

Neuroglial Activation and Neuroinflammation in the Brain of Patients with Autism

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Autism is a neurodevelopmental disorder characterized by impaired communication and social interaction and may be accompanied by mental retardation and epilepsy. Its cause remains unknown, despite evidence that genetic, environmental, and immunological factors may play a role in its pathogenesis. To investigate whether immune-mediated mechanisms are involved in the pathogenesis of autism, we used immunocytochemistry, cytokine protein arrays, and enzyme-linked immunosorbent assays to study brain tissues and cerebrospinal fluid (CSF) from autistic patients and determined the magnitude of neuroglial and inflammatory reactions and their cytokine expression profiles. Brain tissues from cerebellum, midfrontal, and cingulate gyrus obtained at autopsy from 11 patients with autism were used for morphological studies. Fresh-frozen tissues available from seven patients and CSF from six living autistic patients were used for cytokine protein profiling. We demonstrate an active neuroinflammatory process in the cerebral cortex, white matter, and notably in cerebellum of autistic patients. Immunocytochemical studies showed marked activation of microglia and astroglia, and cytokine profiling indicated that macrophage chemoattractant protein (MCP)-1 and tumor growth factor- β 1, derived from neuroglia, were the most prevalent cytokines in brain tissues. CSF showed a unique proinflammatory profile of cytokines, including a marked increase in MCP-1. Our findings indicate that innate neuroimmune reactions play a pathogenic role in an undefined proportion of autistic patients, suggesting that future therapies might involve modifying neuroglial responses in the brain.

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Autism is a common neurodevelopmental disorder characterized by impairments in social, behavioral, and communicative functions.^{1,2} Symptoms appear before 36 months of age, and regression or loss of skills occurs in 30% of affected children, usually between 18 and 24 months.¹ The syndrome is clinically heterogeneous and can be associated in up to 10% of patients with well-described neurological and genetic disorders, such as tuberous sclerosis, fragile X, and Rett's and Down's syndromes, although in most patients the causes are still unknown.^{3,4} Recent epidemiological studies suggest that the prevalence of the autistic syndromes has increased in recent years to 1 in 250 to 500 children, perhaps as a result of improved diagnostic approaches.^{5–7}

Although the neurobiological basis for autism re-

mains poorly understood, several lines of research now support the view that genetic, environmental, neurological, and immunological factors contribute to its development.^{3,4,8–10} Neuropathological studies have shown that abnormalities in cytoarchitectural organization of the cerebral cortex and subcortical structures, as well as reduced numbers of Purkinje cells in the cerebellum, are the most consistent findings in postmortem brain tissues from autistic patients. They suggest that defects in neuronal maturation and cortical organization may be responsible for some of the neurological problems seen in autism.^{11–13}

Immune dysfunction has been proposed as a potential mechanism for the pathogenesis of autism.¹⁴ Several studies in peripheral blood have shown various abnormalities such as T-cell dysfunction, autoantibody

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production, and increased proinflammatory cytokines.^{9,15–18} The potential role for maternal antibodies as a pathogenic factor also has been proposed.¹⁹ Cerebrospinal fluid (CSF) studies demonstrated no evidence of inflammation by standard cell counts, protein electrophoresis, or measurements of quinolinic acid and neopterin.²⁰ Despite the growing interest in possible immune mechanisms in its pathogenesis, there has been no direct evidence linking findings in the peripheral blood to immune activity in the brain of autistic patients.²¹ Neuropathological studies have given little attention to immune and neuroglial activity in autism, and the most comprehensive postmortem study reported no inflammatory changes or astroglial reactions.¹¹ Only a few reports have described gliosis and inflammatory changes.^{12,22} Such neuroinflammation, if present in the brain, might both participate in and result from dysfunctional CNS development and activity in autism. To investigate whether immune-mediated mechanisms are involved in the pathogenesis of autism with respect to the central nervous system (CNS), we

studied brain tissues and CSF from autistic patients and determined the magnitude of neuroglial and inflammatory reactions and their cytokine expression profiles.

Materials and Methods

Patient Information

Brain tissues from autistic patients and nonneurological control cases were obtained through the Autism Tissue Program of the Harvard, University of Miami, and University of Maryland Brain Banks. All autistic cases fit the diagnostic criteria established in the Diagnostic and Statistical Manual–IV and confirmed by the Autism Diagnostic Interview–Revised (ADI-R).^{23,24} The ADI-R was administered previously by researchers at the Autism Tissue Program (ATP) as a criterion for inclusion in the repository. Additional clinical and neurological information also was obtained from the ATP. The demographic characteristics of all autistic patients and controls included in the study are described in Table 1. Information about history of epilepsy, mental retardation, and developmental regression for the autistic patients is also included in Tables 1 and 2. Mental retardation was defined

Table 1. Patient Brain Tissue Information

Case No. ^a	Diagnosis	Age ^b (yr)	Sex	PMD Hours	Cause of Death	Epilepsy	Developmental Regression	Mental Retardation
1349 ^c	Autism	5	M	39	Drowning	No	No	Yes
1174 ^c	Autism	7	F	14	Sudden death	Yes	No	Yes
B5013 ^d	Autism	7	M	40	Drowning	No	No	Yes
2004 ^d	Autism	8	M	23	Drowning	No	No	Yes
1182 ^c	Autism	9	F	24	Respiratory failure	Yes	Yes	Yes
797 ^c	Autism	9	M	13	Drowning	No	Unknown	Unknown
B4925 ^c	Autism	9	M	27	Sudden death	Yes	No	Yes
3714 ^d	Autism	10	M	30	Drowning	No	Unknown	Yes
B4323 ^d	Autism	14	M	10	Hyperthermia	No	No	No
1638 ^c	Autism	20	F	50	Sudden death	Yes	Yes	Yes
B5144 ^c	Autism	20	M	23	Trauma	No	Yes	Yes
3711 ^d	Autism	25	M	26	Found dead, unknown	Yes	Unknown	Unknown
3663 ^d	Autism	27	M	30	Neuroleptic syndrome	Yes	No	Yes
2802 ^d	Autism	29	M	24	Aspiration	No	Unknown	Yes
B4541 ^d	Autism	44	M	30	Acute myocardial infarction	No	No	Yes
1377 ^c	Control	5	F	20	Drowning	No		No
1706 ^c	Control	8	F	20	Allograft rejection	No		No
1860 ^c	Control	8	M	5	Sudden death, cardiac arrhythmia	No		No
629 ^d	Control	7	M	18	Accidental	No		No
1407 ^c	Control	9	F	20	Asthma	No		No
2149 ^d	Control	16	M	13	Gunshot wound	No		No
1862 ^c	Control	20	M	6	Trauma	No		No
3706 ^d	Control	27	M	21	Hanging	No		No
3231 ^d	Control	37	M	24	Asphyxia	No		No
2845 ^d	Control	37	M	21	Heart disease	No		No
B3706 ^c	Control	40	M	28	Trauma	No		No
B4192 ^c	Control	46	M	25	Sudden death, unknown	No		No

^aAutism Tissue Program (ATP) identifier.

^bMean age in the autism group: 16 yr; mean PMD: 26 hr; mean age in the control group: 20 yr; mean PMD: 18 hr.

^cCases in which only frozen tissues were available for cytokine protein array studies.

^dCases in which only fixed tissues were available for morphological studies.

^eCases with frozen and fixed tissues were available for morphological and protein array studies.

PMD = postmortem delay.

Table 2. Patient CSF Information

Case No.	Age (yr)	Sex	Group	Diagnosis	Regression	Mental Retardation
5	4	F	Autism	Autism	Yes	Yes
6	4	M	Autism	Autism	Yes	No
8	6	M	Autism	Autism	Yes	Yes
9	10	M	Autism	Autism	Yes	Yes
11	6	F	Autism	Autism	Yes	Yes
12	3	M	Autism	Autism	Yes	No
5061	36	F	Control	Headaches	N/A	No
2484	45	F	Control	Spondilosis	N/A	No
3121	38	F	Control	Headaches	N/A	No
3685	26	F	Control	Headaches	N/A	No
7108	42	M	Control	Depression	N/A	No
7384	45	M	Control	Delirium	N/A	No
150	35	F	Control	Pseudotumor cerebrii	N/A	No
400	26	F	Control	Pseudotumor cerebrii	N/A	No
500	12	M	Control	Pseudotumor cerebrii	N/A	No

^aIdentifier number from the CSF repository at the Kennedy Krieger Institute, Baltimore, MD or CSF Repository at Johns Hopkins Department of Neurology, Baltimore, MD

CSF = cerebrospinal fluid; N/A = not applicable.

as full-scale IQ less than 70 with impairments in adaptive functions; developmental regression was defined as loss of previously acquired language and social skills, both with onset during early childhood.

Brain Tissue Processing

Fixed and frozen brain tissue samples were obtained from the ATP-affiliated brain banks (see Table 1). Fixed brain tissues from the middle frontal gyrus (MFG), anterior cingulate gyrus (ACG), and cerebellar hemisphere (CBL) were selected from brains obtained at autopsy of autistic ($n = 11$) and control ($n = 6$) patients (see Table 1). Only 3 of the 11 brains from autistic patients had fresh-frozen tissues available for protein analysis. Fresh-frozen tissues from four other cases of autism, and six control cases in which only frozen tissue was available were included for protein analysis. MFG and ACG tissues were available in 9 of 11 fixed brains, and cerebellar tissue in 10 of the 11 brains, from autistic patients. Fixed tissues were paraffin-processed, and 10 μ m sections were obtained for histological and immunocytochemical studies. Frozen tissue samples from the CBL, MFG, and ACG of brains from autistic ($n = 7$) and control patients ($n = 7$) were homogenized with triple-detergent lysis buffer containing 50nM Tris-HCl (pH 7.4), 150nM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1% Igepal (Sigma-Aldrich, Inc., St. Louis, MD), 0.5% sodium deoxycholate, and protease inhibitor cocktail (0.2U/ml aprotinin, 100 μ g/ml phenylmethyl sulfonyl fluoride), then centrifuged at 4°C and stored at -80°C . Total protein concentration was calculated using the BCA protein assay kit (Pierce, Rockford, IL) following the specific protocol described in the kit.

Cerebrospinal Fluid

CSF samples from six living autistic patients (aged 3–10 years) were collected by lumbar puncture during conscious sedation and then immediately frozen at -80°C and kept frozen until used for protein analysis. Similarly, CSF samples

from control patients (aged 12–45 years) were obtained from the Johns Hopkins Department of Neurology CSF repository. Only CSF from patients without evidence of CNS inflammatory disorders or pathological processes was included in the control group (see Table 2).

Immunocytochemical Staining

Immunohistochemical staining was conducted using the avidin-biotin-peroxidase complex method according to established protocols or recommendations by the manufacturers. The primary antibodies and dilutions are described in Table 3.

Quantitative Analysis of Immunoreactivity

Assessment of astroglia (glial fibrillary acidic protein [GFAP]) and activated microglial (Human leukocyte antigen-DR [HLA-DR]) immunostaining was conducted by the unbiased method of fractional area quantification as described previously.^{25,26} The cerebral cortex of the MFG and ACG and the granular cell layer (GCL) and white matter of the cerebellum were outlined for quantitative analysis with the help of a video-microscope controlled by Stereo Investigator Software (MicroBrightfield, Williston, VT). A group of 30 points was systematically placed in random positions, at 20 μ m intervals, within the boundary of each region. The sum of the points falling over structures of interest (eg, astroglia or microglia) was divided by the total number of grid points sampled to estimate the fraction of the area of the region occupied by a particular type of cell. The fractional area was defined according to the Delesse principle²⁵ as equal to the fraction of the volume occupied by the cell type being quantified. This method measures the percentage of the area of interest that is immunoreactive for a specific antibody. One individual, who was blinded to the diagnostic groups, performed the counting procedure.

Table 3. Antibody Information

Antibody	Type	Epitope/Specificity	Dilution	Source
GFAP	Polyclonal	Astrocytes	1:100	Dako
HLA-DR	Monoclonal	MHC class II, activated microglia	1:100	Dako
CD68	Monoclonal	Macrophages, monocytes	1:100	Dako
MRP-8, calgranulin A	Monoclonal	Macrophages in late or chronic infiltrates	1:100	BACHEM
CD3	Polyclonal	T cells	1:50	Dako
CD20	Monoclonal	B cells	1:200	Dako
C9neo (B7)	Monoclonal	Complement, membrane attack complex	1:20	Dr P. Morgan, UK
IL-6	Polyclonal	IL-6	1:750	Novus
MCP-1	Polyclonal	MCP-1	1:200	Peptotech
TGF- β 1	Polyclonal	TGF- β 1	1:200	Santa Cruz
IGFBP1	Polyclonal	IGFBP-1	1:200	Santa Cruz

GFAP = glial fibrillary acidic protein; MRP = migration inhibitory factor (MIF)-related protein; IL = interleukin; MCP = macrophage chemoattractant protein; TGF = tumor growth factor; IGFBP = insulin-like growth factor binding protein.

Confocal Microscopy

Formalin-fixed brain tissues were cryoprotected with sucrose solutions and then cut with a sliding microtome to yield 40 μ m sections. The sections were incubated with primary antibodies (GFAP+HLADR) and incubated with the appropriate fluorogen-tagged secondary antibody (Cy3 or Alexa). Specimens were examined in a Zeiss LSM 5.0 confocal laser microscope (Zeiss, Thornwood, NY).

Protein Tissue Arrays

To further characterize the nature of the inflammatory responses in autistic brains, we studied the relative expression of 79 proteins: cytokines associated with innate and adaptive immunity, chemokines, and growth and differentiation factors by human cytokine protein array methods.^{27,28} Human cytokine array kits (5.1 and V; Raybiotech, Norcross, GA) were used, consisting of 79 different cytokines, chemokines, and growth factors (Table 4) imprinted on a nitrocellulose membrane. The protocol for analysis followed the manufacturer's instructions: Membranes were blocked for 1 hour and incubated with 500 μ g of human tissue homogenate or 1ml of CSF for 2 hours at room temperature and then washed for 30 minutes and incubated with a 1 to 250 dilution of biotin-conjugated antibody mix for 2 hours. After consecutive washes, a 1 to 1,000 dilution of streptavidin-conjugated peroxidase was added and incubated for 1 hour at room temperature. The membranes were washed thoroughly and exposed to peroxidase substrate (ECL chemiluminescence; Amersham, Arlington Heights, IL), followed by apposition of the membranes with autoradiographic film (Hyperfilm ECL; Amersham) for a standard exposure of 1 minutes. The film was scanned, and spots were digitized into pixel densities using the NIH imaging software (Image J). The ratio of relative expression was established after subtraction of the background intensity and comparison with the positive spots available in the membrane.

Enzyme-Linked Immunosorbent Assay Techniques

Tumor growth factor (TGF)- β 1, macrophage chemoattractant protein (MCP)-1, interleukin (IL)-6 (R&D Systems, Minneapolis, MN), and insulin-like growth factor binding protein (IGFBP)-1 (Alpha Diagnostics, San Antonio, TX)

were quantified in tissue homogenates by sandwich enzyme immunoassay using commercially available kits according to the manufacturers' protocols. Values were calculated from a

Table 4. Proteins Included in the Cytokine Protein Array Study

Cytokines	Chemokines	Growth and Differentiation Factors
IL-2	ENA-78	GCSF
IL-4	GRO	GM-CSF
IL-5	GRO- α	IL-3
IL-13	I-309	IL-7
IFN- γ	IL-8	MCSF
TGF- β 1	MCP-1	SCF
IL-16	MCP-2	EGF
TGF- β 2	MCP-3	IGF-I
TGF- β 3	MDC	Ang
IL-1 α	MIG	OSM
IL-1 β	MIP-1 β	Tpo
IL-6	MIP-1 δ	VEGF
IL-10	RANTES	PDGF-B
IL-12	SDF-1	Leptin
IL-15	TARC	BDNF
TNF- α	BLC	FGF-4
TNF- β	Ck β 8-1	FGF-6
	Eotaxin	FGF-7
	Eotaxin-2	FGF-9
	Eotaxin-3	Flt-3 ligand
	Fractalkine	GDNF
	GCP-2	HGF
	IP-10	IGFBP-1
	MCP-4	IGFBP-2
	MIF	IGFBP-3
	MIP-3 α	IGFBP-4
	NAP-2	LIF
		LIGHT
		NT-3
		NT-4
		Osteoprotegerin
		PARC
		PIGF
		TIMP-1
		TIMP-2

standard curve generated for each enzyme-linked immunosorbent assay (ELISA). Samples were diluted 1 to 10, and results were standardized according to previously established protein concentrations, with the final concentration expressed as picograms per micrograms protein.

Statistical Analysis

SPSS 11.0 was used for all statistical analyses. Because of the nonparametric nature of the data (as determined by tests of normality), nonparametric tests were used to increase the robustness of the results. Group differences between autistic cases and controls in the fractional area of immunoreactivity for astroglia and activated microglia in the various brain regions were compared using the Mann–Whitney *U* test because of the non-Gaussian appearance of the data. The Mann–Whitney *U* test also was used to compare group differences in protein tissue arrays and ELISA quantification. Significance was assessed at the 0.05 level. For multiple test comparisons, a Bonferroni correction was performed, and correlations were assessed by Spearman's rank correlation coefficient because of the ordinal nature of the data. These tests were used because they make no assumptions about the distribution of the data (eg, normality).

Results

Increased Microglial and Astroglial Activation Are Observed in the Postmortem Brains of Autistic Patients

Our analysis of the neuropathological changes in brain tissues of autistic patients showed extensive neuroglial responses characterized by microglial and astroglial activation. In the brains of autistic patients, the most prominent histological changes were observed in the cerebellum, characterized by a patchy loss of neurons in the Purkinje cell layer (PCL) and GCL in 9 of 10 cerebella (Fig 1); one of these cerebella also showed an almost complete loss of Purkinje cells from the PCL as well as a marked loss of granular cells (Patient 3711, a 25-year-old male patient with epilepsy, see Fig 1B–D). Only one cerebellum showed no evidence of Purkinje cell loss (Patient 2004, a 8-year-old male patient; see Table 1). In contrast, no significant histological changes were observed in either region in the control brains. As compared with normal controls, GFAP immunostaining in all three regions of the autistic brains showed increased astroglial reactions characterized by an increase in the volume of perikarya and glial processes. In the brains of autistic patients, GFAP immunostaining of the cerebellum showed a marked reactivity of the Bergmann's astroglia in areas of Purkinje cell loss within the PCL, as well as a marked astroglial reaction in the GCL and cerebellar white matter (see Fig 1G–I). In the MFG and ACG, astroglial reactions were prominent in the subcortical white matter, and in some cases panlamellar astrogliosis was observed (Fig 2). Quantitative assessment of astroglial immunoreactivity by fractional area methods^{25,26} showed a signifi-

cant increase in GFAP immunoreactivity in the GCL ($p \leq 0.001$) and white matter ($p = 0.007$) compartments of the cerebellum (see Fig 2I) but did not reach statistical significance in the MFG ($p = 0.076$) or ACG ($p = 0.119$). Astroglial activation and reactivity were further analyzed by Western blotting of GFAP expression in protein homogenates obtained from a subset of autistic ($n = 7$) and control patients ($n = 7$) from whom fresh-frozen brain tissue had been obtained (see Table 1). These blots showed a significantly increased expression of GFAP in the cerebellum ($p = 0.001$), MFG ($p = 0.007$), and ACG ($p = 0.038$) of autistic patients as compared with controls.

Microglial activation in autistic brains was further characterized by immunocytochemical staining for major histocompatibility complex (MHC) class II markers (HLA-DR). Marked microglial activation was observed in the cerebellum (see Fig 1C, E–G), cortical regions (see Fig 2A, E, F), and white matter of autistic patients. The most prominent microglial reaction was observed in the cerebellum, where the immunoreactivity for HLA-DR showed a significantly higher fractional area of immunoreactivity in both the GCL ($p \leq 0.001$) and cerebellar white matter ($p \leq 0.001$) of autistic subjects than in controls (see Fig 2J). Differences in microglial activation in the MFG ($p = 0.106$) and ACG ($p = 0.109$) did not reach statistical significance. In the cerebellum, occasional microglial nodules were seen in the GCL and white matter. Further immunocytochemical studies, including confocal microscopy, showed that microglia and astroglia reactions in the cerebellum were both closely associated with degenerating Purkinje cells, granule cells, and axons (see Fig 1f, g). In the MFG and ACG, microglial activation was prominent at the junction of the cortex and white matter, and in four of nine cases a panlamellar distribution was also seen. In addition to the presence of activated microglia, we observed a marked accumulation of perivascular macrophages and monocytes in the cerebella of 4 of 10 autistic patients when we used antibodies that recognize CD68 (see Fig 1K) or migration inhibitory factor [MIF]-related protein-8 (MRP-8) antigens, markers of monocytes and macrophages in chronic stages of inflammation. We observed no differences in microglial or astroglial activation as a function of age or clinical profile including history of developmental regression or mental retardation in the autistic patients. The presence of microglial activation in the cerebellar white matter of autistic patients with history of epilepsy appeared to be significantly elevated ($p = 0.025$) as compared with those without epilepsy, but no differences were observed in the GCL or other regions. The magnitude of astroglia reaction measured by area fraction of immunoreactivity or Western blot was similar in autistic brain tissues from patients with and without history of epilepsy.

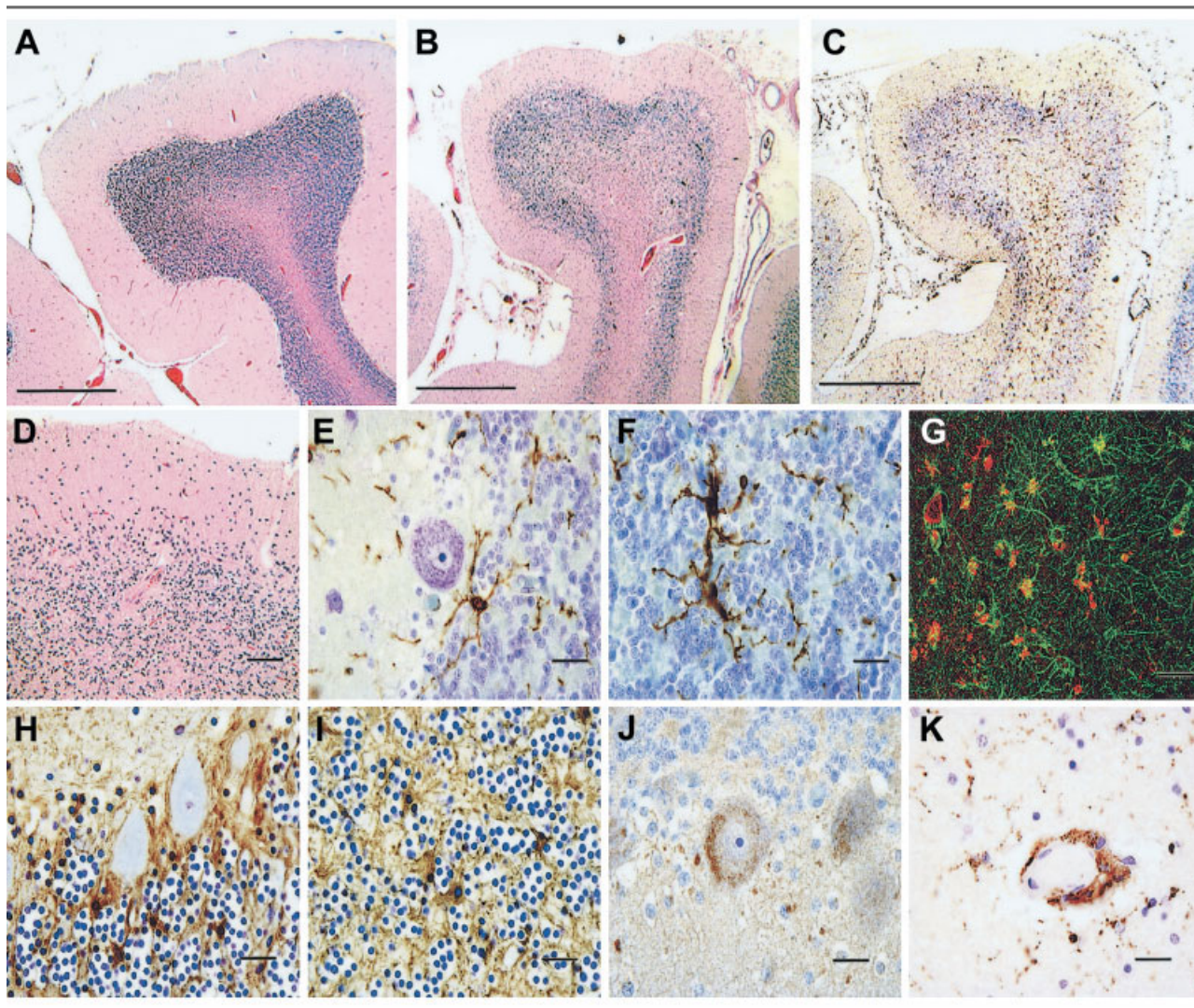


Fig 1. Cerebellar pathology in autism. (A) Normal cerebellar folia in a control brain (H and E staining). (B) Patchy loss of Purkinje cell layer (PCL) and granular cell layer (GCL) neurons (H and E) and (C) marked activation of microglia (immunostained with anti-HLA-DR antibody) are seen in the cerebellar folia of a patient with autism. Bar in A–C = 500 μm. (D) High-magnification detail of a cerebellar region with marked PCL and GCL neuronal loss (H and E). Bar = 50 μm. (E, F) Activated microglia around a Purkinje cell (E) and in the GCL (F), immunostained with anti-HLA-DR. Bar in E and F = 20 μm. (G) Close relationship of reactive astroglia (green) and activated microglia (red) in the GCL of the cerebellum, as seen by double immunocytochemical staining for glial fibrillary acidic protein (GFAP) (green) and HLA-DR (red) and laser confocal microscope imaging. (H, I) Increased Bergmann's astroglia around Purkinje cells of the PCL in H and reactive astrogliosis in the GCL in I, both immunostained with anti-GFAP antibodies. (J) Identification of complement membrane attack complexes by immunocytochemical staining with anti-C9neo antibody (granular pattern) in Purkinje cells and other surrounding cells that appear to be microglia/macrophages. (K) Accumulation of perivascular macrophages and microglia identified with anti-CD68 antibodies. Bar in H–K = 20 μm.

Lack of Evidence of Adaptive Immune Reactions in Autistic Brains

To examine more closely the immunopathological reactions associated with adaptive immunity in the brain of autistic patients, we performed immunocytochemical studies to identify T- and B-lymphocyte infiltration and deposition of immunoglobulin and complement, as indicators of cellular and humoral immune responses. We observed a few isolated perivascular CD3⁺ and CD20⁺ cells in both autistic and control brains but saw no evi-

dence of leptomeningeal, parenchymal, or perivascular inflammatory infiltration in autistic brains in any of the regions studied. Immunostaining with antibodies recognizing IgG, IgA, or IgM showed no deposition of any of these immunoglobulins in neuronal or neuroglial cell populations. In cerebella from autistic brains, we observed deposition of complement membrane attack complexes (MACs) in the perineuronal compartments of the PCL and GCL by immunostaining with an antibody against the C9neo antigen²⁹ (see Fig 1L). The pattern of

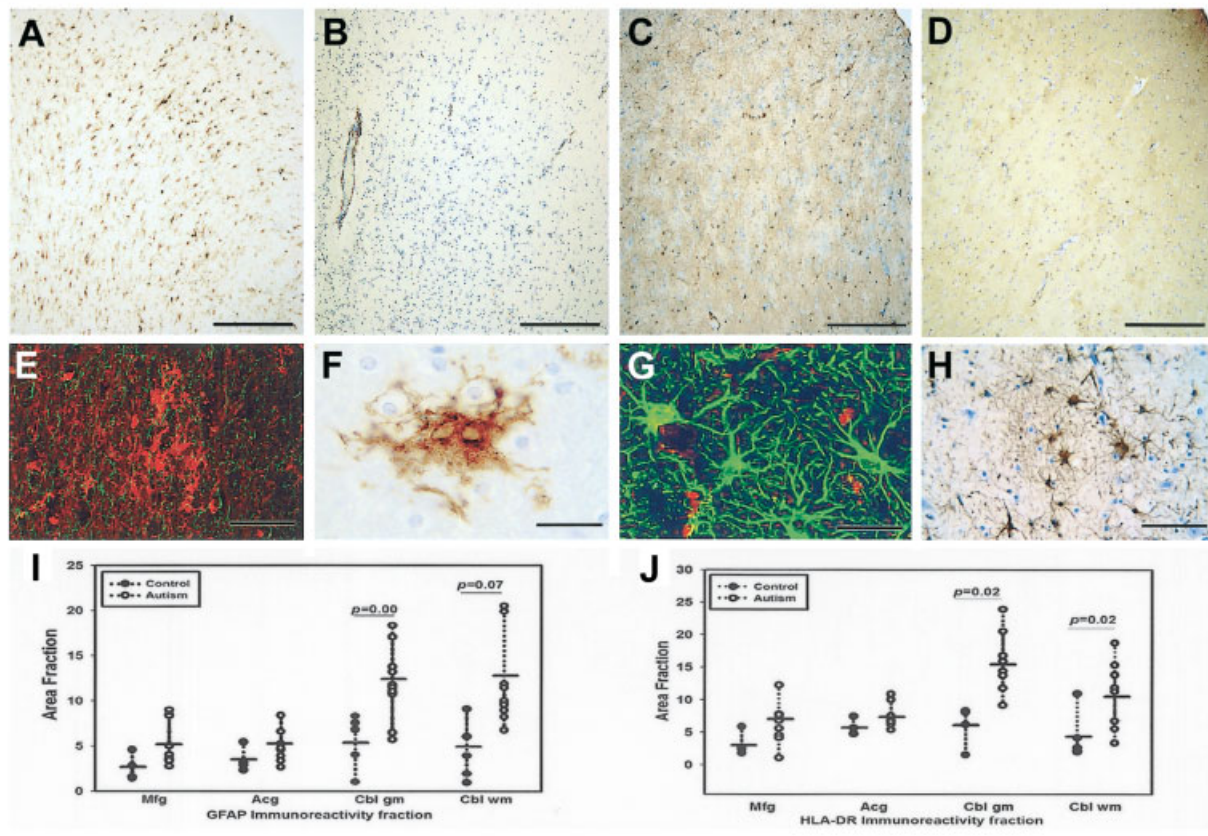


Fig 2. Neuroglial reactions in the cerebral cortex of autistic patients. (A–D) Panlaminar activated microglia and panlaminar astroglia are seen in the middle frontal gyrus (MFG) from an autistic patient in A and C, respectively. MFG from a control brain immunostained for microglia is seen in B and for astroglia in D. Immunostaining in A and B with anti-HLA-DR antibodies and in C and D with anti-GFAP. Bar in A–D = 200 μ m. (E–H) A microglial nodule (E) and a cluster of reactive astrocytes (G) in the cerebral cortex of an autistic patient, as seen with double immunocytochemical staining for microglia (red) and astroglia (green) and laser confocal imaging. Similar clusters of microglia (F) and astrocytes (H) visualized with diaminobenzidine tetrahydrochloride chromogen. (I, J) Fractional area of immunoreactivity for GFAP (I) and HLA-DR (J) in the middle frontal gyrus (Mfg), anterior cingulate gyrus (Acg), cerebellar PCL and GCL (Cbl gcl), and cerebellar white matter (Cbl-wm) compartments. Mann–Whitney U test, significance level $p < 0.05$.

immunoreactivity suggested that some Purkinje cells and cells with macrophage-like morphology had been labeled with the anti-C9neo antibody.

Increased Levels of Proinflammatory Cytokines Are Present in Brain Tissues from Autistic Patients

We assessed the profiles of expression of proteins involved in inflammatory pathways by cytokine protein array methodology^{27,28} in brain tissue homogenates in a subset of autistic ($n = 7$) and control ($n = 7$) patients from whom fresh-frozen brain tissues were available (see Table 1). A statistical analysis of the relative expression of cytokines in autistic and control tissues showed a consistent and significantly higher level of subsets of cytokines in the brains of autistic patients (Table 5 and Fig 3): the antiinflammatory cytokine tumor growth factor- β 1 (TGF- β 1) was increased in the MFG ($p = 0.026$), ACG ($p = 0.011$) and CBL ($p =$

0.035), and the proinflammatory chemokines, MCP-1 and thymus and activation-regulated chemokine (TARC), were increased in the ACG ($p = 0.026$ and 0.035, respectively) and CBL ($p = 0.026$ and 0.035, respectively). Only IGFBP-1, a growth and differentiation factor involved in immune and cellular growth pathways, was consistently increased in the cortical regions (MFG, $p = 0.038$; ACG, $p = 0.011$), but the difference did not reach statistical significance in the cerebellum ($p = 0.11$). Interestingly, a larger spectrum of increased proinflammatory and modulatory cytokines was seen in the ACG, where there was a significant increase in interleukin-6 (IL-6), interleukin-10 (IL-10), macrophage chemoattractant protein-3 (MCP-3), eotaxin, eotaxin 2, macrophage-derived chemokine (MDC), chemokine- β 8 (Ck β 8.1), neutrophil activating peptide-2 (NAP-2), monokine induced by

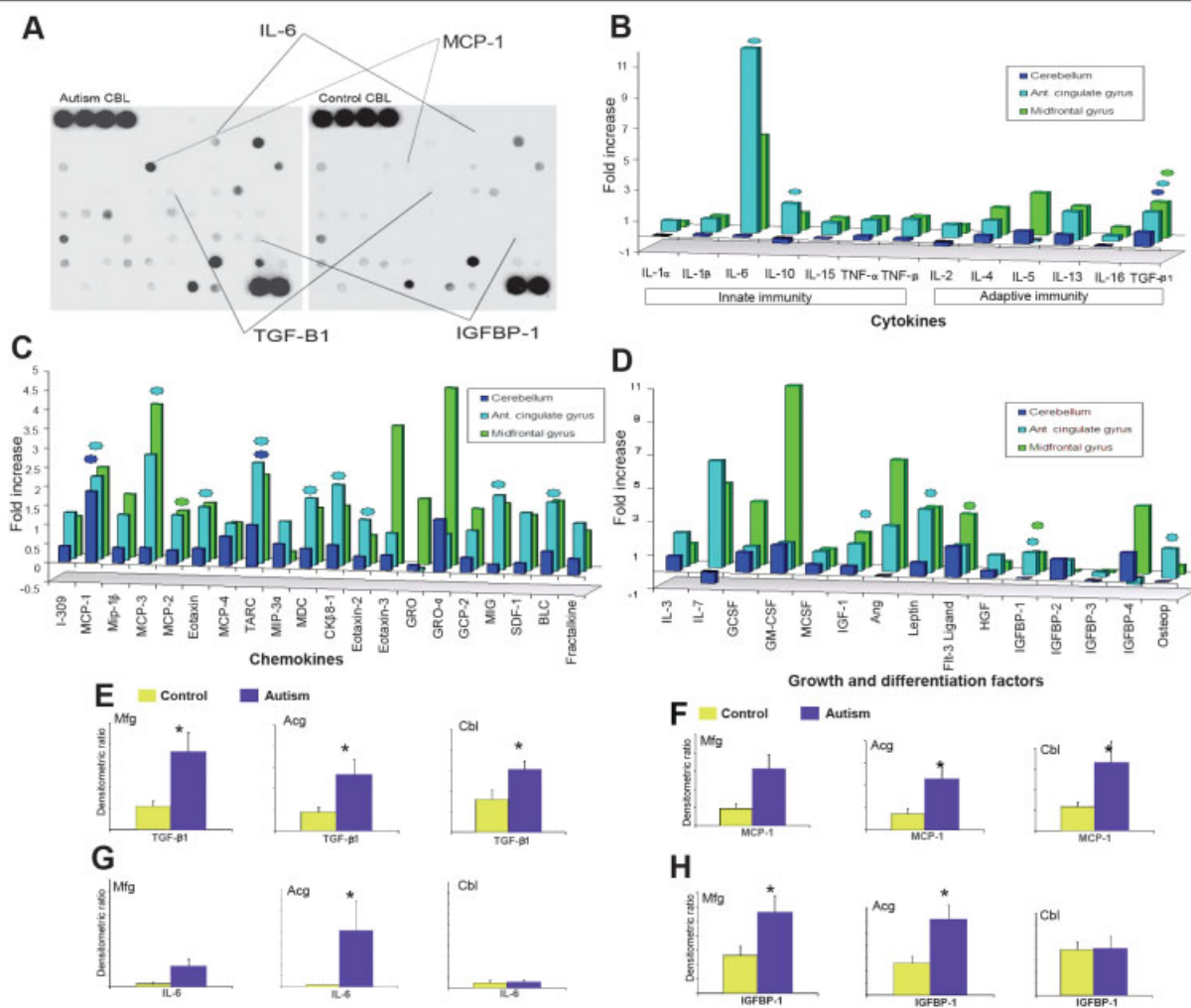


Fig 3. Cytokine profile in brain tissues obtained using cytokine protein array methods. (A) Two cytokine protein array membranes protein homogenates from the cerebellum of an autistic and a control patient. Each spot represent a cytokine for which the ratio of expression (arbitrary units) was obtained between the cytokine and the positive control present in the membrane. The location in the membrane of macrophage chemoattractant protein (MCP)-1, interleukin (IL)-6, tumor growth factor (TGF)-β1, and IGFBP-1 is shown in both membranes for comparison. (B-D) Profiles of expression of (B) cytokine, (C) chemokines, and (D) growth and differentiation factors in the cerebellum (Cbl), anterior cingulate gyrus (Acg), and middle frontal gyrus (Mfg). (E-H) Ratio of expression (arbitrary units) of TGF-β1, MCP-1, IGFBP-1 in the various brain regions of autistic and control cases. The statistical significance (*) was obtained at level $p < 0.05$ by a Mann-Whitney U test.

interferon-γ (MIG), B-lymphocyte chemoattractant (BLC), leptin and osteoprotegerin (Fig 3 and Table 5).

To confirm our observations derived from the cytokine protein array studies, we used ELISA assays to quantify the most significant cytokines, TGF-β1, MCP-1, IGFBP-1, and IL-6, in the same set of brain tissues from autistic and control brains (Fig 4 and Table 6). We found that in the three regions studied, the anti-inflammatory cytokine TGF-β1 was consistently and significantly higher in the autistic group than in the controls; the MCP-1 protein was also significantly elevated in the ACG ($p = 0.017$) and CBL ($p = 0.001$) in the autistic patients, and the levels almost reached significance in the MFG ($p = 0.057$). Similarly, IGFBP-1

concentrations were significantly elevated in the MFG ($p = 0.032$) and ACG ($p = 0.01$) in the autistic patients, but no significant difference was found in the CBL ($p = 0.42$). In contrast, we found no significant differences in the concentration of IL-6 in the MFG ($p = 0.45$), ACG ($p = 0.12$), or CBL ($p = 0.32$) of autistic and control brains.

Reactive Astrocytes Are the Main Source of Cytokines in the Brains of Autistic Patients

To determine the cellular sources of the most significantly increased cytokines in the brains of autistic patients, we conducted immunocytochemical staining for TGF-β1, MCP-1, IGFBP-1, and IL-6 in the MFG,

Table 5. Cytokines Increased in Brain Tissues of Patients with Autism

Cytokine	Middle Frontal Gyrus		Anterior Cingulate Gyrus		Cerebellum	
	Fold Increase	<i>p</i> ^a	Fold Increase	<i>p</i> ^a	Fold Increase	<i>p</i> ^a
IL-6	6.10	ns	31.4	0.011	0.11	ns
IL-10	1.11	ns	2.00	0.007	−0.33	ns
TGF-β1	2.33	0.026	2.00	0.011	0.92	0.035
MCP-1	2.32	ns	2.20	0.026	1.90	0.035
MCP-2	1.24	0.017	1.20	ns	0.37	ns
MCP-3	4.00	ns	2.80	0.038	0.42	ns
Eotaxin	1.46	ns	1.40	0.017	0.44	ns
TARC	2.23	ns	2.63	0.026	1.09	0.035
MDC	1.41	ns	1.74	0.004	0.51	ns
Ckβ8.1	1.47	ns	2.11	0.017	0.62	ns
Eotaxin 2	0.73	ns	1.22	0.038	0.34	ns
MIG	1.65	ns	1.94	0.026	0.22	ns
BLC	1.75	ns	1.80	0.026	0.60	ns
IGF-1	2.09	ns	1.63	0.026	0.51	ns
Leptin	3.72	ns	3.8	0.007	0.84	ns
Flt3-lig	3.34	ns	1.71	ns	1.86	0.022
IGFBP1	1.14	0.038	1.36	0.011	0.02	ns
Osteoprotegerin	0.057	ns	1.78	0.017	0.03	ns

^aMann–Whitney *U* test.

IL = interleukin; TGF = tumor growth factor; MCP = macrophage chemoattractant protein; MDC = macrophage-derived chemokine; insulin-like growth factor IGFBP = IGF binding protein; ns = not significant.

ACG, and CBL. The staining patterns observed indicated that astrocytes were the main source of both MCP-1 and IL-6. Both cytokines were prominently expressed in reactive astrocytes in the cerebellum and cortical and subcortical white matter regions. Confocal microscope studies of sections that had been double-immunostained for GFAP and MCP-1 or GFAP and IL-6 further confirmed these observations and the colocalization of these cytokines within astrocytes (see Fig 4). It is noteworthy that TGF-β1 and IGFBP-1 expression was seen not only in reactive astrocytes but also in the Purkinje cell population and in subsets of GCL cells in the CBL. Some microglial cells were also labeled with the antibodies recognizing TGF-β1 and IGFBP-1. Purkinje cells with degenerative changes appeared strongly immunoreactive for TGF-β1 (see Fig 4).

The Cerebrospinal Fluid from Patients with Autism Shows a Proinflammatory Profile

Because brain tissues from patients with autism showed a prominent proinflammatory profile, we considered the possibility that CSF from autistic patients might have a similar profile. Cytokine protein arrays were used to compare the cytokine profiles of CSF from six autistic patients with that of CSF from a pool of donors without CNS pathology or inflammatory disorders (eg, pseudotumor cerebri or headaches; see Table 2). As we had observed in brain tissue, CSF from autistic patients showed a significant increase in MCP-1 (12-fold increase) when compared with controls (Table

7 and Fig 5). There were no differences in expression of TARC or TGF-β1 in the CSF. However, other proinflammatory and modulatory cytokines such as IL-6, interferon (IFN)-γ, IL-8, macrophage inflammatory protein-1β (MIP1β), NAP-2, interferon-γ inducing protein-10 (IP-10) and angiogenin, as well as growth factors such as mesoderm inducing factor (MIF), vascular endothelial growth factor (VEGF), leukemia inhibitory factor (LIF), osteoprotegerin, hepatic growth factor (HGF), PARC, FGF-4, FGF-9, IGFBP3, and IGFBP4, were all significantly increased when compared with control CSF (see Fig 5 and Table 7).

Discussion

Microglial and Astroglial Reactions Characterize Innate Immune Responses in Autism

In this study, we have demonstrated a marked increase in neuroglial responses, characterized by activation of microglia and astroglia, in the brains of autistic patients. These increased neuroglial responses are likely part of neuroinflammatory reactions associated with the CNS innate immune system in which microglial activation is the main cellular response to CNS dysfunction^{30,31} as compared with adaptive immune responses in which lymphocyte- and/or antibody-mediated reactions are the dominant responses.^{32,33} In our sample of autistic cases, microglial and astroglial activation was present in the absence of lymphocyte infiltration or immunoglobulin deposition in the CNS and was associated with increased production of proin-

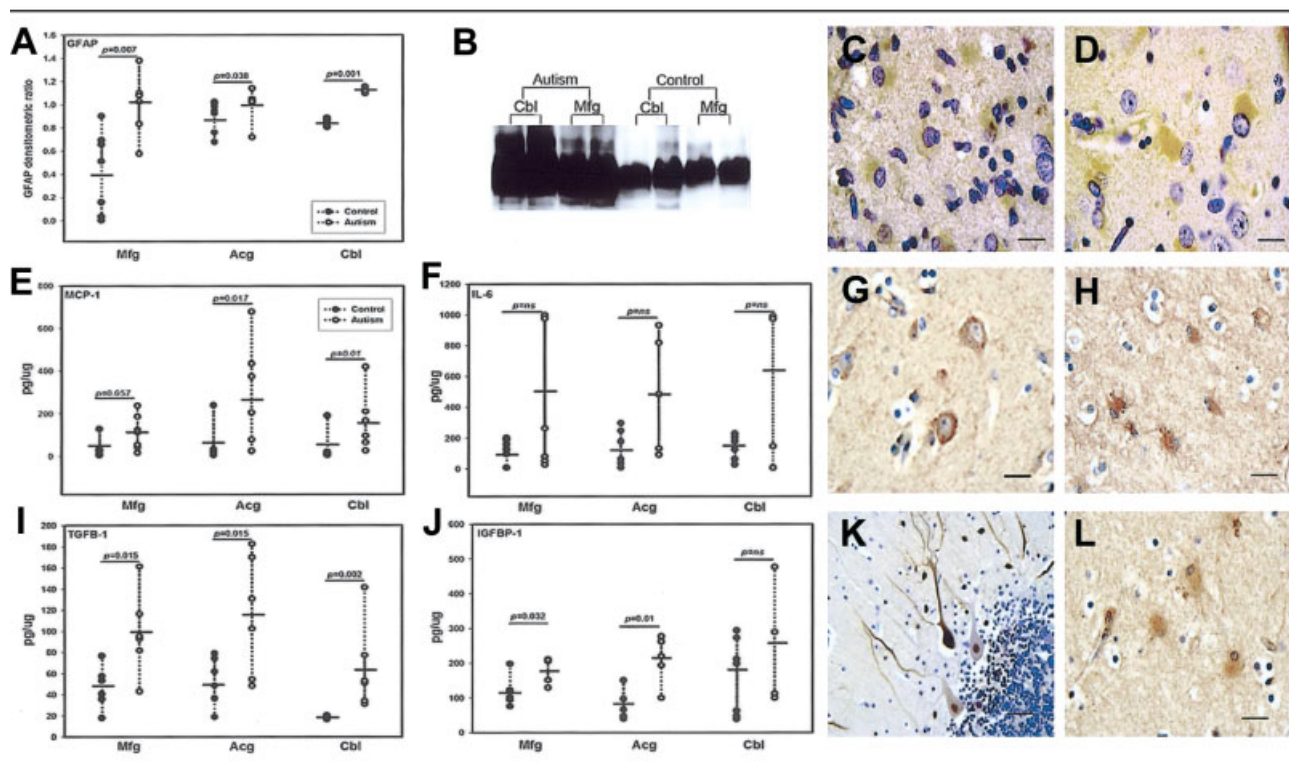


Fig 4. Quantitative validation analysis by enzyme-linked immunosorbent assay and immunolocalization of cytokine expression in the brain. (A) The expression of glial fibrillary acidic protein (GFAP) measured by immunoblot analysis (arbitrary units) was significantly increased in all brain regions of autistic patients. (B) A Western blot for GFAP in the cerebellum (Cbl) and midfrontal gyrus (MFG) from autistic and control cases. (C) Macrophage chemoattractant protein (MCP)–1 immunolocalization in astrocytes. (D) Interleukin (IL)–6 was immunolocalized in reactive astrocytes. (E) MCP-1 concentration was significantly increased in Acg and Cbl in autistic cases. (F) IL-6 was elevated in all three regions, but its increase did not reach statistical significance. (G) IGFBP-1 was localized in subsets of cortical neurons, Purkinje cells, and (H) reactive astrocytes. (I) TGF-β1, and (j) IGFBP-1 quantification. (K) TGF-β1 was also localized in Purkinje cells, (L), reactive astrocytes, and cortical neurons (not shown). Subsets of Purkinje cells with morphological changes consistent with degeneration were intensely immunostained with anti-TGF-β1 antibodies. The statistical significance was obtained at level $p < 0.05$ by Mann–Whitney U test.

flammatory and antiinflammatory cytokines such as MCP-1 and TGF-1 by neuroglia.

Because autism is a heterogeneous disorder that may be associated with multiple causative factors, it is pos-

sible that our sample of cases does not represent the entire autistic spectrum, as some of our patients had other associated neurological disorders frequently found in autism, such as epilepsy and mental retarda-

Table 6. ELISA Quantitative Analysis of Selected Cytokines in Brain Tissue Homogenates

Brain Region	MCP-1 (pg/μg)		IL-6 (pg/μg)		TGF-β1 (pg/μg)		IGFBP-1 (pg/μg)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Cerebellum								
Autism	163.6	57.94	618.4	221.9	64.17	16.75	244.8	88.74
Controls	38.51	23.74	137.5	119.8	18.37	0.57	161.1	60.71
MFG								
Autism	110.8	30.5	482.7	180.3	98.62	15.96	210.6	27.95
Controls	36.77	14.8	130.7	26.48	47.11	8.24	85.89	18.65
ACG								
Autism	260.6	93.15	492.6	171.6	114.8	23.18	181.43	17.06
Controls	50.82	29.67	138.9	48.99	53.35	9.42	118.9	21.23

ELISA = enzyme-linked immunosorbent assay; MCP = macrophage chemoattractant protein; IL = interleukin; TGF = tumor growth factor; IGFBP = insulin-like growth factor binding protein; SEM = standard error of the mean; MFG = middle frontal gyrus; ACG = anterior cingulate gyrus.

Table 7. Cytokines with Significant Increase in the Cerebrospinal Fluid of Patients with Autism

Cytokine	Fold Increase	<i>p</i> ^a
IFN- γ	232.5	0.008
TGF- β 2	30.9	<0.001
MCP-1 ^b	12.2	<0.001
IL-8	6.0	<0.001
IP-10	18.2	0.018
Angiogenin	3.3	0.003
VEGF	81.8	0.001
IGFBP-1 ^b	0.4	0.036
IGFBP-3	26.3	<0.001
IGFBP-4	13.3	0.003
LIF	1.0	<0.001
FGF-4	0.23	0.005
FGF-9	70.0	0.012
PARC	11.3	0.002
Osteoprotegerin ^b	5.2	0.002
HGF	0.3	0.005
IGFBP-3	26.3	<0.001
IGFBP-4	13.3	0.003

^aMann-Whitney *U* test.

^bFound significantly increased also in one or more brain regions in brain tissue analysis.

IFN = interferon; TGF = tumor growth factor; MCP = macrophage chemoattractant protein; IL = interleukin; VEGF = vascular endothelial growth factor; IGFBP = insulin-like growth factor binding protein; LIF = leukemia inhibitory factor; HGF = hepatic growth factor.

tion. However, the presence of morphological and immunological findings demonstrative of neuroimmune reactions in the sample of autistic patients included in this study as well as the CSF findings support a potential role for neuroglia and neuroinflammation as pathogenic mechanisms in an undefined proportion of individuals with autism.

The neuroglial activation in the autism brain tissues was particularly striking in the cerebellum, and the changes were associated with upregulation of selective cytokines in this and other regions of the brain. Immunocytochemical analysis of microglial and astroglial reactions in the brains of these patients showed that regardless of age, history of epilepsy, developmental regression, or mental retardation, marked morphological changes consistent with chronic and sustained neuroglial inflammatory responses were present in cortical and subcortical white matter as well as in the cerebellum. These changes may be involved in mechanisms associated with neuronal and synaptic dysfunction in autism. Microglia, the resident macrophages and the primary immunocompetent cells of the nervous system^{30,34} were consistently activated in all brain regions of autistic patients, but particularly in the cerebellum. Similarly, Western blot analysis showed an increase in GFAP expression (an indicator of the magnitude of astroglial activation) in all regions studied, when compared with normal controls.

Microglial responses in autism cases were diffusely distributed in the cortex and subcortical areas, as well as the cerebellum, or were present as microglial nodules or as part of a prominent accumulation of perivascular macrophages. These responses in autism resemble those seen in neurodegenerative disorders such as Alzheimer's disease (AD),²⁶ Parkinson's disease (PD), and amyotrophic lateral sclerosis.^{35–37} and are similar to those seen in dementia associated with human immunodeficiency virus (HIV) infection.^{38,39} In these conditions, chronic microglial activation appears to be responsible for a sustained neuroinflammatory response that facilitates the production of multiple neurotoxic mediators.^{40,41} Neuroinflammatory activation may be a common pathway leading to CNS dysfunction in all these disorders. In the case of autism, the presence of microglial activation supports the view that innate immune responses are present in cortical and subcortical regions and that a state of chronic activation and reactivity

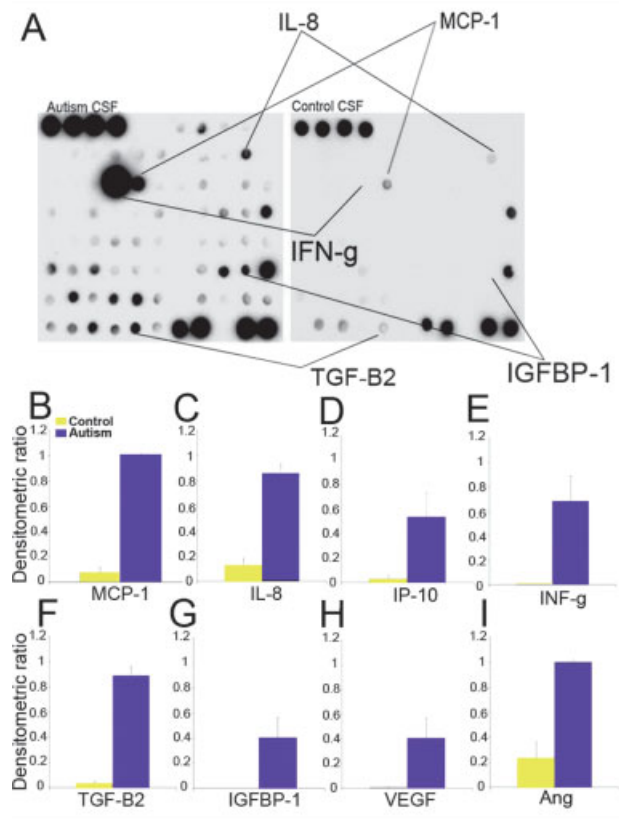


Fig 5. Cytokine profiles in cerebrospinal fluid (CSF) from autistic and control patients. (A) Cytokine protein arrays in CSF samples from an autistic patient and a control. The spots for macrophage chemoattractant protein (MCP)-1, interferon- γ , TGF- β 2, interleukin-8, and IGFBP-1 showed a marked density increase as compared with the CSF control. (B–I) Profile of expression (fold increase) of cytokines that were found markedly increased in autistic patients as compared with controls. $p < 0.05$, Mann-Whitney *U* test.

may be involved in the mechanisms of neuronal and synaptic dysfunction.

The presence of increased neuroglial responses is relevant to the neurobiological mechanisms involved in autism, because both microglia and astroglia are essential for neuronal activity and synaptic function,⁴² neuronal–glial interactions,⁴³ as well as for cortical modeling, organization, and remodeling during brain development.⁴⁴ Furthermore, microglial and astroglial activation seems to play a major role in the neuroimmune mechanisms of disease in the CNS,³⁴ because these cells are part of the first-line response of the innate immune system of the CNS³⁰ and contribute to the modulation of immune responses by producing both proinflammatory and antiinflammatory cytokines as well as growth and differentiation factors.⁴⁵ The microglial and astroglial responses in the CNS may then have a dichotomous role in the inflammatory responses of the brain: as a direct effector of injury and on the other hand as neuroprotectant.⁴⁶ An issue that remains unclear is how and when microglia and astroglia become activated in the brain of autistic patients. Neuroglial responses in autism may be part of both primary (intrinsic) neuroglial responses that result from disturbances of neuroglial function or neuronal–neuroglial interactions during brain development and secondary (extrinsic), resulting from unknown factors that disturb prenatal or postnatal CNS development. Both astrocytes and microglia are critical for brain development and MHC class II (HLA-DR antigen)–positive microglia colonize the developing CNS during the second trimester.^{47,48} It is possible that the presence of activated microglia in the brain in autism may reflect abnormal persistence of fetal patterns of development in response to genetic or environmental (eg, intrauterine, maternal) factors. Even though our studies did not show any difference in neuroglial activation among autistic cases with history of developmental regression or mental retardation, further studies that include larger series of cases are needed to clarify these issues.

Previous neuropathological studies in autism showed abnormalities in cortical organization and neuronal packing and reduced cerebellar Purkinje cell numbers.¹¹ Our findings may indicate that at some point during cortical and neuronal organization, unknown factors influence both neuronal and neuroglial cell populations, disturbing neurodevelopment and producing the neurocytoarchitectural changes seen in autism as well as inducing CNS dysfunction that results in neuroinflammation. An alternative explanation is that extrinsic causative factors (eg, nongenetic, neurotoxic, or environmental) involved in the pathogenesis of autism may produce neuronal and cortical abnormalities, to which neuroglial reactions are only secondary responses. Although the meaning of the neuroinflammation in our sample is unknown at this time, these pri-

mary or secondary responses may be valuable clinical biomarkers and targets for therapy, if it can be demonstrated that they are causing injury to the developing CNS.

Lack of Adaptive Immune Reactions in Brain of Patients with Autism

In contrast with the prominent presence of activated microglia and astrocytes, features that characterize innate immune responses within the CNS, an important finding of our study was the lack of specific T-cell responses and the absence of antibody-mediated reactions in any of the brain regions studied in autistic subjects. These observations suggest that the adaptive immune system does not play a significant pathogenic role in this disorder, at least not during its chronic phase, and that the main immune mechanism involves predominantly innate immune reactions. Because our study focused on autopsy tissues, we cannot exclude the possibility that specific immune reactions, mediated by T-cell and/or antibody responses, occurred at the onset of disease, during prenatal or postnatal stages of development. An interesting finding in our immunocytochemical studies was the observation of complement membrane attack complex in cerebella. The localization and immunoreactivity of C9neo, a marker for the membrane attack complex,²⁹ in the perineuronal Purkinje cell compartment and focal areas of the GCL, suggest that microglial activation may trigger complement activation, and the complement system may play a role in the destructive process that occurs in the cerebellum of autistic patients. The lack of immunoglobulin deposition, however, suggests that complement activation may occur in the absence of antibody-mediated pathways and may resemble the immunopathogenic mechanisms observed in AD, PD, and other neurodegenerative disorders, in which autotoxic phenomena play a role in neuronal injury and neurodegeneration.⁴⁹ Further clarification of the role of these autotoxic reactions (mediated by complement and associated with other innate immune reactions in the cerebellum) is required, because degeneration of the PCL and GCL seems to occur in the absence of adaptive immune responses.

Cerebellum Is a Main Focus of Neuroinflammation in Autism

Our quantitative analysis of neuroglial reactions showed that among the brain regions studied, the cerebellum showed the most prominent neuroglial responses. This marked neuroglial activity in the cerebellum is consistent with previous observations that the cerebellum is one of the foci of pathological abnormalities in morphological^{11,12} and neuroimaging^{50–52} studies of autistic patients. Based on our observations, a selective process of neuronal degeneration and neuroglial activation appear

to occur predominantly in the PCL and GCL of cerebellum in autistic subjects, findings that are consistent with an active and ongoing postnatal process of neurodegeneration and neuroinflammation. These observations do not support the previously proposed hypothesis that the changes in the cerebellum in autism result solely from developmental abnormalities in olivary-cerebellar circuits and a reduced number of Purkinje cells.¹¹ Instead, our observations suggest that the pathological changes observed in the cerebellum in autistic patients do not occur exclusively during prenatal development but appear to involve an ongoing chronic neuroinflammatory process that involves both microglia and astroglia. Furthermore, this process continues beyond early neurodevelopment and is present even at very late stages in the life of patients with autism. These findings also support the hypothesis that selective vulnerability of Purkinje cells plays a role in the etiopathogenesis of autism.⁵³

Macrophage Chemoattractant Protein-1 and Tumor Growth Factor- β 1 Are the Most Prominent Cytokines in the Brain of Autistic Patients

Our study has also demonstrated the presence of unique profiles of cytokine expression in the brain and CSF of autistic subjects. Two proinflammatory chemokines, MCP-1 and TARC, and an antiinflammatory and modulatory cytokine, TGF- β 1, were consistently elevated in the brain regions studied. MCP-1, a chemokine involved in innate immune reactions and important mediator for monocyte and T-cell activation and trafficking into areas of tissue injury,⁵⁴ appeared to be one of the most relevant proteins found in cytokine protein array studies because it was significantly elevated in both brain tissues and CSF. The presence of MCP-1 is of particular interest, because it facilitates the infiltration and accumulation of monocytes and macrophages in inflammatory CNS disease.⁵⁵ As shown by our immunocytochemical studies of the cerebral cortex and cerebellum, MCP-1 is produced by activated and reactive astrocytes, a finding that demonstrate the effector role of these cells in the disease process in autism. The increase expression of MCP-1 has relevance to the pathogenesis of autism because we believe its elevation in the brain is linked to microglial activation and perhaps to the recruitment of monocytes/macrophages to areas of neurodegeneration, such as those we observed in the cerebellum. Our observations resemble findings in other neurological disorders in which elevation of MCP-1 is associated with the pathogenesis of neuroinflammation and neuronal injury such as HIV dementia,⁵⁶ amyotrophic lateral sclerosis,³⁷ stroke,⁵⁷ and multiple sclerosis.⁵⁵ It remains unclear whether MCP-1 plays a more pleiotropic role in the CNS or whether its presence is associated only with inflammatory conditions. It has been speculated

that MCP-1 may be involved in neuronal survival and neuroprotective mechanisms other than monocyte activation and trafficking⁵⁸ or even in nonlymphocytic-mediated neuronal injury.⁵⁹ Expression of MCP-1 in the CNS appears to be developmentally regulated, and previous studies have shown its expression in the cerebellum during prenatal development, a finding that may suggest an association with maturation of Purkinje cells.⁶⁰ Like MHC class II expression in microglia during CNS modeling,⁴⁷ MCP-1 elevation in the brain of autistic patients may reflect persistent fetal patterns of brain development.

Our observation that TGF- β 1 was increased in the cortex and cerebellum of autistic brains may have important implications for the neurobiology of autism. TGF- β 1 is a key antiinflammatory cytokine and is involved in tissue remodeling after injury. It can suppress specific immune responses by inhibiting T-cell proliferation and maturation and downregulates MHC class II expression.⁶¹ Importantly, cells undergoing cell death have been shown to secrete TGF- β 1, possibly to reduce local inflammation and prevent degeneration of additional surrounding cells.⁶² In our immunocytochemical studies, TGF- β 1 was localized mostly within reactive astrocytes and neurons in the cerebellum. Purkinje cells that exhibited morphological features of degeneration showed marked immunoreactivity for TGF- β 1. These findings suggest that the elevation of this cytokine in autism may reflect an attempt to modulate neuroinflammation or remodel and repair injured tissue. Although TGF- β 1, MCP-1, TARC, and IGFBP-1 were consistently elevated in at least two of the three regions examined in the autistic brains, a more remarkable profile of cytokine upregulation was observed in the ACG, a region in which several cytokines, chemokines, and growth factors were markedly elevated when compared with controls. Both proinflammatory cytokines (eg, IL-6) and antiinflammatory cytokines (eg, IL-10) as well as subsets of chemokines were markedly elevated in the ACG, an important cortical region involved in dysfunctional brain activity in autism.⁶³ These findings support the conclusion that an active, ongoing immunological process was present in multiple areas of the brain but at different levels of expression in each area.

Marked Expression of Proinflammatory Cytokines in Cerebrospinal Fluid of Autistic Patients

CSF studies also confirmed a prominent inflammatory cytokine profile in patients with autism. The presence of a marked increase of MCP-1 in CSF supports the hypothesis that proinflammatory pathways are activated in the brain of autistic patients and that its presence may be associated with the mechanisms of macrophage/microglia activation observed in the brain tissue studies. The elevation of MCP-1 in the CSF re-

semble observations in other conditions in which microglia/macrophage activation play an important role such as HIV dementia⁵⁶ and multiple sclerosis.⁶⁴ In addition to the marked elevation in MCP-1, the presence of elevated levels of IFN- γ , IL-8, IP-10, and other proinflammatory molecules such as angiogenin and LIF strongly supports the view that active neuroinflammatory reactions and a network of multiple cytokines are likely involved in immune-mediated mechanisms in the CNS of autistic patients. These cytokines play important roles in immune-mediated processes, and their presence in the CSF in autistic patients may reflect an ongoing stage of inflammatory reactions likely associated with neuroglial activation and/or neuronal injury. Reasons for the relatively greater increases in these cytokines in CSF compared with brain are unknown. It could be that cytokines derive from neuroglial and neuronal sources as demonstrated by our immunocytochemical assessment. The differences we observed in cytokines in CSF compared with brain could result from other sources of production, such the leptomeninges or choroid plexus or might represent a persistent elevation of cytokines as a result of a stage of neurodevelopmental arrest because some of the cytokines are normally elevated during phases of neurodevelopment. Because the CSF is easily accessible for clinical studies, CSF cytokine profiling may be useful in the future to diagnose, characterize, and follow the clinical course of autistic disorders.

Conclusion

Taken together, our observations suggest that neuroglial reactions, in the form of innate immune responses, are important in the mechanisms associated with neural dysfunction in autism and that the cerebellum is the focus of an active and chronic neuroinflammatory process in autistic patients. The presence of proinflammatory chemokines such as MCP-1 as well as antiinflammatory cytokines such as TGF- β 1 supports the idea that a chronic state of specific cytokine activation occurs in autism. This hypothesis is also supported by our finding of marked increase in a larger set of cytokines in the CSF that are usually involved in inflammatory pathways. In view of the heterogeneity of clinical symptoms and possible causes for autism, the presence of neuroinflammatory changes among the cases we examined suggests that this may be a common pathogenic mechanism in some patients with autism. Because neuroimmune responses are influenced by the genetic background of the host, the role of neuroinflammation in the context of the genetic and other factors that determine the autism phenotype remains an important issue to be investigated. Because this neuroinflammatory process appears to be associated with an ongoing and chronic mechanism of CNS dysfunction, potential therapeutic interventions should focus

on the control of its detrimental effects (while preserving reparative benefits) and thereby eventually modify the clinical course of autism.

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References

1. Rapin I. Autism. *N Engl J Med* 1997;337:97–104.
2. Lord C, Cook EH, Leventhal BL, et al. Autism spectrum disorders. *Neuron* 2000;28:355–363.
3. Rapin I, Katzman R. Neurobiology of autism. *Ann Neurol* 1998;43:7–14.
4. Newschaffer CJ, Fallin D, Lee NL. Heritable and nonheritable risk factors for autism spectrum disorders. *Epidemiol Rev* 2002; 24:137–153.
5. Fombonne E. Epidemiological surveys of autism and other pervasive developmental disorders: an update. *J Autism Dev Disord* 2003;33:365–382.
6. Bertrand J, Mars A, Boyle C, et al. Prevalence of autism in a United States population: the Brick Township, New Jersey, investigation. *Pediatrics* 2001;108:1155–1161.
7. Yeargin-Allsopp M, Rice C, Karapurkar T, et al. Prevalence of autism in a US metropolitan area. *JAMA* 2003;289:49–55.
8. Folstein SE, Rosen-Sheidley B. Genetics of autism: complex aetiology for a heterogeneous disorder. *Nat Rev Genet* 2001;2: 943–955.
9. Korvatska E, Van de WJ, Anders TF, et al. Genetic and immunologic considerations in autism. *Neurobiol Dis* 2002;9: 107–125.
10. Lipkin WI, Hornig M. Microbiology and immunology of autism spectrum disorders. *Novartis Found Symp* 2003;251: 129–143.
11. Kemper TL, Bauman M. Neuropathology of infantile autism. *J Neuropathol Exp Neurol* 1998;57:645–652.
12. Bailey A, Luthert P, Dean A, et al. A clinicopathological study of autism. *Brain* 1998;121:889–905.
13. Bauman ML, Kemper TL. The neuropathology of the autism spectrum disorders: what have we learned? *Novartis Found Symp* 2003;251:112–122.
14. Licinio J, Alvarado I, Wong ML. Autoimmunity in autism. *Mol Psychiatry* 2002;7:329.
15. Gupta S, Aggarwal S, Roshanravan B, et al. Th1- and Th2-like cytokines in CD4+ and CD8+ T cells in autism. *J Neuroimmunol* 1998;85:106–109.
16. Singh VK, Warren R, Averett R, et al. Circulating autoantibodies to neuronal and glial filament proteins in autism. *Pediatr Neurol* 1997;17:88–90.
17. Vojdani A, Campbell AW, Anyanwu E, et al. Antibodies to neuron-specific antigens in children with autism: possible cross-reaction with encephalitogenic proteins from milk, *Chlamydia pneumoniae* and *Streptococcus* group A. *J Neuroimmunol* 2002; 129:168–177.

18. Jyonouchi H, Sun S, Le H. Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. *J Neuroimmunol* 2001;120:170–179.
19. Dalton P, Deacon R, Blamire A, et al. Maternal neuronal antibodies associated with autism and a language disorder. *Ann Neurol* 2003;53:533–537.
20. Comi AM, Varsou A, Heyes MP, et al. Quinolinic acid and neopterin in children with autism: an analysis of cerebrospinal fluid. *Ann Neurol* 1999;46:528–529.
21. Zimmerman AW. Commentary: immunological treatments for autism: in search of reasons for promising approaches. *J Autism Dev Disord* 2000;30:481–484.
22. Guerin P, Lyon G, Barthelemy C, et al. Neuropathological study of a case of autistic syndrome with severe mental retardation. *Dev Med Child Neurol* 1996;38:203–211.
23. Lord C, Pickles A, McLennan J, et al. Diagnosing autism: analyses of data from the Autism Diagnostic Interview. *J Autism Dev Disord* 1997;27:501–517.
24. Scahill L, Lord C. Subject selection and characterization in clinical trials in children with autism. *CNS Spectr* 2004;9:22–32.
25. Gundersen HJ, Bendtsen TF, Korbo L, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS* 1988;96:379–394.
26. Vehmas AK, Kawas CH, Stewart WF, et al. Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease. *Neurobiol Aging* 2003;24:321–331.
27. Huang RP. Cytokine protein arrays. *Methods Mol Biol* 2004;278:215–232.
28. Lin Y, Huang R, Chen LP, et al. Profiling of cytokine expression by biotin-labeled-based protein arrays. *Proteomics* 2003;3:1750–1757.
29. Storch MK, Piddlesden S, Haltia M, et al. Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. *Ann Neurol* 1998;43:465–471.
30. Aloisi F. Immune function of microglia. *Glia* 2001;36:165–179.
31. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 1996;19:312–318.
32. Bauer J, Rauschka H, Lassmann H. Inflammation in the nervous system: the human perspective. *Glia* 2001;36:235–243.
33. Medzhitov R, Janeway CA Jr. Innate immune recognition and control of adaptive immune responses. *Semin Immunol* 1998;10:351–353.
34. Aloisi F. The role of microglia and astrocytes in CNS immune surveillance and immunopathology. *Adv Exp Med Biol* 1999;468:123–133.
35. McGeer PL, Kawamata T, Walker DG, et al. Microglia in degenerative neurological disease. *Glia* 1993;7:84–92.
36. Teismann P, Tieu K, Cohen O, et al. Pathogenic role of glial cells in Parkinson's disease. *Mov Disord* 2003;18:121–129.
37. Henkel JS, Engelhardt JI, Siklos L, et al. Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue. *Ann Neurol* 2004;55:221–235.
38. Glass JD, Wesselingh SL. Microglia in HIV-associated neurological diseases. *Microsc Res Tech* 2001;54:95–105.
39. Gartner S. HIV infection and dementia. *Science* 2000;287:602–604.
40. Banati RB, Gehrmann J, Schubert P, et al. Cytotoxicity of microglia. *Glia* 1993;7:111–118.
41. Gebicke-Haerter PJ. Microglia in neurodegeneration: molecular aspects. *Microsc Res Tech* 2001;54:47–58.
42. Auld DS, Robitaille R. Glial cells and neurotransmission: an inclusive view of synaptic function. *Neuron* 2003;40:389–400.
43. Fields RD, Stevens-Graham B. New insights into neuron-glia communication. *Science* 2002;298:556–562.
44. Nedergaard M, Ransom B, Goldman SA. New roles for astrocytes: redefining the functional architecture of the brain. *Trends Neurosci* 2003;26:523–530.
45. Allan SM, Rothwell NJ. Cytokines and acute neurodegeneration. *Nat Rev Neurosci* 2001;2:734–744.
46. Nguyen MD, Julien JP, Rivest S. Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nat Rev Neurosci* 2002;3:216–227.
47. Rezaie P, Male D. Colonisation of the developing human brain and spinal cord by microglia: a review. *Microsc Res Tech* 1999;45:359–382.
48. Wierzbica-Bobrowicz T, Kosno-Kruszewska E, Gwiazda E, et al. Major histocompatibility complex class II (MHC II) expression during the development of human fetal cerebral occipital lobe, cerebellum, and hematopoietic organs. *Folia Neuropathol* 2000;38:111–118.
49. McGeer PL, McGeer EG. Innate immunity, local inflammation, and degenerative disease. *Sci Aging Knowledge Environ* 2002;2002:re3.
50. Courchesne E, Yeung-Courchesne R, Press GA, et al. Hypoplasia of cerebellar vermal lobules VI and VII in autism. *N Engl J Med* 1988;318:1349–1354.
51. Courchesne E. Neuroanatomic imaging in autism. *Pediatrics* 1991;87:781–790.
52. Carper RA, Courchesne E. Inverse correlation between frontal lobe and cerebellum sizes in children with autism. *Brain* 2000;123:836–844.
53. Kinnear KJ. Purkinje cell vulnerability and autism: a possible etiological connection. *Brain Dev* 2003;25:377–382.
54. Leonard EJ, Yoshimura T. Human monocyte chemoattractant protein-1 (MCP-1). *Immunol Today* 1990;11:97–101.
55. Mahad DJ, Ransohoff RM. The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). *Semin Immunol* 2003;15:23–32.
56. Kelder W, McArthur JC, Nance-Sproson T, et al. Beta-chemokines MCP-1 and RANTES are selectively increased in cerebrospinal fluid of patients with human immunodeficiency virus-associated dementia. *Ann Neurol* 1998;44:831–835.
57. Losy J, Zaremba J. Monocyte chemoattractant protein-1 is increased in the cerebrospinal fluid of patients with ischemic stroke. *Stroke* 2001;32:2695–2696.
58. Eugenin EA, D'Aversa TG, Lopez L, et al. MCP-1 (CCL2) protects human neurons and astrocytes from NMDA or HIV-tat-induced apoptosis. *J Neurochem* 2003;85:1299–1311.
59. Peterson KE, Errett JS, Wei T, et al. MCP-1 and CCR2 contribute to non-lymphocyte-mediated brain disease induced by Fr98 polytropic retrovirus infection in mice: role for astrocytes in retroviral neuropathogenesis. *J Virol* 2004;78:6449–6458.
60. Meng SZ, Oka A, Takashima S. Developmental expression of monocyte chemoattractant protein-1 in the human cerebellum and brainstem. *Brain Dev* 1999;21:30–35.
61. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. *Annu Rev Immunol* 1998;16:137–161.
62. Chen W, Frank ME, Jin W, et al. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* 2001;14:715–725.
63. Mundy P. Annotation: the neural basis of social impairments in autism: the role of the dorsal medial-frontal cortex and anterior cingulate system. *J Child Psychol Psychiatry* 2003;44:793–809.
64. Franciotta D, Martino G, Zardini E, et al. Serum and CSF levels of MCP-1 and IP-10 in multiple sclerosis patients with acute and stable disease and undergoing immunomodulatory therapies. *J Neuroimmunol* 2001;115:192–198.