this was a clinic center-based study and the results may not represent the general autistic population. In line with this, similar findings were reported during the BBOW-APSO conference

(71). Our study is an exploratory analysis, therefore, we fully acknowledge that the complexity of ASD requires a larger population-based study in order to achieve an integrated and clear understanding of autism-related complex cellular pathways and interactions. The CD57$^+$ NK cell subset along with further evaluation of other NK and T-cell subsets is a promising research topic that could ultimately result in targeted therapy and diagnostic subgrouping in ASD.

Conflicts of Interest

The Authors TM and EB are R.E.D. Laboratories employees. The Authors report no other conflicts of interest in this work.

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