Table 2 *Continued*

Disease	NMO-C			NMO-W			PD			ALS		
Rank	GEL	Symbol	Name	GEL	Symbol	Name	GEL	Symbol	Name	GEL	Symbol	Name
14	16.00	BTK	Bruton agammaglobulinemia tyrosine kinase	16.62	TLR2	Toll-like receptor 2	2.37	GSTT2	Glutathione S-transferase theta 2	2.13	FGF9	Fibroblast growth factor 9 (glioactivating factor)
15	15.00	CD86	CD86 molecule	15.31	IL6	Interleukin 6 (interferon, beta 2)	2.36	ALDH4A1	Aldehyde dehydrogenase 4 family, member A1	2.11	COX5B	Cytochrome c oxidase subunit Vb
16	14.73	CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	15.13	CXCL5	Chemokine (C-X-C motif) ligand 5	2.36	SOD3	superoxide dismutase 3, extracellular	2.10	HSD11B2	Hydroxysteroid (11-beta) dehydrogenase 2
17	13.22	IL6	interleukin 6 (interferon, beta 2)	14.03	BTK	Bruton agammaglobulinemia tyrosine kinase	2.35	POLR2I	Polymerase (RNA) II (DNA directed) polypeptide I, 14.5*TH* kDa	2.04	GSTM2	Glutathione S-transferase M2 (muscle)
18	12.60	FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	12.76	SOD2	Superoxide dismutase 2, mitochondrial	2.29	CTGF	Connective tissue growth factor	2.02	GSTP1	Glutathione S-transferase pi
19	11.80	NNMT	Nicotinamide N-methyltransferase	12.51	BCL2A1	BCL2-related protein A1	2.28	RBBP6	Retinoblastoma binding protein 6	2.01	COX7A2	Cytochrome c oxidase subunit VIIa polypeptide 2 (liver)
20	11.67	SLC7A7	Solute carrier family 7 (cationic amino acid transporter, y + system), member 7	12.12	E2F4	E2F transcription factor 4, p107/p130-binding	2.24	TCEB2	Transcription elongation factor B (SIII), polypeptide 2 (18*TH* kDa, elongin B)	2.01	ABCC8	ATP-binding cassette, subfamily C (CFTR/MRP), member 8
Total of up-regulated genes (n)		225			234			31			20	
Total of down-regulated genes (n)		173			158			71			64	

Gene expression profile was analyzed by using a cDNA microarray of 1258 genes. Total RNA isolated from the frontal lobe brain tissues of PD, ALS, the cortex-enriched sample of NMO (NMO-C), and the white matter-enriched sample of NMO (NMO-W) was labeled with Cy5, while pooled human frontal lobe total RNA (Clontech) labeled with Cy3 was utilized as the universal reference. The gene expression level (GEL), representing fluorescence intensity (FI) (Cy5) of the sample/FI (Cy3) of the universal reference, equal to or greater than 2.0 or smaller than 0.5 was considered as significant up-regulation or down-regulation. Top 20 up-regulated genes are listed with GEL, gene symbol, and gene name.

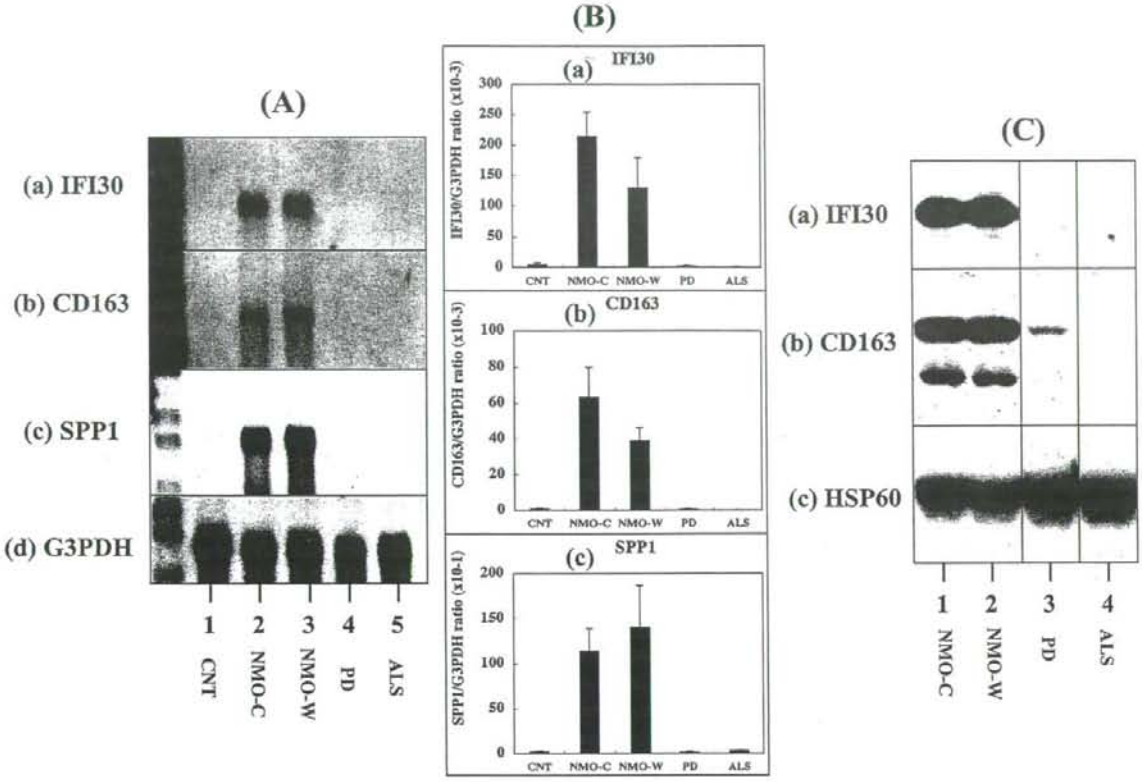

Fig. 2 Validation of microarray data. The levels of expression of IFI30, CD163 or secreted phosphoprotein 1 (SPP1) were studied in the frozen tissues of the neuromyelitis optica (NMO) cortex-enriched sample (NMO-C), the white matter-enriched sample (NMO-W), and the whole brain of Parkinson disease (PD) and ALS. (A) Northern blot analysis. The panels (a–d) represent (a) IFI30 (1.0 kb), (b) CD163 (4.0 kb), (c) SPP1 (1.6 kb), and (d) glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (1.3 kb) as an internal control. Three microgram of RNA was loaded on each lane. The lanes (1–5) indicate (1) pooled human frontal lobe total RNA as a control, (2) NMO-C, (3) NMO-W, (4) PD, and (5) ALS. (B) Real-time RT-PCR analysis. The panels (a–c) represent (a) IFI30, (b) CD163, and (c) SPP1. The levels of expression of target genes were quantified by standardizing them against those of G3PDH. (C) Western blot analysis. The panels (a–c) represent (a) IFI30 (29-kDa), (b) CD163 (70-kDa, 50-kDa), and (c) HSP60 (60-kDa) as an internal control. Sixty microgram of protein was loaded on each lane. The lanes (1–4) indicate (1) NMO-C, (2) NMO-W, (3) PD and (4) ALS.

and by interferon-regulatory factor (IRF) at the third rank where the score was 14.3 and the score (P) = 5.071E-005. These results suggest that a battery of transcriptional factors essential for immune regulation might control the expression of up-regulated genes in NMO brains, and thereby constitute the highly complex molecular network.

Up-regulated expression of IFI30 and CD163 on macrophages and microglia in NMO lesions

Finally, we studied pathologically the expression pattern of IFI30 and CD163 in NMO lesions by immunohistochemistry. Multifocal, patchy and diffuse lesions characterized by inflammatory demyelination, axonal loss, necrosis, cavity formation, and prominent thickening of vascular walls were found in both cerebral hemispheres, midbrain, pons, medulla oblongata, and the bilateral optic nerves and tracts. The distribution of inflammatory lesions was so extensive that they mostly involved both the cerebral cortex and the white matter. In active demyelinating lesions with a complete loss of neurofilament-positive axons, inflammatory infiltrates were composed of numerous $CD68^+$ macrophages, and not so many $CD3^+$ T cells, eosinophils and granulocytes (Fig. 4, panels a, c–e; Fig. 5, panels, a, c, f). Importantly, the number of $GFAP^+$ astrocytes was markedly reduced in the center of active demyelinating lesions, along with decreased immunoreactivities for both AQP4 and AQP1 (Fig. 4, panels b, f; Fig. 5, panel b; not shown for AQP1). In contrast, virtually all of $CD68^+$ macrophages and microglia expressed intense immunore-

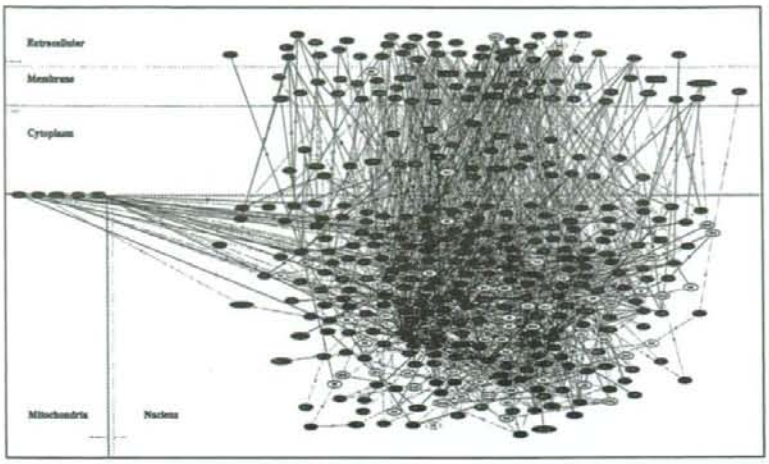

Fig. 3 Molecular network of up-regulated genes in neuromyelitis optica (NMO) brains. The list of Gene ID of 234 genes up-regulated in white matter-enriched sample (NMO-W) was imported into KeyMolnet, a bioinformatics tool for analyzing molecular interaction on the curated knowledge database. KeyMolnet extracted 413 molecules directly linked to 234 genes. The common upstream search of 413 molecules generated a complex network composed of 418 fundamental nodes and 1326 molecular relations. It is shown with respect to subcellular location of the molecules. The statistical evaluation of the extracted network showed the significant relationship with transcriptional regulation by the nuclear factor-kappaB (NF-κB), B-lymphocyte-induced maturation protein-1 (Blimp-1), and interferon-regulatory factor (IRF), as described in the text. Red nodes represent starting point molecules, whereas blue nodes represent common upstream molecules. Purple nodes express characteristics of both starting point and common upstream molecules. White nodes exhibit additional molecules extracted automatically from KeyMolnet core and secondary contents, and incorporated in the network to establish molecular connections. The direction of molecular relation is indicated by dash line with arrow (transcriptional activation) or dash line with arrow and stop (transcriptional repression). Thick lines indicate the core contents, while thin lines indicate the secondary contents of KeyMolnet.

activities for IFI30 and CD163 in these lesions (Fig. 4, panels g, h; Fig. 5, panels d, e). In NMO lesions, we hardly found the cells expressing CD20, a human B-lymphocyte surface molecule widely expressed during B-cell ontogeny from early pre-B-cell developmental stages until final differentiation into plasma cells (data not shown).

In PD, ALS, and neurologically normal brains, IFI30 was expressed in a small population of perivascular macrophages and microglia, and CD163 was chiefly located in perivascular macrophages (Fig. 5, panels g–j). In contrast, both IFI30 and CD163 expression was greatly enhanced on numerous $CD68^+$ macrophages and microglia and occasionally identified in $GFAP^+$ reactive astrocytes in active demyelinating lesions of MS, which are associated with an infiltrate of $CD3^+$ T cells and a loss of neurofilament-positive axons, accompanied by increased immunoreactivities for both AQP4 and AQP1 (Fig. 5, panels k, l; Fig. 6, panels a–h). These observations indicate that up-regulated expression of IFI30 and CD163 on both macrophages and microglia is a pathologically common event shared between active lesions of NMO and MS.

DISCUSSION

To identify molecular events responsible for development of NMO brain lesions, we studied the gene expression profiles of frozen brains of a patient with NMO by using DNA microarray. We identified more than 200 genes up-regulated in NMO brains. The top 20 genes were composed of the molecules closely associated with immune regulation. Importantly, among them, SPP1 (osteopontin), CD163, FCER1G, HLA-DRA, CD86, IL6 and SOD2 are a group of the genes up-regulated promptly in the human peripheral blood mononuclear cells in response to IFNβ in culture.[20] A previous study showed that the expression of the genes encoding IgG Fc receptor I and high affinity IgE receptor β chain is elevated in chronic MS plaques.[15] It is worthy to note that the gene encoding hypoxia-inducible factor 1, alpha subunit (HIF1A), a key regulator for hypoxia-induced gene regulation, was identified as a 6-fold up-regulated gene in NMO lesions (data not shown), suggesting an active involvement of hypoxic and ischemic insults in development of NMO lesions.[17,26] In the present study, the top three up-regulated genes include IFI30, CD163 and SPP1. Pathologically, $CD68^+$ macrophages and microglia expressed intense immunoreactivities for IFI30 and CD163 in NMO lesions, which are characterized by inflammatory demyelination, axonal loss, necrosis, cavity formation, and vascular fibrosis. Consistent with previous studies,[4-6] we found a profound decrease in immunoreactivities for AQP4, AQP1, and GFAP in the center of inflammatory demyelinating lesions of NMO, by contrast to enhancement of those in MS.[4-6,24] KeyMolnet, a bioinformatics tool for analyzing molecular interaction on the curated knowledge database,[22] suggested that the molecular network of up-regulated genes in NMO brains involves transcriptional regulation by NF-κB and Blimp-1. These results suggest that fulminant activation of the macrophage-mediated proinflammatory immune mechanism plays an important role in development of NMO brain lesions.

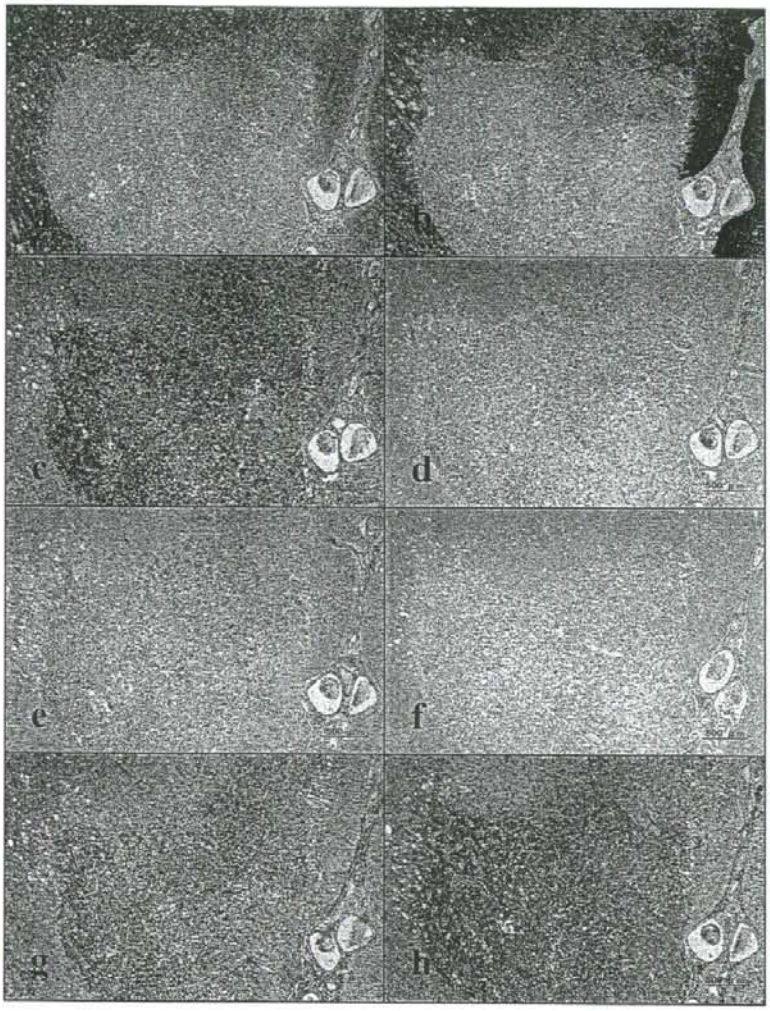

Fig. 4 Up-regulated expression of IFI30 and CD163 in neuromyelitis optica (NMO) lesions. The serial sections prepared from the frontal lobe brain tissues of NMO were immunolabeled with antibodies against (a) MBP, (b) GFAP, (c) CD68, (d) CD3, (e) neurofilament (NF), (f) aquaporin-4 (AQP4), (g) IFI30, and (h) CD163. An active demyelinating lesion is located in the center in panels a–h.

Previously, SPP1 (osteopontin) has been identified as one of the most abundant transcripts by large-scale sequencing of cDNA libraries prepared from MS plaques.[27] Subsequently, microarray analysis of spinal cords of rats with experimental autoimmune encephalomyelitis (EAE) verified increased SPP1 transcripts.[27] The clinical severity of EAE is attenuated in SPP1-deficient mice.[27] The expression of osteopontin is enhanced in astrocytes in active demyelinating lesions of MS,[6] and the plasma osteopontin levels are elevated in active relapsing-remitting MS.[28] Osteopontin promotes the survival of activated T cells by inhibiting the transcription factor Foxo3a, by activating NF-kB, and by altering expression of proapoptotic regulators Bim, Bak and Bax.[29] All of these observations suggest that osteopontin acts as a proinflammatory Th1 cytokine. Although SPP1 might play a key role in the pathogenesis of NMO as well as of MS, in the present study, we have focused on IFI30 and CD163, both of which are heretofore unreported markers in MS lesions by previous DNA microarray studies.[15-17]

IFI30, also designated a gamma interferon-inducible lysosomal thiol reducase (GILT), is expressed constitutively in antigen-presenting cells, and further induced by exposure to IFNγ, IL1β and TNF in other cell types via a Stat1-dependent pathway or a NF-κB signaling pathway.[30,31] This enzyme that catalyzes reduction of protein inter- and intrachain disulfide bonds plays a pivotal role in the first step of MHC class II-restricted antigen processing of the proteins containing disulfide bonds.[32] T cells also express GILT, which serves as an inhibitor of T cell receptor engagement-mediated activation.[33]

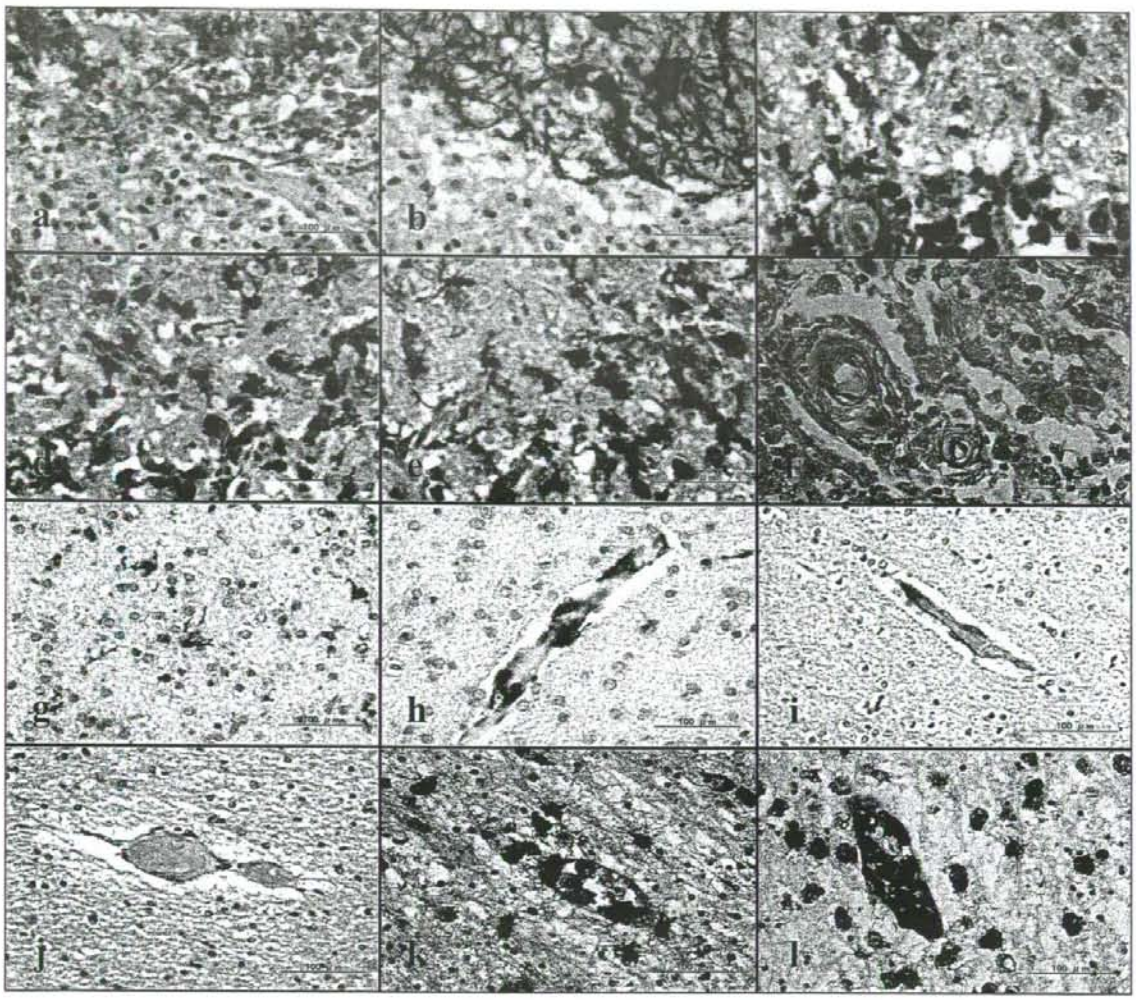

Fig. 5 IFI30 and CD163 expression in neuromyelitis optica (NMO), MS, Parkinson disease (PD), ALS, and neurologically normal brains. The frontal or parietal lobe brain tissues of (a–f) active demyelinating lesisons of NMO, (g) PD, (h) ALS, (i) neurologically normal case (NNC)#3, (j) NNC#2, and (k and l) MS#3 were immunolabeled with antibodies against (a) MBP, (b) GFAP, (c) CD68, (d, g, i, k) IFI30, (e, h, j, l) CD163, and (f) HE.

CD163 is a glycoprotein that belongs to the scavenger receptor cysteine-rich (SRCR) family group B. CD163 is expressed exclusively on subpopulations of monocytes and macrophages, and acts as a cell-surface scavenger receptor capable of internalizing the haptoglobin-hemoglobin complex for clearance of the potent oxidant hemoglobin from circulation.[34] Proinflammatory cytokines such as IFNγ and TNFα reduce CD163 expression, while anti-inflammatory mediators such as glucocorticoids and IL-10 up-regulate the expression of CD163 on moncytes/macropages.[35] Membrane-bound CD163 is actively shed from the cell surface via a metalloprotease-dependent mechanism.[36] Consequently, soluble CD163 inhibits T cell proliferation, suggesting that it plays a key role in anti-inflammatory immune responses.[34] A previous study showed that CD163 expression is enhanced on the majority of perivascular macrophages and some microglia in MS lesions, being consistent with our observations on MS lesions.[37] The levels of the plasma sCD163 are elevated, while those of the membrane CD163 are reduced in MS patients.[36] CD163 is expressed in both macrophages and microglia in HIV encephalitis lesions.[38]

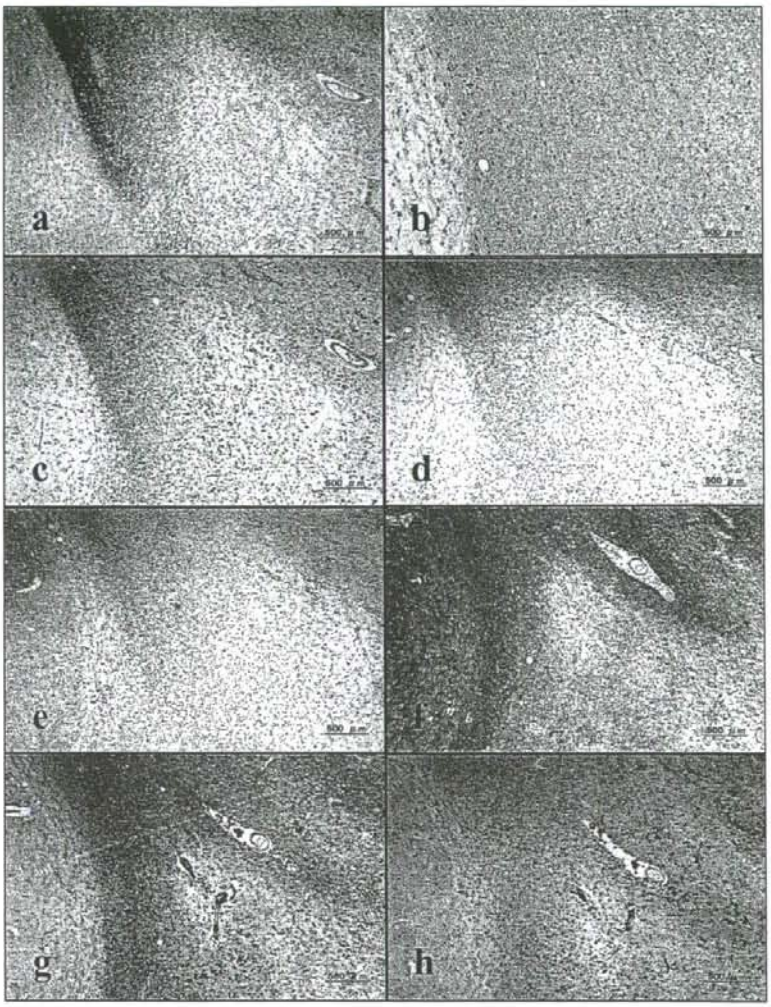

Fig. 6 Up-regulated expression of IFI30 and CD163 in MS lesions. The serial sections prepared from the parietal lobe brain tissues of MS#3 were immunolabeled with antibodies against (a) MBP, (b) GFAP, (c) CD68, (d) CD3, (e) neurofilament (NF), (f) AQP4, (g) IFI30 and (h) CD163. An active demyelinating lesion is located in the center and the right half in panels a-h.

We have attempted to identify the most relevant molecular network associated with up-regulated genes in NMO lesions by using KeyMolnet.[22] It stores the highly reliable content database of human proteins, small molecules, molecular relations, diseases, and drugs, carefully curated by experts from the literature and public databases. This software makes it possible to effectively extract the most relevant molecular interaction from large quantities of gene expression data.[23] Thus, for the first time we identified a central involvement of the complex transcriptional regulation by NF-κB, Blimp-1, and IRF from the microarray data of NMO. Our results indicate that the combination of DNA microarray and molecular network analysis is more effective to establish a biologically relevant logical working model than the conventional microarray data analysis alone.[23]

NF-κB is a central regulator of innate and adaptive immune responses, cell proliferation, and apoptosis.[39] The NF-κB family consists of five members, such as NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel. NF-κB p50 and NF-κB p52 are synthesized as large precursors p105 and p100, which are post-translationally cleaved into the DNA-binding subunits termed p50 and p52. NF-κB, commonly composed of a heterodimer of p50 and p65, exists in an inactive state in unstimulated cells, being sequestrated in the cytoplasm via non-covalent interaction with the inhibitor of NF-κB (IκB) proteins. A wide variety of extracellular stimuli, including cytokines, viral and bacterial products, and stress-inducing agents, activate specific IκB kinases (IKK) that phosphorylate N-terminal serine residues on IκB proteins. Then, phosphorylated IκBs are ubiquitinated and processed for proteasome-mediated

degradation, thereby induce nuclear translocation of an activated form of NF-κB that regulates the expression of a wide variety of proinflammatory target genes by binding to the consensus promoter sequence.[40] Interestingly, RelA, c-Rel, and p50 subunits of NF-κB are overexpressed in macrophages in active demyelinating lesions of MS,[41] while RelA is activated in oligodendrocytes survivng in the edge of demyelinating lesions of MS.[42] A recent study showed that the CNS-restricted inactivation of NF-κB ameliorates EAE owing to a defect in induction of proinflammatory genes in astrocytes.[43]

Blimp-1 is originally identified as a master regulator of the terminal differentiation of B cells into antibody-secreting plasma cells.[44] The molecular network of up-regulated genes in NMO lesions on KeyMolnet indicated an active involvement of Blimp-1 in the pathogenesis of NMO. It is unexpected but potentially important, because recent studies suggest that an autoantibody directed to AQP4, produced by plasma cells outside the CNS, triggers the activation of complements, vasculocentric inflammatory demyelination, and necrosis found in NMO lesions.[45,46] Furthermore, Blimp-1 induces the terminal differentiation of macrophages.[47] The expression of Blimp-1, also identified in effector and memory T cells, controls their homeostatic expansion.[48] IL-2 induces Blimp-1 expression in $CD4^+$ T cells, which suppresses transcription of IL-2, providing a negative feedback loop, possibly acting for resolution of inflammation.[48] Thus, the markedly up-regulated genes in NMO lesions are closely associated with key molecules involving a wide range of immunoregulatory pathways.

In conclusion, the gene expression profile on DNA microarray, combined with immunohistochemical studies, indicated that severe fulminant activation of the macrophage-mediated proinflammatory immune mechanism plays a fundamental role in development of NMO brain lesions. Although the brain materials we studied are small because of their limited availability, our observations warrant further investigations that include a large number of brain tissues.

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REFERENCES

1. Wingerchuk DM, Lennon VA, Lucchinetti CF, Pittock SJ, Weinshenker BG. The spectrum of neuromyelitis optica. *Lancet Neurol* 2007; **6**: 805–815.
2. Wingerchuk DM, Lennon VA, Pittock SJ, Lucchinetti CF, Weinshenker BG. Revised diagnostic criteria for neuromyelitis optica. *Neurology* 2006; **66**: 1485–1489.
3. Lucchinetti CF, Mandler RN, McGavern D *et al.* A role for humoral mechanisms in the pathogenesis of Devic's neuromyelitis optica. *Brain* 2002; **125**: 1450–1461.
4. Roemer SF, Parisi JE, Lennon VA *et al.* Pattern-specific loss of aquaporin-4 immunoreactivity distinguishes neuromyelitis optica from multiple sclerosis. *Brain* 2007; **130**: 1194–1205.
5. Misu T, Fujihara K, Kakita A *et al.* Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. *Brain* 2007; **130**: 1224–1234.
6. Sinclair C, Kirk J, Herron B, Fitzgerald U, McQuaid S. Absence of aquaporin-4 expression in lesions of neuromyelitis optica but increased expression in multiple sclerosis lesions and normal-appearing white matter. *Acta Neuropathol* 2007; **113**: 187–194.
7. Weinschenker BG. Neuromyelitis optica is distinct from multiple sclerosis. *Arch Neurol* 2007; **64**: 899–901.
8. Poppe AY, Lapierre Y, Melancon D *et al.* Neuromyelitis optica with hypothalamic involvement. *Mult Scler* 2005; **11**: 617–621.
9. Nakamura M, Endo M, Murakami K, Konno H, Fujihara K, Itoyama Y. An autopsied case of neuromyelitis optica with a large cavitary cerebral lesion. *Mult Scler* 2005; **11**: 735–738.
10. Pittock SJ, Lennon VA, Krecke K, Wingershuk DM, Lucchinetti CF, Weinshenker BG. Brain abnormalities in neuromyelitis optica. *Arch Neurol* 2006; **63**: 390–396.
11. Cabrera-Gómez JA, Quevedo-Sotolongo L, González-Quevedo A *et al.* Brain magnetic resonance imaging findings in relapsing neuromyelitis optica. *Mult Scler* 2007; **13**: 186–192.
12. Hengstman GJD, Wesseling P, Frenken CWGM, Jongen PJH. Neuromyelitis optica with clinical and histopathological involvement of the brain. *Mult Scler* 2007; **13**: 679–682.
13. Guimarães J, Sá MJ. Devic disease with abnormal brain magnetic resonance image findings: the first Portuguese case. *Arch Neurol* 2007; **64**: 290–291.

14. Steinman L, Zamvil S. Transcriptional analysis of targets in multiple sclerosis. *Nat Rev Immunol* 2003; **3**: 483–492.
15. Lock C, Hermans G, Pedotti R *et al.* Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* 2002; **8**: 500–508.
16. Tajouri L, Mellick AS, Ashton KJ *et al.* Quantitative and qualitative changes in gene expression patterns characterize the activity of plaques in multiple sclerosis. *Mol Brain Res* 2003; **119**: 170–183.
17. Zeis T, Graumann U, Reynolds R, Schaeren-Wiemers N. Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection. *Brain* 2008; **131**: 288–303.
18. Satoh J, Nakanishi M, Koike F *et al.* Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis. *Neurobiol Dis* 2005; **18**: 537–550.
19. Satoh J, Nakanishi M, Koike F *et al.* T cell gene expression profiling identifies distinct subgroups of Japanese multiple sclerosis patients. *J Neuroimmunol* 2006; **174**: 108–118.
20. Satoh J, Nanri Y, Tabunoki H, Yamamura T. Microarray analysis identifies a set of CXCR3 and CCR2 ligand chemokines as early IFNβ-responsive genes in peripheral blood lymphocytes: an implication for IFNβ-related adverse effects in multiple sclerosis. *BMC Neurol* 2006; **6**: 18; online.
21. Takahashi T, Fujihara K, Nakashima I *et al.* Anti-aquaporin-4 antibody is involved in the pathogenesis of NMO: a study on antibody titre. *Brain* 2007; **130**: 1235–1243.
22. Sato H, Ishida S, Toda K *et al.* New approaches to mechanism analysis for drug discovery using DNA microarray data combined with KeyMolnet. *Curr Drug Discov Technol* 2005; **2**: 89–98.
23. Satoh J, Illes Z, Peterfalvi A, Tabunoki H, Rozsa C, Yamamura T. Aberrant transcriptional regulatory network in T cells of multiple sclerosis. *Neurosci Lett* 2007; **422**: 30–33.
24. Satoh J, Tabunoki H, Yamamura T, Arima K, Konno H. Human astrocytes express aquaporin-1 and aquaporin-4 *in vitro* and *in vivo*. *Neuropathology* 2007; **27**: 245–256.
25. Satoh J, Tabunoki H, Nanri Y, Arima K, Yamamura T. Human astrocytes express 14-3-3 sigma in response to oxidative and DNA-damaging stresses. *Neurosci Res* 2006; **56**: 61–72.
26. Graumann U, Reynolds R, Steck AJ, Schaeren-Wiemers N. Molecular changes in normal appearing white matter in multiple sclerosis are characteristic of neuroprotective mechanisms against hypoxic insult. *Brain Pathol* 2003; **13**: 554–573.
27. Chabas D, Baranzini SE, Mitchell D *et al.* The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science* 2001; **294**: 1731–1735.
28. Vogt MH, Lopatinskaya L, Smits M, Polman CH, Nagelkerken L. Elevated osteopontin levels in active relapsing-remitting multiple sclerosis. *Ann Neurol* 2003; **53**: 819–822.
29. Hur EM, Youssef S, Haws ME, Zhang SY, Sobel RA, Steinman L. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat Immunol* 2007; **8**: 74–83.
30. O'Donnell PW, Haque A, Klemsz MJ, Kaplan MH, Blum JS. Cutting edge: induction of the antigen-processing enzyme IFN-gamma-inducible lysosomal thiol reductase in melanoma cells Is STAT1-dependent but CIITA-independent. *J Immunol* 2004; **173**: 731–735.
31. Lackman RL, Cresswell P. Exposure of the promonocytic cell line THP-1 to Escherichia coli induces IFN-gamma-inducible lysosomal thiol reductase expression by inflammatory cytokines. *J Immunol* 2006; **177**: 4833–4840.
32. Maric M, Arunachalam B, Phan UT *et al.* Defective antigen processing in GILT-free mice. *Science* 2001; **294**: 1361–1365.
33. Barjaktarevic I, Rahman A, Radoja S *et al.* Inhibitory role of IFN-gamma-inducible lysosomal thiol reductase in T cell activation. *J Immunol* 2006; **177**: 4369–4375.
34. Moestrup SK, Møller HJ. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann Med* 2004; **36**: 347–354.
35. Buechler C, Ritter M, Orsó E, Langmann T, Klucken J, Schmitz G. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J Leukoc Biol* 2000; **67**: 97–103.
36. Fabriek BO, Møller HJ, Vloet RP *et al.* Proteolytic shedding of the macrophage scavenger receptor CD163 in multiple sclerosis. *J Neuroimmunol* 2007; **187**: 179–186.
37. Fabriek BO, Van Haastert ES, Galea I *et al.* CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. *Glia* 2005; **51**: 297–305.
38. Roberts ES, Masliah E, Fox HS. CD163 identifies a unique population of ramified microglia in HIV encephalitis (HIVE). *J Neuropathol Exp Neurol* 2004; **63**: 1255–1264.
39. Li Q, Verma IM. NF-κB regulation in the immune system. *Nat Rev Immunol* 2002; **2**: 725–734.

40. Kumar A, Takada Y, Boriek AM, Aggarwal BB. Nuclear factor-κB: its role in health and disease. *J Mol Med* 2004; **82**: 434–448.
41. Gveric D, Laltschmidt C, Cuzner ML, Newcombe J. Transcription factor NF-κB and inhibitor IκBα are localized in macrophages in active multiple sclerosis lesions. *J Neuropathol Exp Neurol* 1998; **57**: 168–178.
42. Bonetti B, Stegagno C, Cannella B, Rizzuto N, Moretto G, Raine CS. Activation of NF-κB and c-*jun* transcription factors in multiple sclerosis lesions. Implications for oligodendrocyte pathology. *Am J Pathol* 1999; **155**: 1433–1438.
43. Van Loo G, De Lorenzi R, Schmidt H *et al.* Inhibition of transcription factor NF-κB in the central nervous system ameliorates autoimmune encephalomyelitis in mice. *Nat Immunol* 2006; **7**: 954–961.
44. Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 2003; **19**: 607–620.
45. Pittock SJ, Weinshenker BG, Lucchinetti CF, Wingerchuk DM, Corboy JR, Lennon VA. Neuromyelitis optica brain lesions localized at sites of high aquaporin 4 expression. *Arch Neurol* 2006; **63**: 964–968.
46. Hinson SR, Pittock SJ, Lucchinetti CF *et al.* Pathogenic potential of IgG binding to water channel extracellular domain in neuromyelitis optica. *Neurology* 2007; **69**: 2221–2231.
47. Chang DH, Angelin-Duclos C, Calame K. BLIMP-1: trigger for differentiation of myeloid lineage. *Nat Immunol* 2000; **1**: 169–176.
48. Nutt SL, Kallies A, Belz GT. Blimp-1 connects the intrinsic and extrinsic regulation of T cell homeostasis. *J Clin Immunol* 2008; **28**: 97–106.

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Detection of anti-Nogo receptor autoantibody in the serum of multiple sclerosis and controls

Onoue H, Satoh J-I, Ogawa M, Tabunoki H, Yamamura T. Detection of anti-Nogo receptor autoantibody in the serum of multiple sclerosis and controls.
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Objectives – A myelin-associated neurite outgrowth inhibitor Nogo-A plays a key role in inhibition of axonal regeneration. Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits, although its molecular mechanism remains unknown. The aim was to study the prevalence of autoantibodies against Nogo-A and Nogo receptor (NgR) in the serum of MS. *Methods* – The antibodies were identified in the serum of 30 MS patients, 22 patients with non-MS other neurological diseases (OND), and 22 healthy control (HC) subjects by Western blot using recombinant human Nogo-A-specific segment (NAS), the shared segment of Nogo-A and -B (NAB), Nogo-66 (N66), the non-glycosylated form of NgR, the glycosylated NgR (NgR-Fc), and myelin oligodendrocyte glycoprotein (MOG). *Results* – None showed immunoglobulin G (IgG) antibodies against NAS or NAB. In contrast, 30% of MS, 23% of OND and 32% of HC subjects exhibited anti-N66 IgG, while 27% of MS, 27% of OND and 18% of HC showed anti-MOG IgG. None of HC but 33% of MS and 14% of OND showed anti-non-glycosylated NgR IgG. Furthermore, 60% of MS, 18% of OND and 14% of HC showed anti-NgR-Fc IgG. *Conclusions* – Because IgG autoantibodies against N66, NgR and MOG are often detected in the serum of MS and controls, they do not serve as an MS-specific marker.

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Introduction

Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits and progression of clinical disability (1, 2). Although the underlying molecular mechanism remains unknown, the degree of inflammatory demyelination correlates with the extent of axonal damage, suggesting an involvement of proinflammatory mediators in development of axonal degeneration in MS (3). The alternative possibility could be proposed that axonal regeneration is severely impaired in MS lesions, because an extensive accumulation of neurite outgrowth inhibitors and glial scar provides a non-permissive environment for axonal regrowth (4).

Nogo constitutes a family of myelin-associated inhibitors of axonal regeneration (5, 6). The Nogo gene encodes three distinct isoforms named A, B and C, derived by alternative splicing and promoter usage, all of which share a C-terminal 66 amino acid segment named Nogo-66 located between the two transmembrane domains. Nogo-A, the longest isoform, is the major neurite outgrowth inhibitor expressed on oligodendrocytes and myelin sheath and a subpopulation of neurons in the central nervous system (CNS) (7, 8). Nogo-A has at least two discrete regions with neuronal growth-inhibitory activities. One is located in the Nogo-A-specific region that restricts neurite outgrowth, while another is Nogo-66 that induces growth cone collapse, both of which assume different membrane topologies depending on cell types (9). Nogo-66

binds to the Nogo receptor (Nogo-66 receptor-1; NgR), a glycosylphosphatidylinositol-anchored membrane protein (10). In contrast to Nogo-A, NgR is not identified on oligodendrocytes but expressed in a subpopulation of neurons and their axons, including cerebral cortical pyramidal neurons and cerebellar Purkinje cells (10, 11). Signal transduction mediated by NgR depends on its association with a coreceptor $p75^{NTR}$ or TROY in combination with an adaptor molecule LINGO-1 (12–14). Not only Nogo-66 but also myelin-associated glycoprotein and oligodendrocyte-myelin glycoprotein bind to NgR and transduce neurite growth-inhibitory signals via $p75^{NTR}$ by activating RhoA and inhibiting Rac1 (15). *In vivo* blockade of interaction between NgR and its ligands, by neutralizing anti-Nogo-A antibodies, NgR antagonistic peptides, or soluble truncated NgR, induces extensive axonal regeneration and enhances functional recovery after injury in the CNS (16–18). Furthermore, NgR-deficient mice exhibit an enhanced axonal plasticity after ischemic stroke, accompanied by improved functional recovery (19).

Increasing evidence indicates that autoantibodies against CNS antigens play a key role in the immunopathogenesis of MS. The detection of anti-myelin oligodendrocyte glycoprotein (MOG) antibody in the patients with clinically isolated syndrome (CIS) predicts the early conversion of CIS to clinically definite MS (20). The detection of immunoglobulin G (IgG) autoantibody against aquaporin-4 discriminates the patients with neuromyelitis optica from those with the conventional MS (21). Recently, autoantibodies against the Nogo-A segment spanning amino acid residues 1–979 are identified in the serum of relapsing–remitting MS (RRMS) patients, although they show no significant correlation to disease course, duration, relapse rate and disability of the patients (22). More recently, we found that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is upregulated in reactive astrocytes at the edge of chronic demyelinating lesions of MS, suggesting that Nogo-A/NgR mediates glial–glial interaction under demyelinating environments (23). Inflammatory demyelination and axonal damage are less severe in Nogo-A-deficient mice affected with experimental autoimmune encephalomyelitis, an animal model of MS (24). These observations propose a possible scenario that the Nogo-A/NgR interaction restricts axonal regeneration in MS brain.

The present study is designed to investigate the prevalence of autoantibodies against a panel of Nogo and NgR fragments in the serum of MS and controls by Western blot analysis, and to determine the possible association of these antibodies to clinical profiles of MS.

Patients and methods

Study population

The serum samples were obtained from randomly selected 30 MS patients, 22 patients with other neurological diseases (OND) who visited the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan in 2004, and 22 healthy control (HC) subjects. Written informed consent was obtained from all the subjects. MS was diagnosed following the established criteria (25) by qualified neurologists of the hospital. The MS population was composed of 10 men and 20 women showing the mean age of 41.5 ± 12.2 years, the mean disease duration of 7.1 ± 6.3 years, and the mean Expanded Disability Status Scale (EDSS) score of 3.6 ± 2.0. The MS group included 17 patients with RRMS during remission, nine with RRMS in acute relapse, three with secondary progressive MS (SPMS) and one with primary progressive MS (PPMS). Among all MS patients, nine were under IFNβ treatment, 11 were treated with corticosteroids, and two with azathioprine at the time of blood sampling. The patients received no other immunosuppressive or immunomodulatory drugs. The OND population included 13 men and nine women with the mean age of 57.7 ± 13.8 years, composed of six patients with spinocerebellar degeneration, five with Parkinson's disease, three with myopathy, two with neuropathy, one with myasthenia gravis, Fisher syndrome, adrenoleukodystrophy, spastic paraplegia, epilepsy, or cerebral infarction. The HC population was composed of nine men and 13 women presenting with the mean age of 41.7 ± 12.4 years. After sampling, the serum samples were stored immediately at −30°C before use.

Recombinant human Nogo and NgR fragments

The human Nogo-A gene encoding the Nogo-A-specific segment (NAS; amino acids 186–1004), the segment shared between Nogo-A and Nogo-B (NAB; amino acids 1–185), and the Nogo-66 segment (N66; amino acids 1053–1118), the human NgR gene encoding the full-length NgR following a cleavage of the N-terminal signal peptide (amino acids 27–473), and the human MOG gene encoding the full-length MOG

Table 1 Primers utilized for PCR-based cloning

Genes	Proteins (symbol, amino acid residues)	GenBank accession no.	Sense primers	Antisense primers
RTN4	Nogo-A-specific segment (NAS, 186–1044)	NM_020532	5′-gatgagacccttttttgctcttcct-3′	5′-tcatgaagttttactcagctctgctga-3′
RTN4	Nogo-A/B-shared segment (NAB, 1–185)	NM_020532	5′-atggaagaactggaccagtctcct-3′	5′-tcacactgagcccgaggagcccct-3′
RTN4	Nogo-66 segment (N66, 1053–1118)	NM_020532	5′-agctttaggatatacaagggtgtg-3′	5′-tcaagaatcaactaaatcatcaactaa-3′
RTN4R	Nogo receptor without an N-terminal signal peptide (NgR, 27–473)	NM_023004	5′-tgcccaggtgcctgcgtatgctacaat-3′	5′-tcagcagggcccaagcactgtccacag-3′
MOG	Myelin oligodendrocyte glycoprotein without an N-terminal signal peptide (MOG, 30–247)	NM_206809	5′-gggcagttcagagtgataggaccaaga-3′	5′-tcagaagggatttcgtagctcttcaagg-3′

The PCR product was cloned into a prokaryotic expression vector pTrcHis-TOPO to produce a fusion protein with an N-terminal Xpress tag in *E. coli*.

following a cleavage of the N-terminal signal peptide (amino acids 30–247) were amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) from cDNA of NTera2-derived human neurons (26) or human brain using a panel of sense and antisense primer sets listed in Table 1. The PCR product was cloned into a prokaryotic expression vector pTrcHis-TOPO (Invitrogen, Carlsbad, CA, USA). After transformation of the vector into *Escherichia coli*, the expression of recombinant proteins was induced by exposing the culture of *E. coli* to isopropyl β-thiogalactoside. All recombinant proteins having an N-terminal Xpress tag for detection were purified from the *E. coli* lysate by isolating the specific bands immunoreactive with monoclonal anti-Xpress antibody (Invitrogen) separated on an 8%, 12% or 15% SDS-PAGE gel. The recombinant proteins were eluted from the gels, precipitated by cold acetone, and dissolved in distilled water containing 0.1% SDS. The protein concentration was determined by a Bradford assay kit (BioRad, Hercules, CA, USA). For control, the pTrcHis-TOPO vector containing a *lacZ* gene fragment (Invitrogen) was transformed into *E. coli* in parallel. Recombinant human NgR (Met1-Ser447)-IgG Fc (Pro100-Lys330) fusion protein (95 kDa), a glycosylated form expressed in a mouse myeloma cell line, was obtained from R&D Systems (Minneapolis, MN, USA).

Western blot analysis

Six different recombinant proteins, including NAS, NgR, LacZ, NAB, MOG and N66, were separated on a single 12% SDS-PAGE gel. After gel electrophoresis, they were transferred onto a nitrocellulose membrane. This membrane is designated the miniarray. Then, the array was incubated at room temperature (RT) overnight with the serum diluted at a concentration of 1:1000 in phosphate-buffered saline containing 5% skim milk. It was followed by incubation at RT for 60 min with horseradish peroxidase (HRP)-conjugated anti-human IgG (160 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with an HRP-conjugated antibody specific for human IgM $Fc_{5\mu}$ fragment (8 ng/ml; Jackson ImmunoResearch, West Grove, PA, USA). To identify autoantibodies against NgR-Fc, the serum was diluted at a concentration of 1:15,000, and either an HRP-conjugated antibody specific for human IgG $F(ab')_2$ fragment (20 ng/ml; Jackson ImmunoResearch) or a HRP-conjugated antibody against human IgM $Fc_{5\mu}$ fragment (120 ng/ml; Jackson ImmunoResearch) was utilized as a secondary antibody. The specific reaction was visualized at 15 min after incubating the array for 5 min with a chemiluminescent substrate (Pierce, Rockford, IL, USA). The optimal concentration of the serum, secondary antibodies, and incubation time prior to development was determined to obtain the maximum signal/background intensity by preliminary experiments that include varying combinations of serially diluted sera and secondary antibodies, and incubation periods. The signal intensity was graded as (–) none, (+) weak, (++) strong, and (+++) extremely strong.

Statistical analysis

The statistical difference in clinical profiles between the serum autoantibody-positive and negative groups of MS patients was evaluated by Mann–Whitney *U*-test. A P value of <0.05 was considered statistically significant.

Results

Miniarray of Nogo and NgR fragments

A panel of recombinant proteins, such as NAS, NgR, LacZ, NAB, MOG and N66, all of which have an N-terminal Xpress tag, were separated on a single 12% SDS-PAGE gel and transblotted onto

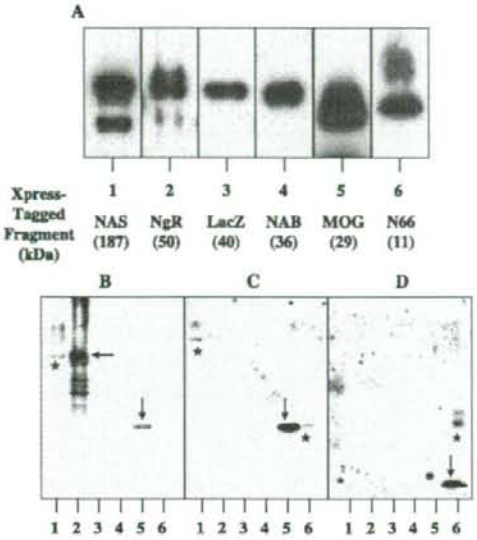

Figure 1. Miniarray of Nogo and NgR fragments. A panel of six different recombinant proteins, all of which have an N-terminal Xpress tag, were purified from *E. coli* transformed by PCR-based cloning strategy using primer sets listed in Table 1. The recombinant proteins, whose amounts range from 60 ng to 11.6 μg per lane, were separated on a single 12% SDS-PAGE gel and transblotted onto the identical nitrocellulose membrane, designated the miniarray. (A) The miniarray immunolabeled with anti-Xpress antibody. The lanes (1–6) represent (1) NAS (187 kDa), (2) NgR (50 kDa), (3) LacZ (40 kDa), (4) NAB (36 kDa), (5) MOG (29 kDa), and (6) N66 (11 kDa). The panels of the miniarray (B–D) represent the serum of (B) SPMS exhibiting both anti-non-glycosylated NgR IgG (lane 2, extremely strong; + + +) and anti-MOG IgG (lane 5, weak; +), (C) RRMS in remission exhibiting anti-MOG IgG (lane 5, extremely strong; + + +), and (D) HC subject presenting with anti-N66 IgG (lane 6, extremely strong; + + +). The specific bands are indicated by arrows, while non-specific reactions, possibly derived from the cross-reaction with contaminating *E. coli* proteins, are indicated by asterisks.

the identical nitrocellulose membrane, designated the miniarray. Then, the array was immunolabeled with anti-Xpress antibody (Fig. 1A). To verify whether the same amount of recombinant proteins was blotted on each array, the signal intensity of Xpress-immunoreactive bands was normalized between individual arrays onto which different serum samples were exposed. The profile of serum IgG autoantibodies varied from case to case, eliminating the possible cross-reactivity of secondary antibodies with recombinant proteins (Fig. 1B–D). This is further confirmed by the observation that negative controls including all the steps except for incubation with the serum detected no immunoreactive bands (data not shown).

Detection of autoantibodies against MOG, N66, and NgR in the serum of MS and controls

None of the subjects examined showed IgG autoantibodies against NAS or NAB (Table 2). In contrast, 30% of MS patients, 23% of OND patients, and 32% of HC subjects exhibited anti-N66 IgG expressing a specific band with varying intensities (Table 2 and Fig. 2A, lanes 1–7). Furthermore, 27% of MS patients, 27% of OND patients, and 18% of HC subjects showed anti-MOG IgG (Table 2 and Fig. 2B, lanes 1–7). IgG antibody against non-glycosylated NgR was identified in 33% of MS patients and 14% of OND patients, whereas it was undetected in any HC subject (Table 2 and Fig. 3A, lanes 1–7). In several cases, minor bands with lower molecular weights were detected in the blots, which possibly represent immunoreactivity against a small amount of degraded NgR proteins (Fig. 3A). No subjects showed IgG antibody against LacZ fragment tagged with Xpress (Table 2), excluding the possibility that the serum IgG autoantibodies against N66, MOG, and NgR were directed to a potentially immunogenic epitope of the tag. In contrast to the frequent detection of anti-NgR IgG, only two OND patients showed IgM antibody against non-glycosylated NgR (Table 2 and Fig. 3B, lanes 1, 2).

A larger proportion (60%) of MS patients, 18% of OND patients, and 14% of HC subjects showed IgG antibody against glycosylated NgR-Fc (Table 2 and Fig. 3C, lanes 1–7). All progressive MS patients ($n = 4$, Table 2) expressed anti-NgR-Fc IgG. In contrast, no subjects showed anti-NgR-Fc IgM (Table 2). Among RRMS patients positive for anti-NgR-Fc IgG, the patients in remission (12/17) greatly outnumbered those in acute relapse (2/9), while among RRMS patients with anti-MOG IgG, the patients in acute relapse (4/9) outnumbered those during remission (3/17) (Table 2), raising the possibility that anti-NgR-Fc IgG is overexpressed in RRMS patients in remission. However, no significant differences were found between MS patients positive or negative for anti-NgR-Fc IgG in age, sex, disease duration, EDSS score, the number of recent relapses, the number of patients with spinal cord involvement, and the treatment given at blood sampling (Table 3).

Discussion

The present study using recombinant human proteins as target antigens on Western blot showed that a substantial proportion of MS patients, OND patients, and HC subjects exhibited the serum IgG

Table 2 Detection of autoantibodies against Nogo and NgR fragments in the serum of MS and controls

		MS subtypes					
	MS ($n = 30$)	RRMS (total) ($n = 26$)	RRMS (in remission) ($n = 17$)	RRMS (in relapse) ($n = 9$)	SP/PPMS ($n = 4$)	OND ($n = 22$)	HC ($n = 22$)
NAS IgG	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
NAB IgG	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
N66 IgG	9 (30)	7 (27)	4 (24)	3 (33)	2 (50)	5 (23)	7 (32)
NgR IgG	10 (33)	8 (31)	7 (41)	1 (11)	2 (50)	3 (14)	0 (0)
NgR IgM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (9)	0 (0)
NgR-Fc IgG	18 (60)	14 (54)	12 (71)	2 (22)	4 (100)	4 (18)	3 (14)
NgR-Fc IgM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
MOG IgG	8 (27)	7 (27)	3 (18)	4 (44)	1 (25)	6 (27)	4 (18)
LacZ IgG	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The number of subjects with immunoreactive bands is shown with the percentage in parenthesis. RRMS, relapsing–remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; OND, non-MS other neurological diseases; HC, healthy controls; NAS, Nogo-A-specific fragment; NAB, Nogo-A and Nogo-B-shared fragment; N66, Nogo-66 fragment; NgR, non-glycosylated Nogo receptor fragment; NgR-Fc, glycosylated Nogo receptor-IgG Fc fusion protein; MOG, myelin-oligodendrocyte glycoprotein.

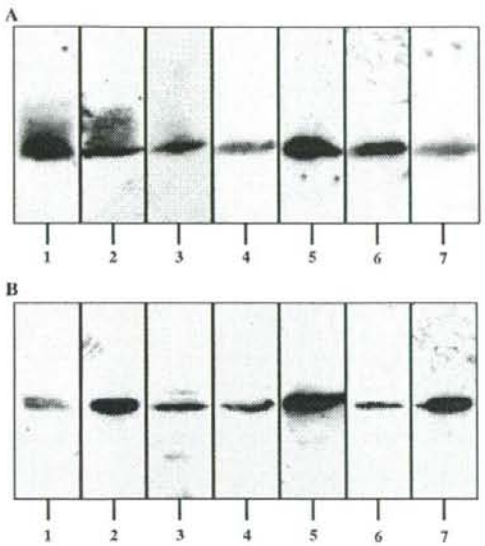

Figure 2. Detection of IgG autoantibodies against N66 and MOG in the serum of MS and controls. The miniarray was incubated with the serum of MS patients, the patients with non-MS neurological diseases (OND) or healthy control (HC) subjects. It was followed by incubation with HRP-conjugated anti-human IgG. (Panel A) Anti-N66 IgG. The lanes (1–7) represent the serum of (1) RRMS in acute relapse, (2) SPMS, (3) Parkinson's disease, (4) adrenoleukodystrophy, (5) HC, (6) HC, and (7) HC. (Panel B) Anti-MOG IgG. The lanes (1–7) represent the serum of (1) RRMS in remission, (2) RRMS in remission, (3) Parkinson's disease, (4) spinocerebellar degeneration, (5) spinocerebellar degeneration, (6) HC, and (7) HC.

autoantibodies against N66, MOG, and NgR. These results agree with a previous study reporting the substantial prevalence of the serum anti-MOG IgG in MS (20). In contrast, no subjects showed IgG antibodies against NAS or NAB, excluding the possibility that the detection of anti-N66, anti-MOG, and anti-NgR antibodies in our study is simply attributable to non-specific binding. However, our observations do not support a recent study showing that both IgG and IgM autoantibodies against the Nogo-A 1–979 fragment, corresponding to a segment overlapping NAS and NAB in the present study, is often found in the serum of RRMS and OND patients (22). This discrepancy might be derived from following differences between the previous study and our own. First, the study populations were different between both of them. The previous study enrolled 55 patients with RRMS and 38 patients with chronic progressive (CP) MS (22). Ten of them were under treatment with intravenous immunoglobulins (IVIG), which potentially affects the autoantibody profile. In contrast, our study included 26 patients with RRMS, three with SPMS and one with PPMS. None of them received IVIG treatment. Second, the previous study focused on the ELISA-based immunoassay (22), which has more chance of detecting non-specific binding as positive. In contrast, we utilized Western blot throughout the study. Third, the previous study did not include in their assay irrelevant recombinant proteins having the same epitope tag that target fusion proteins have (22), whereas our miniarray included Xpress-tagged LacZ as a negative control.

The present study for the first time showed that 60% of MS patients, 18% of OND patients, and 14% of HC subjects have IgG antibody against glycosylated NgR-Fc. The DNAQLR motif located in the third leucine-rich repeat domain of NgR is the principal epitope recognized by a monoclonal anti-NgR antibody with a capacity to block binding of all NgR ligands (27). The

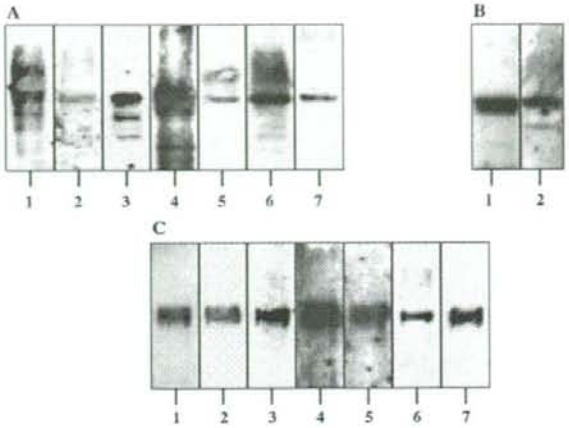

Figure 3. Detection of IgG and IgM autoantibodies against NgR and NgR-Fc in the serum of MS and controls. The miniarray and the NgR-Fc blot were incubated with the serum of MS patients, OND patients or HC subjects. It was followed by incubation with HRP-conjugated anti-human IgG (panel A), IgM $Fc_{5\mu}$ (panel B), or IgG $F(ab')_2$ (panel C). (Panel A) IgG antibody against non-glycosylated NgR. The lanes (1–7) represent the serum of (1) RRMS in remission, (2) RRMS in remission, (3) RRMS in remission, (4) SPMS, (5) Parkinson's disease, (6) spinocerebellar degeneration, and (7) Charcot–Marie–Tooth disease CMT1A. (Panel B) IgM antibody against non-glycosylated NgR. The lanes (1,2) represent the serum of (1) spastic paraplegia of unknown etiology and (2) Fisher syndrome. (Panel C) IgG antibody against glycosylated NgR-Fc. The lanes (1–7) represent the serum of (1) RRMS in remission, (2) RRMS in remission, (3) RRMS in remission, (4) spastic paraplegia of unknown etiology, (5) mitochondrial encephalomyopathy, (6) HC, and (7) HC.

Table 3 Clinical characteristics of MS patients positive or negative for IgG antibodies against NgR-Fc in the serum

Clinical characteristics	NgR-Fc IgG-positive MS ($n = 18$)	NgR-Fc IgG-negative MS ($n = 12$)
Age (years)[a]	42.2 ± 10.6	40.4 ± 14.8
Male vs female (number)	8 vs 10	2 vs 10
Disease duration (years)[a]	6.6 ± 5.7	7.9 ± 7.2
EDSS score[a]	3.7 ± 2.2	3.5 ± 1.8
Relapses during the recent one year (number)[a]	0.7 ± 0.9	1.0 ± 1.0
Patients with spinal cord involvement (number and %)	8 (44%)	5 (42%)
Patients in remission, in relapse, and in progression (number)	12 vs 2 vs 4	5 vs 7 vs 0
Patients receiving IFNβ, corticosteroids, and azathioprine at blood sampling (number)	5 vs 7 vs 2	4 vs 4 vs 0

[a]No significant differences are found by Mann–Whitney's *U*-test between the two groups of MS in age, disease duration, EDSS score, and relapses during the recent one year.
NgR-Fc, glycosylated Nogo receptor-IgG Fc fusion protein; EDSS, Expanded Disability Status Scale.

Nogo-66 region contains several immunogenic epitopes for mouse T and B cells (28). Although immunogenic epitopes of NgR and Nogo-A in the human immune system remain to be clarified, our observations suggest that both NgR and Nogo-66 are more immunogenic than NAS and NAB for activation of human B cells. Among RRMS patients with anti-NgR-Fc IgG, the patients in remission ($n = 12$) outnumbered those in acute relapse ($n = 2$). Therefore, the possibility exists that this antibody might play a role in the maintenance of remission or the protection against relapse in RRMS, by blocking the interaction between Nogo-66 on oligodendrocytes/myelin and NgR on neurons/axons. This hypothesis should be evaluated by future studies including the larger cohort of MS patients and controls.

Stress-inducing stimuli greatly affect the levels of Nogo-A expression in distinct cell types. Global ischemia enhances Nogo-A expression on the myelin sheath in the adult rat brain (29). Nogo-A expression is enhanced around the lesion site, whereas NgR is maintained at constant levels in the adult mouse and rat spinal cord following injury (7, 11, 30, 31). Kainate-induced seizure elevates Nogo-A mRNA levels in the adult rat hippocampal neurons (32). Nogo-A is induced in hippocampal neurons of the patients with

temporal lobe epilepsy (33). Unexpectedly, we identified the serum IgG autoantibodies against N66, MOG, and NgR-Fc not only in MS but also in non-MS and HC subjects. Our observations indicate that both Nogo-66 and NgR in addition to MOG serve as an autoantigen in the human immune system. Supporting this, a recent study identified anti-MOG IgG in the serum of both RRMS patients and HC subjects at the similar frequency (34). Increasing evidence indicates that Nogo-A, Nogo-B, Nogo-C and NgR are widely distributed in the cells and tissues outside the CNS (35–37). Nogo-A expression is elevated in postmortem and biopsied muscles of amyotrophic lateral sclerosis patients (38, 39). These observations suggest that the Nogo-66/NgR interaction plays an as yet undefined extraneural role, associated with an increased probability of antibody production under pathological conditions, when they are exposed and recognized by the human immune system.

In conclusion, IgG antibodies against N66, NgR, and MOG are frequently detected in the serum of both MS patients and controls. Although the study population is small and heterogeneous, we could definitely conclude that these autoantibodies do not serve as a disease-specific marker for MS.

Acknowledgements

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References

1. Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. Brain 1997;**120**:393–99.
2. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mörk S, Bö L. Axonal transection in the lesions of multiple sclerosis. N Engl J Med 1998;**338**:278–85.
3. Lassmann H. Axonal injury in multiple sclerosis. J Neurol Neurosurg Psychiatry 2003;**74**:695–97.
4. Schwab ME, Bartholdi D. Degeneration and regeneration of axons in the lesioned spinal cord. Physiol Rev 1996;**76**:319–70.
5. Chen MS, Huber AB, van der Haar ME, et al. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 2000;**403**:434–39.
6. GrandPré T, Nakamura F, Vartanian T, Strittmatter SM. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 2000;**403**:439–44.
7. Josephson A, Widenfalk J, Widmer HW, Olson L, Spenger C. NOGO mRNA expression in adult and fetal human and rat nervous tissue and in weight drop injury. Exp Neurol 2001;**169**:319–28.
8. Wang X, Chun S-J, Treloar H, Vartanian T, Greer CA, Strittmatter SM. Localization of Nogo-A and Nogo-66 receptor proteins at sites of axon-myelin and synaptic contact. J Neurosci 2002;**22**:5505–15.
9. Oertle T, van der Haar ME, Bandtlow CE et al. Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. J Neurosci 2003;**23**:5393–406.
10. Fournier AE, GrandPré T, Strittmatter SM. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 2001;**409**:341–46.
11. Josephson A, Trifunovski A, Widmer HR, Widenfalk J, Olson L, Spenger C. Nogo-receptor gene activity: cellular localization and developmental regulation of mRNA in mice and humans. J Comp Neurol 2002;**453**:292–304.
12. Wang KC, Kim JA, Sivasankaran R, Segal R, He Z. p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 2002;**420**:74–8.
13. Mi S, Lee X, Shao Z, et al. LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. Nat Neurosci 2004;**7**:221–28.
14. Park JB, Yiu G, Kaneko S, Wang J, Chang J, He Z. A TNF receptor member, TROY, is a coreceptor with Nogo receptor in mediating the inhibitory activity of myelin inhibitors. Neuron 2005;**45**:345–51.
15. Niederöst B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J Neurosci 2002;**22**:10368–76.
16. Bareyre FM, Haudenschild B, Schwab ME. Long-lasting sprouting and gene expression changes induced by the monoclonal antibody IN-1 in the adult spinal cord. J Neurosci 2002;**22**:7097–110.
17. Fournier AE, Gould GC, Liu BP, Strittmatter SM. Truncated soluble Nogo receptor binds to Nogo-66 and blocks inhibition of axonal growth by myelin. J Neurosci 2002;**22**:8876–83.
18. GrandPré T, Li S, Strittmatter SM. Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 2002;**417**:547–51.
19. Lee J-K, Kim J-E, Sivula M, Strittmatter SM. Nogo receptor antagonism promotes stroke recovery by enhancing axonal plasticity. J Neurosci 2004;**24**:6209–17.
20. Berger T, Rubner P, Schautzer F et al. Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. N Engl J Med 2003;**349**:139–45.
21. Lennon VA, Kryzer TJ, Pittock SJ, Verkman AS, Hinson SR. IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. J Exp Med 2005;**202**:473–77.
22. Reindl M, Khantane S, Ehling R, et al. Serum and cerebrospinal fluid antibodies to Nogo-A in patients with multiple sclerosis and acute neurological disorders. J Neuroimmunol 2003;**145**:139–47.
23. Satoh J-I, Onoue H, Arima K, Yamamura T. Nogo-A and Nogo receptor expression in demyelinating lesions of multiple sclerosis. J Neuropathol Exp Neurol 2005;**64**:129–38.
24. Karnezis T, Mandemakers W, McQualter JL, et al. The neurite outgrowth inhibitor Nogo A is involved in autoimmune-mediated demyelination. Nat Neurosci 2004;**7**:736–44.

25. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the international panel on the diagnosis of multiple sclerosis. Ann Neurol 2001;**50**:121–27.
26. Satoh J-I, Kuroda Y. Differential gene expression between human neurons and neuronal progenitor cells in culture: an analysis of arrayed cDNA clones in NTera2 human embryonal carcinoma cell line as a model system. J Neurosci Methods 2000;**94**:155–64.
27. Li W, Walus L, Rabacchi SA, et al. A neutralizing anti-Nogo66 receptor monoclonal antibody reverses inhibition of neurite outgrowth by central nervous system myelin. J Biol Chem 2004;**279**:43780–88.
28. Fontoura P, Ho PP, DeVoss J, et al. Immunity to the extracellular domain of Nogo-A modulates experimental autoimmune encephalomyelitis. J Immunol 2004;**173**: 6981–92.
29. Zhou C, Li Y, Nanda A, Zhang JH. HBO suppresses Nogo-A, NgR, or RhoA expression in the cerebral cortex after global ischemia. Biochem Biophys Res Commun 2003;**309**:368–76.
30. Hunt D, Mason MRJ, Campbell G, Con R, Anderson PN. Nogo receptor mRNA expression in intact and regenerating CNS neurons. Mol Cell Neurosci 2002;**20**:537–52.
31. Hunt D, Con RS, Prinjha RK, Campbell G, Anderson PN. Nogo-A expression in the intact and injured nervous system. Mol Cell Neurosci 2003;**24**:1083–102.
32. Meier S, Bräuer AU, Heimrich B, Schwab ME, Nitsch R, Savaskan NE. Molecular analysis of Nogo expression in the hippocampus during development and following lesion and seizure. FASEB J 2003;**17**:1153–55.
33. Bandtlow CE, Dlaska M, Pirker S, Czech T, Baumgartner C, Sperk G. Increased expression of Nogo-A in hippocampal neurons of patients with temporal lobe epilepsy. Eur J Neurosci 2004;**20**:195–206.
34. Lampasona V, Franciotta D, Furlan R et al. Similar frequency of anti-MOG IgG and IgM in MS patients and healthy subjects. Neurology 2004;**62**:2092–94.
35. Satoh J-I, Kuroda Y. Cytokines and neurotrophic factors fail to affect Nogo-A mRNA expression in differentiated human neurones: implications for inflammation-related axonal regeneration in the central nervous system. Neuropathol Appl Neurobiol 2002;**28**:95–106.
36. Oertle T, Huber C, van der Putten H, Schwab ME. Genomic structure and functional characterisation of the promoters of human and mouse nogo/rtn4. J Mol Biol 2003;**325**:299–323.
37. Dodd DA, Niederöst B, Bloechlinger S, Dupuis L, Loeer JP, Schwab ME. Nogo-A, -B, and -C are found on the cell surface and interact together in many different cell types. J Biol Chem 2005;**280**:12494–502.
38. Dupuis L, Gonzalez de Aguilar JL, di Scala F, et al. Nogo provides a molecular marker for diagnosis of amyotrophic lateral sclerosis. Neurobiol Dis 2002;**10**:358–65.
39. Jokic N, de Aguilar J-LG, Pradat P-F, et al. Nogo expression in muscle correlates with amyotrophic lateral sclerosis severity. Ann Neurol 2005;**57**:553–56.

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Therapeutic Potential of CD1d-Restricted Invariant Natural Killer T Cell–Based Treatment for Autoimmune Diseases

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CD1d-restricted invariant natural killer T (iNKT) cells are a unique subset of T cells that recognize glycolipid antigens presented by the CD1d molecule. iNKT cells participate in various kinds of immunoregulation due to a potent ability to produce a variety of cytokines. Recent advances in studies of novel synthetic glycolipid ligands has led to new strategies to manipulate the pleiotropic functions of iNKT cells. The molecular mechanism of selective cytokine production by glycolipid ligands will be discussed. We will also focus on the possible therapeutic application of such ligands for the clinical treatment of various autoimmune diseases.

Keywords: autoimmunity, CD1d, CIA, EAE, glycolipid, NKT cell

INTRODUCTION

Autoimmune diseases have been studied for more than four decades, but the mechanisms of initiation and progression of disease have remained a mystery. Recently, however, new biological therapeutics including cytokines, anticytokine reagents, and monoclonal antibodies against surface molecules have shown some clinical efficacy in several autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA). Despite that, these drugs are not curative and are not effective for all patients. Moreover, the use of strong immunomodulatory drugs is sometimes accompanied by adverse side effects. Therefore, further effort for drug development for autoimmune diseases is required.

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Among the major immunologic topics under study, self-tolerance is one of the most fundamentally important and has been extensively studied [1–3]. T cells with high affinity for self-antigens are first eliminated in the thymus through negative selection. However, T cells with lower affinity for self-antigens or with high affinity for determinants that are not expressed in the thymus escape from thymic selection and are found in the periphery. Self-reactive T cells are controlled in the periphery by the physiologic regulatory mechanisms that prevent pathologic autoimmunity. Disruption of these tolerance mechanisms could lead to autoimmune disease. Conversely, maintenance or reestablishment of peripheral tolerance is a therapeutic strategy to restrain destructive autoimmune processes. Immunoregulation by regulatory cells has emerged as an important part of these mechanisms. Thus, it seems attractive to induce or stimulate regulatory cells to control harmful autoimmunity. Among several different regulatory cells, we will focus on CD1d-restricted invariant natural killer T (iNKT) cells that are involved in the regulation of a variety of autoimmune diseases.

iNKT CELLS

iNKT cells are a unique subset of T cells that express an invariant Tcell receptor (TCRα) chain (Vα14-Jα18 segments in mice and Vα24-Jα18 segments in humans) associated with biased β chain (Vβ8.2, Vβ7, or Vβ2 in mice and Vβ11 in human) [4,5]. They recognize glycolipid antigens in the context of CD1d, a non-polymorphic MHC class I-like molecule. Although the tissue distribution of iNKT cells varies, they are most frequent in the liver, thymus, and bone marrow and less abundant in the spleen, lymph nodes, and peripheral blood. Subsets of mouse and human iNKT cells have a similar memory phenotype and can recognize the same antigens such as α-galactosylceramide (α-GalCer) associated with CD1d molecules, which are highly conserved through mammalian evolution. Whereas human and mouse iNKT cells share many characteristics, their frequency is much lower in humans [4,5]. Consistent with the preactivation status, iNKT cells promptly release large amounts of cytokines including IL-4 and IFN-γ upon antigen stimulation and affect the functions of neighboring cell populations such as T cells, B cells, NK cells, and dendritic cells. iNKT cells are composed of two subsets; $CD4^+$ or $CD4^-CD8^-$ (double-negative; DN). $CD4^+$ and DN NKT cells appear different in terms of cytokine production in humans [4,6]. The $CD4^+$ subset of human iNKT cells produces both Th1 and Th2 cytokines upon antigen stimulation, whereas the DN subset produces Th1 cytokines and upregulates

production of perforin after exposure to cytokines. Consistent with their capacity to produce cytokines, iNKT cells have been implicated in a variety of immune responses. Rapid induction of various cytokines and lack of diversity of TCR suggest the bridging role of iNKT cells between innate and adoptive immune responses. iNKT cells appears to be involved in the early phase of immune responses such as a number of infections with microbial organisms including bacteria, viruses, and parasites. iNKT cells are also implicated in immune surveillance for neoplasia. A number of studies have revealed a role for iNKT cells in the protection of various autoimmune diseases [7–10]. In contrast, recent work has demonstrated that iNKT cells may also participate in the pathogenesis of several diseases such as allergic bronchial asthma [11–13], atherosclerosis [14,15], and arthritis [16–19]. Taken together, iNKT cells can be helpful or harmful depending on the local milieu where they accumulate.

NATURAL GLYCOLIPID LIGANDS FOR iNKT CELLS

Recently, glycosylceramides derived from *Sphingomonas* bacterial components have been identified as exogenous antigens for iNKT cells [20,21]. In addition, phosphatidylinositol tetramannoside derived from *Mycobacterium bovis* (BCG) was reported to bind to CD1d and activate a subpopulation of iNKT cells [22], suggesting a defensive role of iNKT cells against these microorganisms. It is, however, speculated that "self-antigen" probably functions as a physiologic antigen for iNKT cells to mediate immunomodulatory functions due to the self-reactivity of iNKT cells and the activated memory phenotype of iNKT cells isolated from human umbilical-cord blood [23,24] and germ-free mice [25]. While several glycolipids such as glycosylphosphatidylinositols [26] and cellular phospholipids [27] have been reported as candidates for endogenous antigens, isoglobotrihexosylceramide (iGb3) has been shown to be an important selecting ligand for iNKT cell development in mice [28]. Deficiency of β-hexoaminidase B, an enzyme thought to produce iGb3, in mice results in a severe impairment of iNKT cell development. However, it is still unclear whether iGb3 functions as an endogenous antigen in immune responses.

IMMUNOMODULATORY FUNCTION OF α-GalCer

α-GalCer is a synthetic glycolipid originally isolated from the marine sponge *Agelas mauritanius* as an anticancer component, and later a synthetic analogue of this compound, KRN7000, was developed for experimental studies and clinical trials (Fig. 1) [29]. α-GalCer has been