the younger generation cannot be explained solely by the inclusion of such antibody-positive patients. Even in the Western literature, there is a recent report describing the presence of diffuse spinal cord lesions in 13% of Caucasian patients with MS in whom the OSMS presentation is rare [50]. Considering the large increase in the total number of patients with MS, it is conceivable that phenotypic changes in clinically definite patients with MS are, for the most part, attributable to the increased occurrence of CMS in the younger generation, which is in keeping with the increased prevalence of Barkhof brain lesions in these populations. However, although small changes in the absolute number should be interpreted cautiously, the emergence of patients with intermediate phenotypes, such as CMS with LESCLs and OSMS+SMS without LESCLs, in place of decreasing numbers of patients with prototypic Asian type MS (OSMS with LESCLs) is assumed to have occurred in the north. The higher frequencies of OBSMS and BSMS and other possible intermediate forms, among northern patients compared with southern patients, may also have occurred in place of decreasing numbers of patients with prototypic Asian type MS. Collectively, these observations suggest the possibility that a shift from the OSMS phenotype to the CMS phenotype through intermediate phenotypes is also occurring among northern patients. The presence of intermediate phenotypes could be partly related to the limitations of the clinical classification on the one hand, while on the other hand, it may indicate an overlap between CMS and OSMS thereby suggesting that CMS and OSMS are within a spectrum of the disease.

Changes in the distributions of factors predisposing people to Western type MS appear to have occurred preferentially in the north. The aboriginal people, known as the Ainu, of the northernmost island (Hokkaido) of the Japanese archipelago are a minor ethnic population distinct from mainland Japanese. They make up approximately 0.01% of the northern Japanese population at present [51]. Although earlier anthropological observations based on morphological features determined that the Ainu were Caucasian descendents, recent genetic studies have shown close relationships to both North Asians and mainland Japanese [52,53]. Moreover, most residents of Hokkaido are descendants of migrants from all over mainland Japan who went to the Hokkaido area during and after the Meiji era (1868–1912), about a century ago. Therefore, the influence of the genetic admixture on phenotypic changes in northern patients appears to be modest, but cannot be completely excluded. Since patients born before the end of World War II have similar clinical features in the north and south, environmental changes that predispose people to MS have preferentially occurred thereafter in the north, or alternatively, northern people are intrinsically more susceptible to such exogenous changes than southern people.

In summary, the temporal changes and geographical differences in MS phenotypes suggest that susceptibility to the CMS phenotype and brain lesion burdens can be altered drastically over a relatively short period and in particular areas by environmental factors. "Westernization," which is likely to have reinforced the Western MS phenotype, has taken place equally in northern and southern parts of Japan. Nonetheless, the emergence of Western type MS appears to be happening faster in the north, suggesting that latitude or latitude-related factors could be influential in determining MS phenotypes, even in races resistant to MS. Interestingly, the MRI features characteristic of Western type and Asian type MS, namely Barkhof brain lesions and LESCLs, respectively, appeared to be differentially influenced by environmental factor changes, since the former, which are markedly enhanced in northern-born northern residents, is also augmented by a "Westernized" environment, in which the younger generation are being raised, whereas development of the latter is less affected or unaffected by such changes. Future nationwide surveys incorporating anti-AQP4 antibody assays and detailed MRI analyses in Japanese will provide further insights into the mechanisms underlying the phenotypic changes in MS induced by the environment.

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Appendix

The chairmen of the previous nationwide survey committees were Professors Yoshigoro Kuroiwa (Department of Neurology, Kyushu University; first survey), Akihiro Igata (Third Department of Internal Medicine, Kagoshima University; second survey), and Hiroshi Nishitani (Department of Neurology, National Utano Hospital; third survey). In the fourth survey, in addition to the authors, the following were members of the Research Committee of Neuroimmunological Diseases: Drs Susumu Chiba (Department of Neurology, School of Medicine, Sapporo Medical University), Dr Yoshitaka Fujii (Department of Surgery II, Nagoya City University Medical School), Susumu Furukawa (Department of Pediatrics, Yamaguchi University School of Medicine), Hideo Hara (Department of Vascular Dementia Research, National Institute for Longevity Sciences, National Center of Geriatrics and Gerontology), Toshirou Hara (Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University), Kinya Hisanaga (Department of Neurology, Miyagi National Hospital), Shu-ichi Ikeda (Department of Neurology, Shinshu University School of Medicine), Shuji Izumo (Division of Molecular Pathology, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University), Ryuji Kaji (Department of Neurology, Graduate School of Medicine, Tokushima University), Takashi Kanda (Department of Neurology and Clinical Neuroscience, Yamaguchi University School of Medicine), Shosei Koh (Department of Biomedical Laboratory Sciences, School of Medicine, Shinshu University), Susumu Kusunoki (Department of Neurology, Kinki University School of Medicine), Satoshi Kuwabara (Department of Neurology, Chiba University School of Medicine), Hidenori Matsuo (Division of Clinical Research, Nagasaki Medical Center of Neurology), Hidehiro Mizusawa (Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University), Tatsufumi Nakamura (Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University), Kyoichi Nomura (Department of Neurology, Saitama Medical School), Mieko Ogino (Department of Internal Medicine III (Neurology), Kitasato University School of Medicine), Yoshiro Ohara (Department of Microbiology, Kanazawa Medical University), Mitsuhiro Osame (Department of Neurology and Geriatrics, Kagoshima University School of Medicine), Kohei Ota (Department of Health Science, Faculty of Science, Tokyo University of Science), Jun Shimizu (Department of Neurology, University of Tokyo), Akio Suzumura (Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University), Takeshi Tabira (Department of Vascular Dementia Research, National Institute for Longevity Sciences, National Center of Geriatrics and Gerontology), Keiko Tanaka (Department of Neurology, Brain Research Institute, Niigata University), Masami Tanaka (Department of Neurology and Clinical Research Center, Nishi-Niigata Chuo National Hospital), Makoto Yoneda (Second Department of Internal Medicine, Faculty of Medical Sciences, University of Fukui), Hiroaki Yoshikawa (Health Service Center, Kanazawa University), and Nobuhiro Yuki (Department of Neurology and Research Institute for Neuroimmunological Diseases, Dokkyo Medical University School of Medicine).

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Molecular network of the comprehensive multiple sclerosis brain-lesion proteome

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Background A recent proteomics study of multiple sclerosis (MS) lesion-specific proteome profiling clearly revealed a pivotal role of coagulation cascade proteins in chronic active demyelination. However, among thousands of proteins examined, nearly all of remaining proteins are yet to be characterized in terms of their implications in MS brain-lesion development.
Methods By the systems biology approach using four different pathway analysis tools of bioinformatics, we studied molecular networks and pathways of the proteome dataset of acute plaques, chronic active plaques (CAP), and chronic plaques (CP).
Results The database search on Kyoto Encyclopedia of Genes and Genomes (KEGG) and protein analysis through evolutionary relationships (PANTHER) indicated the relevance of extracellular matrix (ECM)–mediated focal adhesion and integrin signaling to CAP and CP proteome. KeyMolnet disclosed a central role of the complex interaction among diverse cytokine signaling pathways in brain-lesion development at all disease stages, as well as a role of integrin signaling in CAP and CP. Ingenuity pathway analysis (IPA) identified the network constructed with a wide range of ECM components, such as collagen, type I α1, type I α2, type VI α2, type VI α3, fibronectin 1, fibulin 2, laminin α1, vitronectin, and heparan sulfate proteoglycan, as one of the networks highly relevant to CAP proteome.
Conclusions Although four distinct platforms produced diverse results, they commonly suggested a role of ECM and integrin signaling in development of chronic lesions of MS. These *in silico* observations indicate that the selective blockade of the interaction between ECM and integrins in brain lesions *in situ* would be a target for therapeutic intervention in MS. *Multiple Sclerosis* 2009; 15: 531–541. http://msj.sagepub.com

Key words: extracellular matrix; multiple sclerosis; pathway analysis; proteome; systems biology

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) presenting with relapsing-remitting and progressive clinical courses. An autoimmune process triggered by a complex interplay between genetic and environmental factors may mediate MS, although the causative agents have not yet been identified. Pathologically, MS shows remarkable heterogeneity in inflammatory demyelination, astrogliosis, and axonal degeneration [1]. Even though various drugs are lined up in clinical trials, currently, treatment options with limited efficacies, including interferon-β, glatiramer acetate, and mitoxantrone are available for ordinary clinical practice of MS [2].

The completion of the Human Genome Project in 2003 allows us to systematically characterize the comprehensive disease-associated profiles of the whole human genome [3]. The global analysis of transcriptome, proteome, protein interactome, and metabolome helps us identify disease-specific molecular signatures and biomarkers for diagnosis and prediction of prognosis, and would broaden the spectrum of molecular mechanism-based therapy for MS [4,5]. Actually, the comprehensive gene expression profiling of MS brain tissues and peripheral blood lymphocytes by DNA microarray identified a battery of genes aberrantly regulated in MS, whose role has not been previously predicted during its pathogenesis [6,7]. A recent proteomics study of MS lesion-specific proteome profiling showed

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that overproduction of tissue factor and protein C inhibitor plays a central role in molecular events ongoing in chronic active plaques (CAP) [8]. *In vivo* administration of coagulation cascade inhibitors really reduced the clinical severity in a mouse model of experimental autoimmune encephalomyelitis (EAE), supporting the view that the blockade of the coagulation cascade would be a potential approach for the treatment of MS [8]. However, among thousands of proteins this study examined, nearly all of remaining proteins were left behind to be characterized in terms of their implications in MS brain-lesion development.

Since the global expression analysis of transcriptome and proteome usually produces high-throughput experimental data at a time, it is often difficult to find out the meaningful biological implications of the dataset. Recent advances in systems biology enable us to illustrate the cell-wide map of the complex molecular interactions by using the literature-based knowledgebase of molecular pathways [9,10]. In the scale-free molecular network, targeted disruption of limited numbers of critical components, on which the biologically important molecular connections concentrate, could disturb the whole cellular function by destabilizing the network [11]. From this point of view, the integration of comprehensive transcriptome and proteome data of disease-affected tissues with underlying molecular networks could provide the rational approach not only to characterize disease-relevant pathways but also to achieve the network-based choice of effective drug targets. By using four different pathway analysis tools of bioinformatics, this study was designed to characterize molecular networks and pathways of MS lesion-specific proteome data of Han, *et al.* [8]. Although the analysis by distinct platforms did not lead to fully identical results, they commonly suggested a role of extracellular matrix (ECM) and integrin signaling in chronic lesions of MS. These *in silico* observations indicate that ECM and integrins would be a target candidate for designing therapeutic intervention in MS.

Databases and methods

The dataset of the comprehensive MS brain-lesion proteome

In the original dataset of Han, *et al.* [8], fresh-frozen brain autopsy samples were collected from six MS patients of different clinical subtypes, acute, chronic, progressive, secondary progressive, or chronic progressive, with ages 27–54, and from two age-matched control subjects free of neurological diseases. The postmortem interval of each case ranged from 4 to 24 h. Multiple sclerosis lesions were classified into three distinct categories: acute plaques (AP), CAP (chronic active plaques), or chronic plaques (CP), based on histological evaluation of the disease activity, briefly as follows: AP showed characteristics of acute ongoing inflammation, edema, and active demyelination. CAP was characterized by chronic demyelination with active inflammation at the lesion edges, whereas CP represented chronic inactive demyelination accompanied by profound astrogliosis. Protein samples were prepared from small pieces of brain tissues isolated by laser-captured microdissection, and the tissue pieces were characterized separately by the standard histological examination. The proteins were separated on one-dimensional SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels. Then, the protein bands were dissected and digested in a gel with trypsin, and peptide fragments were processed for mass spectrometric analysis several times to obtain a saturation point. Among 2,574 proteins determined with high confidence, the application of a computational data exploration program named INTERSECT/INTERACT identified 158, 416, and 236 lesion-specific proteins that were detected exclusively in AP, CAP, and CP, respectively. In this study, we tentatively called them as the comprehensive MS brain-lesion proteome dataset.

Conversion of protein IDs into Entrez Gene IDs and KEGG IDs

We converted the protein IDs listed in the dataset described above into the corresponding the National Center for Biotechnology Information (NCBI) Entrez Gene IDs, Gene Symbols, and Kyoto Encyclopedia of Genes and Genomes (KEGG) IDs by searching them on the UniProt knowledgebase (http://www.expasy.org/sprot).

Molecular network analysis

To identify biologically relevant molecular pathways from large-scale proteome data, we have undertaken the systems biology approach. We analyzed them by using four distinct pathway analysis tools endowed with a comprehensive knowledgebase which are as follows: KEGG (http://www.kegg.jp), the protein analysis through evolutionary relationships (PANTHER) classification system (http://www.pantherdb.org), Ingenuity pathways analysis (IPA) (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com), and KeyMolnet (Institute of Medicinal Molecular Design, Tokyo, Japan; http://www.immd.co.jp).

By importing the list of KEGG IDs, we studied molecular pathways on KEGG, a public database that systematically integrates genomic and chemical information to create the whole biological system *in silico*. KEGG contains manually curated reference pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes, and human diseases [12]. Currently, KEGG contains 90,931 pathways generated from 371 reference pathways. PANTHER, a public database generated by computational algorithms that relate the evolution of protein sequence to the evolution of protein functions and biological roles, provides a structured representation of protein function in the context of biological reaction networks [13]. Currently, PANTHER includes the information on 165 regulatory and metabolic pathways, manually curated by expert biologists. PANTHER visualizes pathway maps with the format compatible with the Systems Biology Markup Language (SBML) standard. By uploading the list of Entrez Gene IDs, PANTHER identifies the genes in terms of over- or under-representation in canonical pathways, followed by statistical evaluation by multiple comparison with a Bonferroni correction.

IPA is a commercial tool built upon a knowledgebase that contains approximately 1,600,000 biological and chemical interactions and functional annotations with scientific evidence. They are collected from more than 300 selected articles, textbooks, and other data sources, manually curated by expert biologists. By uploading the list of Entrez Gene IDs, the network-generation algorithm identifies focused genes integrated in a global molecular network [14]. IPA calculates the score *P*-value, the statistical significance of association between the genes and the network by the Fisher's exact test.

KeyMolnet is a commercial database, composed of knowledge-based contents on relationships among human genes, molecules, diseases, pathways, and drugs, curated by expert biologists. They are categorized into the core contents that are collected from selected review articles with the highest reliability or the secondary contents extracted from abstracts of PubMed database and Human Reference Protein database. By importing the list of Entrez gene ID, KeyMolnet automatically provides corresponding molecules as a node on networks [15]. The "N-points to N-points" network-search algorithm identifies the molecular network constructed by the shortest route connecting the start point molecules and the end point molecules. The generated network was compared side by side with 346 human canonical pathways of the KeyMolnet library. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The significance in the similarity between both is scored following the formula, where O = the number of overlapping molecular relations between the extracted network and the canonical pathway, V = the number of molecular relations located in the extracted network, C = the number of molecular relations located in the canonical pathway, T = the number of total molecular relations composed of approximately 90,000 sets, and the X = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score}(p))$$

$$\text{Score}(p) = \sum_{x=0}^{\text{Min}(C,V)} f(x)$$

$$f(x) = {}_C C_x \cdot {}_{T-C} C_{V-x} / {}_T C_V$$

Results

KEGG and PANTHER searches elucidated a role of ECM-mediated cell adhesion in chronic lesions of MS

First of all, we converted all protein IDs listed in the original database [8] into the corresponding NCBI Entrez Gene IDs, Gene Symbols, and KEGG IDs by searching them on the UniProt knowledgebase. After the removal of unaccepted and redundant IDs, we finally identified 155, 407, and 232 Entrez Gene IDs and KEGG IDs from the AP, CAP, and CP-specific proteome data, respectively. They are listed in Supplementary Tables 1–3*.

When the KEGG IDs of the proteome were uploaded onto the 'Search Objects in Pathway' tool of the KEGG database, the vast majority of AP, CAP, or CP-specific proteins was not mapped on any KEGG human reference pathways (Table 1). However, a battery of CAP-specific proteins were categorized as those located in the pathways linked to focal adhesion (KEGG pathway ID: hsa04510), cell communication (hsa01430), ECM-receptor interaction (hsa04512), purine metabolism (hsa00230), and other biological pathways (not shown). Likewise, a panel of CP-specific proteins was found to be involved in the pathways linked to focal adhesion, regulation of actin cytoskeleton (hsa04810), oxidative phosphorylation (hsa00190), and cell communication (Table 1). These results are derived chiefly from enhanced production and deposition of ECM and receptor components, including collagen, fibronectin, vitronection, integrin, and laminin in CAP and CP lesions. In contrast, relatively small numbers of AP-specific proteins were mapped on the

*Supplementary Tables 1–4 are available online at http://msj.sagepub.com/

Table 1 The molecular pathway relevant to multiple sclerosis (MS) brain-lesion proteome suggested by KEGG search

Stage	Rank	Functional category (KEGG Pathway ID)	Genes classified
AP	1	Unclassified	123 genes
	2	Oxidative phosphorylation (hsa00190)	NDUFS7, NDUFB9, ATP4A, ATP6V0C
	3	Regulation of actin cytoskeleton (hsa04810)	FGD1, ITGB4, SSH1, ACTA1
CAP	1	Unclassified	281 genes
	2	Focal adhesion (hsa04510)	COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, LAMA1, MYLK, SHC3, PPP1CA, PARVA, PRKCB1, MYL7, RAC3, SPP1, SRC, THBS1, VTN
	3	Cell communication (hsa01430)	NES, COL1A, COL1A2, COL5A2, COL6A2, COL6A3, KRT78, FN1, GJA1, LAMA1, KRT3, SPP1, THBS1, VTN
	4	ECM-receptor interaction (hsa04512)	COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, LAMA1, HSPG2, SPP1, THBS1, VTN
	5	Purine metabolism (hsa00230)	ADCY5, TYMP, NT5E, PDE2A, PDE3B, PDE4A, PDE4B, PRPS2, GMPS, ENTPD1
CP	1	Unclassified	166 genes
	2	Focal adhesion (hsa04510)	COL4A2, COL6A1, CRK, FYN, ITGA6, LAMB2, LAMC1, PIK3CA, ZYX
	3	Regulation of actin cytoskeleton (hsa04810)	WASF2, BAIAP2, CRK, ITGA6, PIK3CA, TIAM1, MYH14, ARHGEF7
	4	Oxidative phosphorylation (hsa00190)	NDUFB6, NDUFB8, NDUFS5, ATP5I, ATP6V1F
	5	Cell communication (hsa01430)	COL4A2, COL6A1, ITGA6, LAMB2, LAMC1

The list of KEGG IDs of MS brain-lesion proteome was uploaded onto the 'Search Objects in Pathway' tool of the KEGG database. Top 2 for AP and top 4 for CAP and CP of human reference pathways relevant to the proteome data are shown with KEGG pathway IDs and the list of genes classified.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

pathways, such as oxidative phosphorylation and regulation of actin cytoskeleton (Table 1). Thus, the KEGG search suggested that the biological process of ECM and integrin-mediated cell adhesion and communication plays a role in chronic lesions of MS.

When the Entrez Gene IDs of the proteome were imported into the 'Gene Expression Data Analysis' tool of the PANTHER database, the vast majority of AP, CAP, or CP-specific proteins were not mapped on any PANTHER canonical pathways in comparison with a reference set of NCBI human genes (Table 2). However, PANTHER identified a statistically significant relationship between a set of CAP proteins and signaling pathways of chemokines and cytokines, integrin (Figure 1), muscarinic and nicotinic acetylcholine receptors (Table 2). PANTHER suggested an involvement of integrin signaling in CP, but identified no pathways relevant to AP (Table 2). Thus, the PANTHER search indicated that integrin signaling plays a role in both CAP and CP, whereas inflammation mediated by chemokine and cytokine signaling plays a predominant role in CAP.

Table 2 The molecular pathway relevant to MS brain-lesion proteome suggested by PANTHER search

Stage	Rank	Functional category	Number of genes classified	Human reference genes	*P*-value
AP	1	Unclassified	120	22436	6.89E-02 (NS)
CAP	1	Unclassified	321	22436	1,73E-04
	2	Inflammation mediated by chemokine and cytokine signaling pathway	17	315	2,63E-03
	3	Integrin signaling pathway	14	227	3,55E-03
	4	Muscarinic acetylcholine receptor 1 and 3 signaling pathway	7	62	1,17E-02
	5	Nicotinic acetylcholine receptor signaling pathway	8	91	2,03E-02
CP	1	Unclassified	182	22436	9,75E-03
	2	Integrin signaling pathway	9	227	4,33E-02

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Gene Expression Data Analysis' tool of the PANTHER classification system by comparing with a reference set of NCBI human genes. The canonical pathways relevant to the proteome data are shown with the number of genes classified and *P*-value evaluated by multiple comparison with a Bonferroni correction.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; CP, chronic plaques; and NS, not significant.

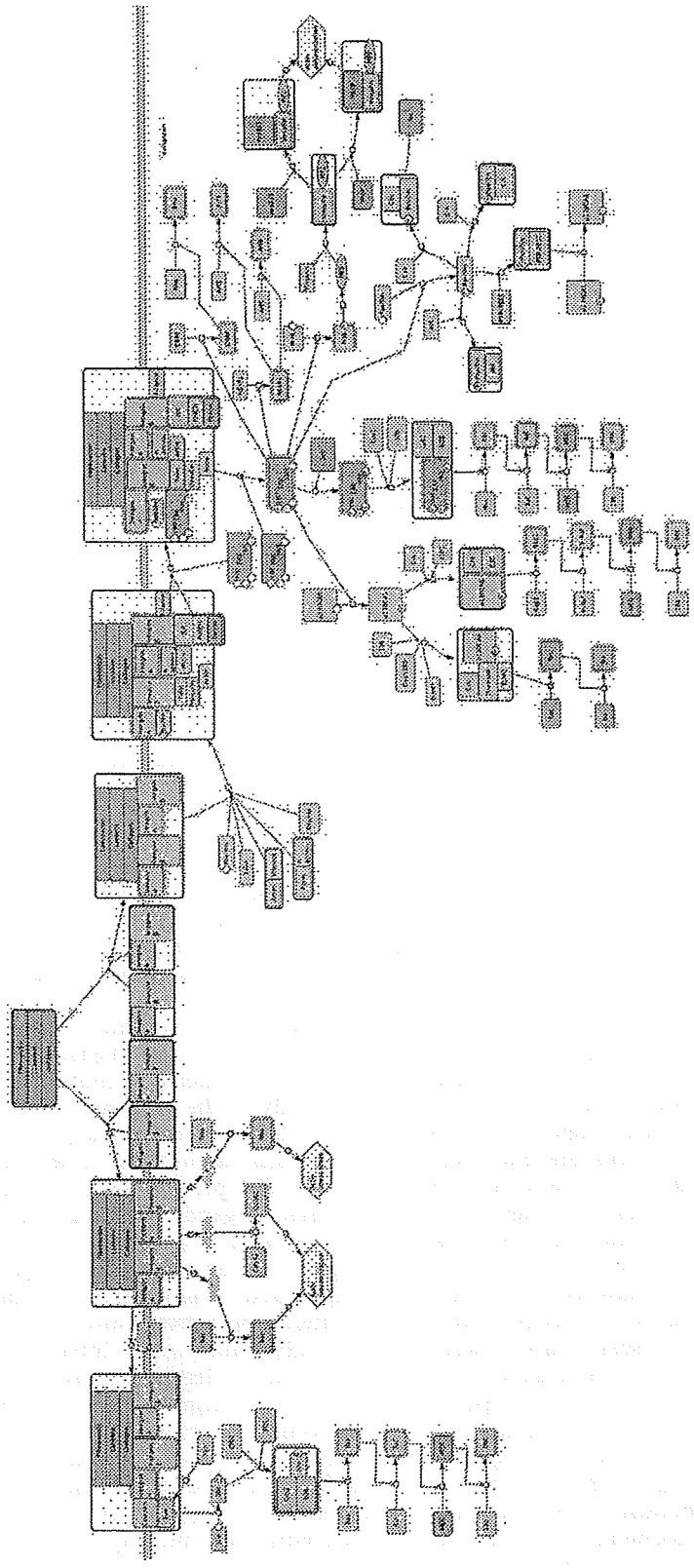

Figure 1 Integrin signaling pathway relevant to CAP proteome suggested by PANTHER. The list of Entrez Gene IDs of CAP-specific proteome was uploaded onto the 'Gene Expression Data Analysis' tool of the PANTHER classification system by comparing them with a reference set of NCBI human genes. Integrin signaling pathway was identified as one of canonical pathways statistically relevant to the CAP proteome (Table 2). The pathway is illustrated as the map compatible with the Systems Biology Markup Language (SBML) standard. The molecules colored in pink represent those included in the gene list (Supplementary Table 2). They are composed of fibronectin (Gene symbol: FN1), laminin (LAMA1), collagen (COL1A1, COL1A2, COL5A2, COL6A2, COL6A3), Rac (RAC3), MEK (MAP2K4), FAK (PTK2B), parvin (PARVA), Src (SRC), Jnk (MAP2K4), Arp2/3 (ARPC1A), and VASP (ENAH).

KeyMolnet and IPA searches disclosed a role of the complex interaction of diverse intracellular signaling pathways in brain lesion development of MS

Next, we investigated molecular networks of MS brain proteome by utilizing two different commercial platforms. When the Entrez Gene IDs of the proteome were uploaded onto the "N-points to N-points" search tool of KeyMolnet, it extracted highly complex large-scale molecular networks of the AP, CAP, and CAP-specific proteome (Figure 2). The network of the AP, CAP, or CP proteome is composed of 777, 1,120, or 952 fundamental nodes with 1,892, 2,772, or 2,279 molecular relations, respectively. The statistical evaluation indicated that the top five most relevant molecular networks include IL-4, IL-6, IL-2, and catenin signaling pathways and transcriptional regulation by STAT (signal transducer and activator of transcription) for the AP proteome, PI3K, IL-4, type I IFN, and IL-6 signaling pathways and transcriptional regulation by STAT for the CAP proteome, and IL-4, hepatocyte growth factor (HGF), TCR (T cell receptor), integrin and IL-6 signaling pathways for the CP proteome (Table 3). It is worthy to note that the integrin signaling pathway was ranked as the sixth relevant pathway to the CAP proteome with *P*-value of the score = 2.13E-012. Considerable overlap existed in the results of PANTHER (Table 2) and KeyMolnet (Table 3). The KeyMolnet search disclosed a central role of the complex interaction of diverse cytokine signaling pathways in brain lesion development at all disease stages of MS, and the role of the integrin signaling pathway in both CAP and CP.

When the Entrez Gene IDs of the proteome were imported into the 'Core Analysis' tool of IPA, it highlighted several units of small-scale molecular networks relevant to the proteome data (Table 4). The network most relevant to the AP proteome was linked to the functional category of cellular assembly and organization, cancer, and cellular movement with the score *P*-value = 1.00E-49, where both ERK (extracellular signal-regulated kinase) and Akt (V-akt murine thymoma viral oncogene homolog) act as a hub of the network with highly connected molecular relations (Figure 3A). The network most relevant to the CAP proteome included two categories with the score *P*-value = 1.00E-47. One is the network of dermatological diseases and conditions, connective tissue disorders, and inflammatory disease. This network is constructed with various ECM components, including collagen, type I α1, type I, α2, type VI α2, type VI α3, fibronectin 1, fibulin 2, laminin α1, vitronectin, and heparan sulfate proteoglycan, where ERK acts as a hub (Figure 3B). The other is the network of lipid metabolism, molecular transport, and small molecule biochemistry, where Akt acts as a hub (Figure 3C). The network most relevant to the CP proteome was linked to cell cycle, cell morphology, and cell-to-cell signaling and interaction with the score *P*-value = 1.00E-50, where NF-κB (nuclear factor-kappa B) serves as a hub (Figure 3D). Overall, the biological processes involved in cellular assembly, organization, growth, proliferation, movement, and development are key functional categories shared by AP and CP molecular networks (Table 4). IPA also identified in the canonical pathways relevant to the proteome data. Both calcium signaling and oxidative phosphorylation were categorized as those relevant to AP and CAP proteome, whereas the actin cytoskeleton signaling pathway was considered as the important pathway in both CAP and CP (Table 5). Considerable overlap existed in the results of KEGG (Table 1) and IPA (Table 5).

Discussion

A recent proteomics study of MS lesion-specific proteome profiling clearly showed a pivotal role of coagulation cascade proteins in chronic active demyelination [8]. However, among thousands of proteins this study examined, nearly all of remaining proteins are left behind to be characterized in terms of their implications in MS brain-lesion development. The present study characterized molecular networks and pathways of the proteome data by using four different pathway analysis tools of bioinformatics. Although distinct platforms produced diverse results, they commonly suggested a role of ECM and integrin-mediated signaling as the pathway relevant to chronic lesion of MS. Therefore, these *in silico* observations warrant experimental validation.

In the CNS, ECM proteins provide a microenvironment for neurons and glial cells to maintain the ionic and nutritional homeostasis. They are localized chiefly to the vascular and the astroglial basement membranes and meninges but scarcely found in the brain parenchyma under physiological conditions. ECM proteins interact with integrins, the cell-surface ligands that support a physical link between ECM and cytoskeletal components [16]. Integrins consist of 24 pairs composed of noncovalently linked heterodimeric αβ subunits. Although the interaction between integrins and ECM proteins is partially redundant, β1 integrins are the principal ligand for collagen, fibronectin, and laminin, whereas αv integrins are the primary ligand for vitronectin. Integrins regulate the cytoskeletal rearrangement required for cell growth, movement, proliferation, and differentiation by transducing bidirectional signals in an 'inside-out' and 'outside-in' fashion [16]. Integrins, expressed on

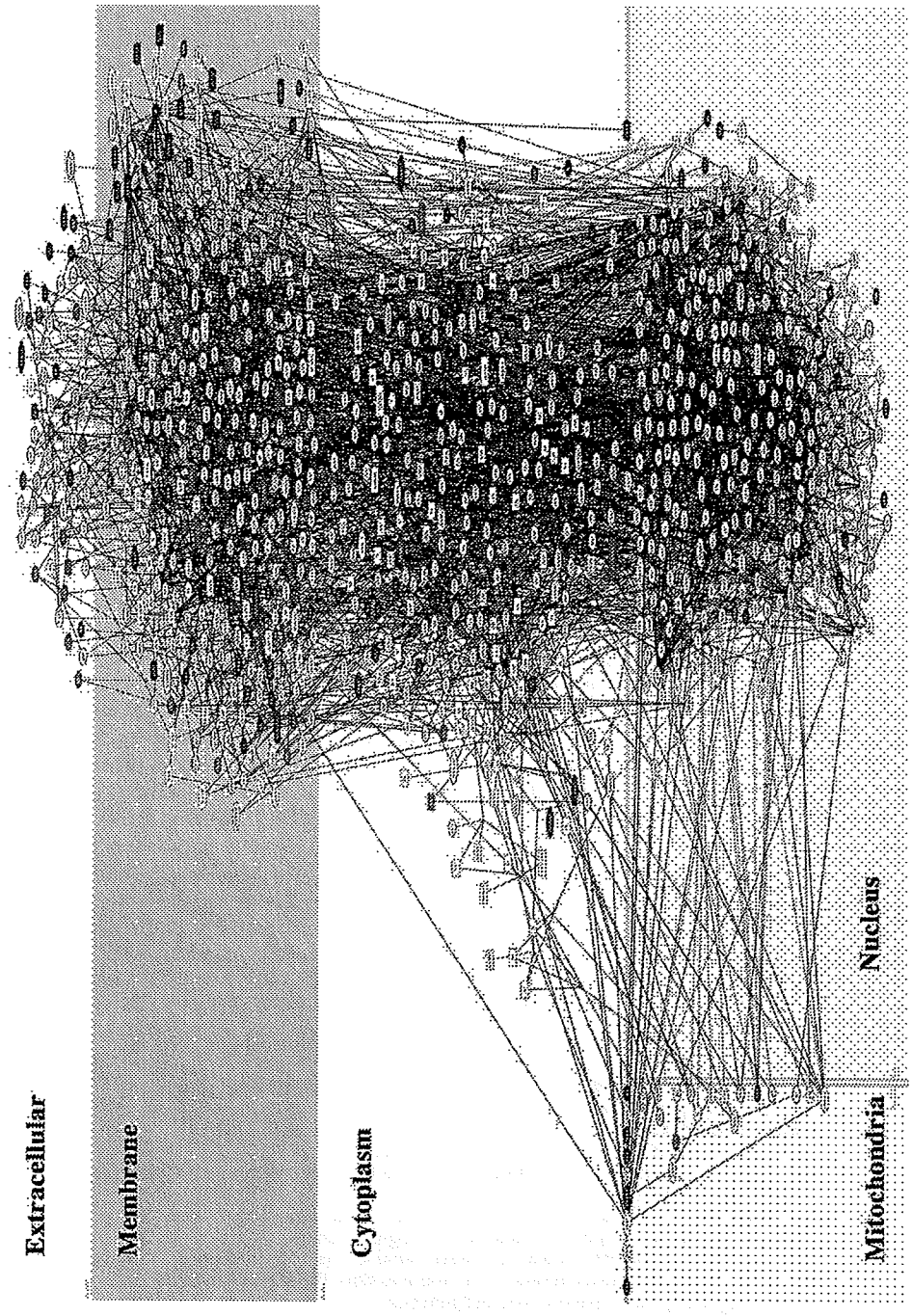

Figure 2 The molecular network of the CAP proteome suggested by KeyMolnet. The list of Entrez Gene IDs of CAP-specific proteome was uploaded onto the 'N-points to N-points search' tool of KeyMolnet. This generated a complex network composed of 1,120 fundamental nodes with 2,772 molecular relations, constructed by the shortest route connecting the start point of 75 MS-linked molecules of the KeyMolnet library (Supplementary Table 4)* and the end point of the CAP-specific proteome. The network is illustrated with respect to subcellular location of molecules. Red nodes represent start point molecules, whereas blue nodes represent end point molecules. Purple nodes express characteristics of both start and end point molecules. White nodes exhibit additional molecules extracted automatically from KeyMolnet core contents to establish molecular connections. The molecular relation is indicated by solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). *Supplementary Tables 1–4 are available online at http://msj.sagepub.com/

Table 3 The molecular network relevant to multiple sclerosis (MS) brain-lesion proteome suggested by KeyMolnet search

Stage	Rank	Functional category	Score	*P*-value
AP	1	IL-4 signaling pathway	42,324	1,794E-13
	2	IL-6 signaling pathway	40,966	4,656E-13
	3	IL-2 signaling pathway	36,684	9,059E-12
	4	Transcriptional regulation by STAT	32,789	1,347E-10
	5	Catenin signaling pathway	32,725	1,408E-10
CAP	1	PI3K signaling pathway	56,937	7,25E-18
	2	IL-4 signaling pathway	46,914	7,541E-15
	3	Transcriptional regulation by STAT	43,694	7,025E-14
	4	IFNα/βsignaling pathway	41,557	3,09E-13
	5	IL-6 signaling pathway	41,274	3,762E-13
CP	1	IL-4 signaling pathway	53,096	1,039E-16
	2	HGF signaling pathway	45,735	1,708E-14
	3	TCRα/βsignaling pathway	43,621	7,39E-14
	4	Integrin signaling pathway	38,501	2,572E-12
	5	IL-6 signaling pathway	38,115	3,359E-12

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'N-points to N-points search' tool of KeyMolnet. The molecular network is constructed by the shortest route connecting the start point of 75 MS-related molecules of the KeyMolnet library (Supplementary Table 4) and the end point of MS lesion–specific proteome. Top 5 networks relevant to the proteome data are shown with the score and *P*-value.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; CP, chronic plaques; PI3K, phosphoinositide-3-kinase; and HGF, hepatocyte growth factor.

immune cells, act as an adhesion receptor for cell trafficking and serve as a scaffold for immunological synapses. By the KEGG search, we identified focal adhesion, cell communication, and ECM-receptor interaction as molecular pathways most relevant to the CAP proteome. They involve a wide range of ECM components, including collagen (COL1A1, COL1A2, COL5A2, COL6A2, COL6A3), fibronectin (FN1), laminin (LAMA1), vitronectin (VTN), heparan sulfate proteoglycan (HSPG2), thrombospondin (THBS1), parvin (PARVA), and osteopontin (SPP1). Furthermore, we found focal adhesion, regulation of actin cytoskeleton, and cell communication as the pathways involved in CP. They include collagen (COL4A2, COL6A1), laminin (LAMB2, LAMC1), and integrin (ITGA6). The relevance of

Table 4 The molecular network relevant to multiple sclerosis (MS) brain-lesion proteome suggested by IPA search

Stage	Rank	Functional category	The number of genes classified	*P*-value
AP	1	Cellular assembly and organization; cancer; cellular movement	24	1,00E-49
	2	Small molecule biochemistry; molecular transport; cellular assembly and organization	15	1,00E-26
	3	Cellular assembly and organization; cellular function and maintenance; skeletal and muscular system	14	1,00E-24
	4	Cellular development; cellular growth and proliferation; hematological system development and function	13	1,00E-22
	5	Cellular compromise; immune and lymphatic system development and function; hair and skin development and function	12	1,00E-19
CAP	1	Dermatological diseases and conditions; connective tissue disorders; inflammatory disease	29	1,00E-47
	2	Lipid metabolism; molecular transport; small molecule biochemistry	29	1,00E-47
	3	Cardiovascular disease; nephrosis; renal and urological disease	25	1,00E-38
	4	Endocrine system disorders; metabolic disease; renal and urological disease	25	1,00E-38
	5	Skeletal and muscular system development and function; tissue morphology; cardiovascular system development and function	22	1,00E-31
CP	1	Cell cycle; cell morphology; cell–cell signaling and interaction	27	1,00E-50
	2	Tissue morphology; cardiovascular disease; cellular development	24	1,00E-43
	3	Cellular assembly and organization; cell morphology; cellular movement	22	1,00E-38
	4	Cellular assembly and organization; cellular development; cellular growth and proliferation	18	1,00E-29
	5	Cell–cell signaling and interaction, Hematological system development and function; Immune and lymphatic system development and function	15	1,00E-22

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Core Analysis' tool of IPA. Top five molecular networks relevant to the proteome data are shown with the number of genes classified and the score *P*-value.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

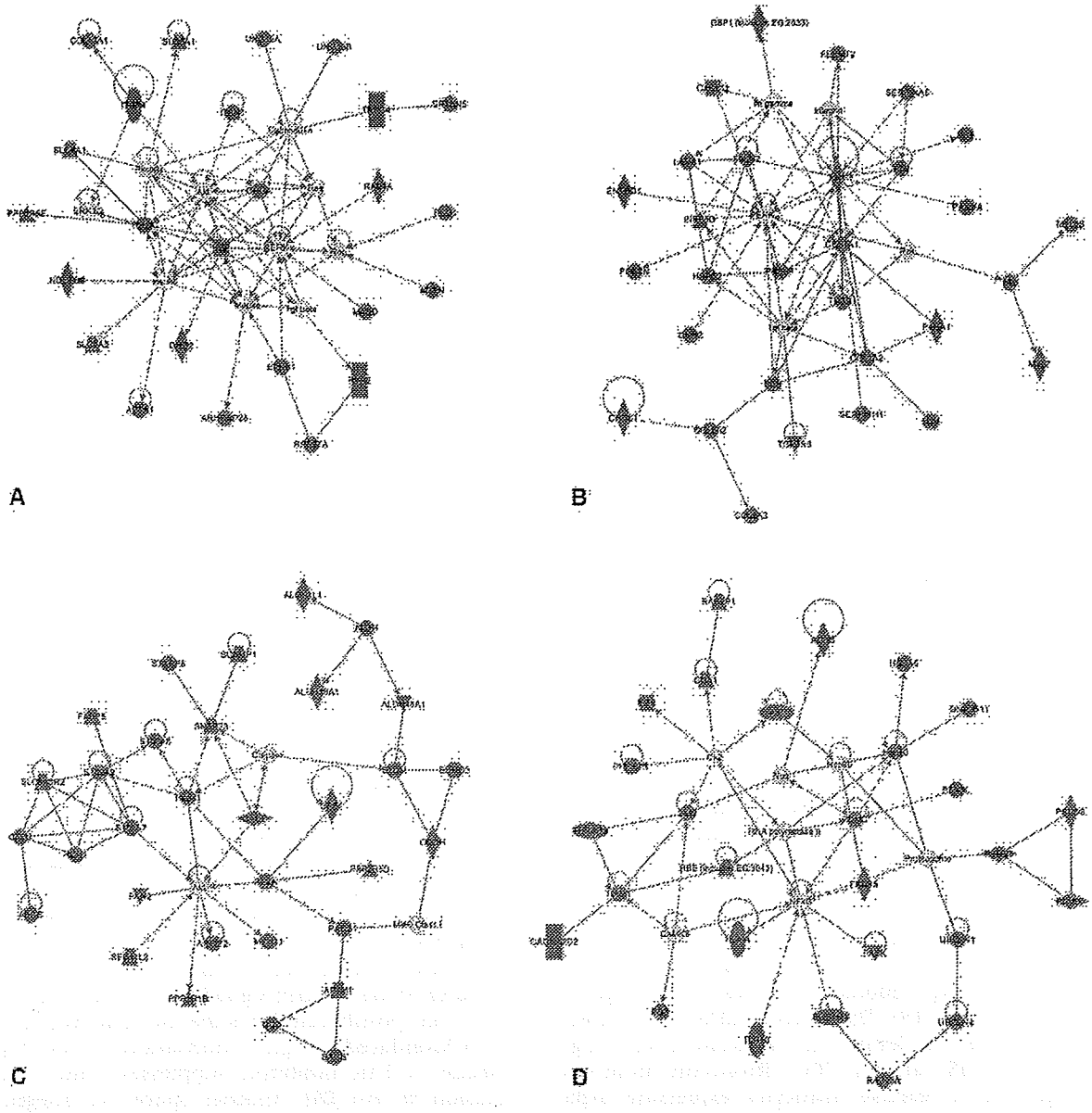

Figure 3 The molecular network of the AP, CAP, and CP proteome suggested by IPA. The list of Entrez Gene IDs of the MS lesion-specific proteome was uploaded onto the 'Core Analysis' tool of Ingenuity pathway analysis (IPA). Molecular networks most relevant to the AP (A), CAP (B and C), or CP (D) proteome are shown. Red nodes represent the molecules included in the gene list (Supplementary Tables 1–3). The molecular network (A) is constructed by 35 nodes, including ACTA1, AGRN, Akt, ARHGAP26, Calmodulin, CHD2, CHGA, COL17A1, EFNB1, ERK, ERK1/2, FGD1, HGF, insulin, ITGB4, ITSN1, MADD, Mapk, NDUFB9, Pkc(s), PP2A, PPP2R5E, RAB1A, Rac, Ras, RPS27A, RYR2, SLC2A3, SLC6A1, SLC8A1, SPTBN5, TGF-β, TRPC4, UNC13A, and UNC13B. The network (B) is constructed by 35 nodes, including BGN, CHI3L1, CNN2, COL1A1, COL1A2, COL6A2, COL6A3, CXCL11, ENTPD1, ERK, FBLN2, FERMT2, FN1, GBP1, HSPG2, IFN-γ, INPP5D, Integrin, LAMA1, LUM, Mlc, MYL7, MYL6B, NES, P4HA1, Pak, PARVA, POSTN, PRELP, SERPINA5, SERPINH1, TGF-β, TGFBR3, THBS1, and VTN. The network (C) is constructed by 35 nodes, including Akt, ALDH, ALDH16A1, ALDH18A1, ALDH1L1, AP1M1, APCS, ARFIP2, Calpain, CALU, CAST, DCD, FABP5, MHC Class I, MYH11, OGDH, PACS1, Pkc(s), PKN2, PP2A, PPP1R1B, PPP2R5D, RCN1, S100A7, S100A8, S100A9, SACS, SCAMP1, SEC14L2, SLC9A3R2, SNAP23, STOM, STXBP5, SUMO3, and UPF1. The network (D) is constructed by 35 nodes, including ADH5, AIP, CACNA2D2, CaMKII, Ck2, DMD, DNAJB11, EIF5, FKBP5, GGA1, HBB, HLA-A, Hsp70, Hsp90, HSPA6, NFkB, Nos, PASK, PEX5L, POMC, PPFIBP1, Proteasome, PSD, PSMB3, PSMB5, PSMD6, RABEP1, RAD23A, RNA polymerase II, SQSTM1, THRAP3, TIAM1, TLR10, UBQLN1, and UBQLN4. The molecular relation is indicated by solid line (direct interaction), dash line (indirect interaction), with filled arrow (acts on), stop (inhibits), stop and filled arrow (inhibits and acts on), and open arrow (translocates to).

Table 5 The molecular pathway relevant to MS brain-lesion proteome suggested by Ingenuity pathway analysis (IPA) search

Stage	Rank	Functional category	The number of genes classified	*P*-value
AP	1	Calcium signaling	7	2,53E-03
	2	Oxidative phosphorylation	4	2,69E-02
CAP	1	Calcium signaling	14	5,14E-04
	2	Hepatic fibrosis and hepatic stellate cell activation	11	1,53E-03
	3	Purine metabolism	16	3,05E-03
	4	Actin cytoskeleton signaling	13	5,77E-03
	5	Oxidative phosphorylation	9	1,12E-02
CP	1	Biosynthesis of steroids	4	7,37E-04
	2	Actin cytoskeleton signaling	8	8,00E-03
	3	Ubiquinone biosynthesis	4	9,54E-03
	4	Axonal guidance signaling	11	1,37E-02
	5	Integrin signaling	7	2,19E-02

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Core Analysis' tool of IPA. The canonical pathways relevant to the proteome data are shown with the number of genes classified and *P*-value.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

the ECM and integrin signaling pathway to CAP and CP was further verified by molecular network analysis using PANTHER, KeyMolnet, and IPA followed by statistical evaluation. These *in silico* observations agree well with *in-vivo* studies, showing remarkable upregulation of diverse ECM constituents in MS brain lesions, where cytokine/chemokine-activated microglia, astrocytes, and infiltrating macrophages release a large amount of proteolytic enzymes bound to ECM molecules, which mediate myelin breakdown [17,18]. Glial scars in chronic lesions of MS include certain ECM proteins that contribute to the failure of regeneration of damaged axons and remyelination of preserved axons [17,18].

In active demyelinating lesions of MS, the expression of vitronectin is greatly enhanced in blood vessel walls, as well as in demyelinated axons and hypertrophic astrocytes at the edge of demyelination [19]. The levels of CD51, a vitronectin receptor, are elevated in the serum of relapsing-remitting MS patients [20]. Vitronectin promotes migration of reactive astrocytes expressing αvβ8 integrin [21]. In active demyelinating lesions of MS, fibronectin is accumulated in the brain parenchyma and is deposited abundantly in blood vessel walls and perivascular infiltrates [22]. Fibronectin facilitates migration of immune cells, promotes proliferation of astrocytes, and inhibits differentiation of oligodendrocyte progenitors [23]. In MS lesions, both vitronectin and fibronectin are derived mainly form plasma protein components passing across the disrupted blood–brain barrier and partly from the local synthesis by endothelial cells, macrophages, astrocytes, and infiltrating immune cells. Vitronectin and fibronectin activate microglia and upregulate MMP-9 production [24]. Thrombospondin produced by reactive astrocytes facilitates macrophage-mediated phagocytosis of apoptotic cells and possible uptake of degraded myelin via the ECM receptors CD36 and αvβ3 integrin [25]. Large-scale sequencing of MS plaque cDNA libraries showed that osteopontin (SPP1), a proinflammatory component of ECM, is one of the most abundant transcripts [26]. The clinical severity of EAE is attenuated in SPP1-deficient mice [26]. The expression of osteopontin is enhanced in astrocytes in active demyelinating lesions of MS [27]. The plasma osteopontin levels are elevated in active relapsing-remitting MS patients [28]. All of these observations support the concept that the selective blockade of the interaction between ECM and integrins in brain lesions *in situ* would be a target candidate for therapeutic intervention in MS.

Because focal adhesion kinase (FAK) is a central mediator of the integrin signaling pathway (see Figure 1), one possible choice is the use of an inhibitor for ECM-induced autophosphorylation of FAK [29]. TAE226, a FAK inhibitor, suppresses tumor cell invasion *in vivo* [29]. Another option for integrin signaling inhibitors is disintegrins, a group of small disulfide-rich peptides containing the arginine–glycine–aspartic acid sequence that mediates the selective binding to integrins [30]. Liposomal delivery of contortrostatin, a snake venome disintegrin, shows a tumor-suppressive anti-angiogenic activity [30]. However, a complete blockade of general function of integrins has a risk for inducing serious side effects [31]. Even in the context of the selective blockade, treatment with a humanized monoclonal antibody against VLA4, α4β1 integrin (natalizumab) reduced relapses 66% in clinical trials of MS but also activated the lethal infection of JC virus in some patients [32].

Acknowledgments

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Immunopathology and Infectious Diseases

Synthetic Retinoid AM80 Inhibits Th17 Cells and Ameliorates Experimental Autoimmune Encephalomyelitis

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Recent evidence suggests that interleukin-17-producing CD4$^+$ T cells (Th17 cells) are the dominant pathogenic cellular component in autoimmune inflammatory diseases, including multiple sclerosis. It has recently been demonstrated that *all-trans* retinoic acid can suppress Th17 differentiation and promote the generation of Foxp3$^+$ regulatory T cells via retinoic acid receptor signals. Here, we investigated the effects of AM80, a synthetic retinoid with enhanced biological properties to *all-trans* retinoic acid, on Th17 differentiation and function and evaluated its therapeutic potential in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. AM80 treatment was more effective than *all-trans* retinoic acid in inhibiting Th17 differentiation *in vitro*. Oral administration of AM80 was protective for the early development of EAE and the down-modulation of Th17 differentiation and effector functions *in vivo*. Moreover, AM80 inhibited interleukin-17 production by splenic memory T cells, *in vitro*-differentiated Th17 cells, and central nervous system-infiltrating effector T cells. Accordingly, AM80 was effective when administered therapeutically after the onset of EAE. Continuous AM80 treatment, however, was ineffective at inhibiting late EAE symptoms despite the maintained suppression of RORγt and interleukin-17 expression levels by central nervous system-infiltrating T cells. We reveal that continuous AM80 treatment also led to the suppression of interleukin-10 production by a distinct T cell subset that expressed both Foxp3 and RORγt. These findings suggest that retinoid signaling regulates both inflammatory Th17 cells and Th17-like regulatory cells. *(Am J Pathol 2009, 174:2234–2245; DOI: 10.2353/ajpath.2009.081084)*

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease affecting the central nervous system (CNS).[1] Previous studies of experimental autoimmune encephalomyelitis (EAE), a murine model of MS,[2,3] indicated that autoimmune responses were initiated by a subset of myelin-specific CD4$^+$ T cells secreting the inflammatory cytokine interferon (IFN)-γ, termed Th1 cells.[4,5] More recently, the identification of an additional subset of differentiated inflammatory helper T cells secreting large amount of the cytokine interleukin (IL)-17 (Th17 cells) have allowed new insights into the pathology of a range of inflammatory autoimmune diseases.[6,7] Indeed, the presence of such Th17 cells among CNS-infiltrating leukocytes has been demonstrated in EAE animals.[7] Furthermore, induction of EAE in IL-17-deficient mice leads to less severe disease[8] and mice that lack IL-23, a cytokine required for Th17 cell survival,[9] are resistant to EAE.[6] Critically, increased levels of IL-17 are detected in MS patients[10] and the presence of IL-17-secreting T cells has been shown to link with acute CNS lesions in MS.[11]

Differentiation of naïve T cells into Th17 cells *in vitro* requires culture with a combination of IL-6, an inflammatory cytokine elaborated by innate immune cells subsequent to ligation of pathogen-associated molecular pattern receptors, and transforming growth factor (TGF)-β,[12–14] a cyto-

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kine classically regarded as anti-inflammatory and also associated with the differentiation of regulatory T cells.[15] The requirement for IL-6 and TGF-β in Th17 differentiation has also been demonstrated *in vivo*[12,16] Phenotypically, Th17 cells express the retinoic acid (RA)-related orphan receptor γt (RORγt) in a Stat3-dependent manner, and produce high levels of many inflammatory cytokines, including IL-17, IL-6, tumor necrosis factor (TNF)-α, IL-21, and IL-22.[7,17–19] Interestingly, naïve T cells receiving TGF-β signaling alone are induced to become a $CD4^+CD25^+$ regulatory T cell population (Treg cells).[13] Treg cells are capable of suppressing inflammation, mediating self-tolerance, and produce suppressive cytokines such as TGF-β and/or IL-10[20] The generation of Treg cells requires the forkhead/winged-helix transcription factor Foxp3 and its functional impairment leads to autoimmunity.[21,22] $Foxp3^+$ Treg cells in the CNS have been shown to ameliorate EAE via the production of IL-10[23], which has recently been associated with restraining Th17-mediated pathology in EAE.[24] Furthermore, IL-10-producing RORγt^+Foxp3^+ T cells have been identified *in vivo*, suggesting the existence of a regulatory Th17-like cell type.[25]

RA, the active metabolite of vitamin A, has multiple effects on cell differentiation and survival through ligation to the two families receptors: retinoic acid receptors (RAR) and retinoid X receptor (RXR), each of which has multiple isoforms.[26] Recently, *all-trans* retinoic acid (ATRA) has been reported to suppress the differentiation of Th17 cells through ligation to the RAR-α,[27,28] accompanied by a down-regulation of RORγt and reciprocal induction of Treg cells expressing Foxp3.[27,29] Possible mechanisms of action of ATRA for the suppression of Th17 cell function have been reported to be a result of reduced expression of IL-6 receptor and IL-23 receptor as well as enhanced TGF-β signaling in a Smad3-dependent manner.[30]

RA has been shown to ameliorate EAE.[31,32] But as these studies were carried through before the discovery of Th17 cells, the amelioration was attributed to suppression of Th1 cells. In addition, the therapeutic application of RA to date has been limited by instability and poor bioavailability of this compound as well as by its nonselective binding to a broad range of retinoid receptors, which conceivably leads to unexpected side effects.[26,33,34] To circumvent these potential problems in the clinical use of RAR agonists, a variety of synthetic RAR agonists with improved biological properties *in vivo* have been developed. One of these synthetic retinoids, AM80, is already available as medication under the trade name Tamibarotene for human diseases such as acute promyelocytic leukemia (APL) and psoriasis.[35–37] AM80 is specific for the RARα/β and characterized by a higher stability, fewer potential side effects, and superior bioavailability compared with ATRA.[35–38] Therefore, we may open up new therapeutic avenues for treating Th17-mediated autoimmune diseases by testing AM80 in an immunoregulatory context.

In this study, we demonstrate for the first time that AM80 inhibits Th17 differentiation *in vitro* with a higher potency than ATRA. Treatment with AM80 ameliorates EAE and inhibits both the differentiation of Th17 cells and the effector function of Th17 cells *in vivo* without generating general immunosuppression. In addition, AM80 proved to be effective in rescue from acute EAE when administered after the onset of the disease. Interestingly, continuous AM80 treatment failed to improve chronic disease despite of apparent suppression of T cell expression of IL-17 and RORγt. We demonstrate that continuous AM80 treatment results in the suppression of IL-10 production by a unique subset of T cells, which is identified as T cells that co-expresses RORγt and Foxp3. We conclude that treatment with the synthetic retinoid AM80 is a considerable intervention strategy for the acute phase of Th17-mediated autoimmune diseases such as MS.

Materials and Methods

Animals and EAE Induction

C57BL/6J (B6) mice (CLEA Laboratory Animal Corp., Tokyo, Japan) were maintained in specific pathogen-free conditions in accordance with institutional guidelines (National Institute of Neuroscience, NCNP, Tokyo, Japan). This study used female mice at 8 to 10 weeks of age. For EAE induction mice were injected subcutaneously with 100 μg of myelin oligodendrocyte glycoprotein (MOG) amino acids 35–55 (MOG_{35-55} peptide MEVGWYRSPFSRVVHLYRNGK)[39] and 1 mg of heat-killed *Mycobacterium tuberculosis* H37RA emulsified in complete Freund's adjuvant (Difco, Lawrence, KS). 200 ng of pertussis toxin (List Biological Laboratories) was injected intraperitoneally on days 0 and 2 after immunization. Some groups of mice also received 3 mg/kg AM80 in 0.5% carboxymethylcellulose (CMC) solution (WAKO Chemicals, Osaka, Japan) by oral gavage.

EAE was clinically scored daily (0, no clinical signs; 1, partial tail paralysis; 2, flaccid tail; 3, partial hindlimb paralysis; 4, total hindlimb paralysis; 5, Hind- and foreleg paralysis).[39] Disease was also assessed using histological examination of CNS tissue as previously described.[40] Briefly, animals were perfused with 20 ml of cold phosphate-buffered saline, and CNS tissue was excised and fixed in formal saline. Paraffin-embedded sections were prepared and stained with either Luxol fast blue or H&E and photomicrographs acquired with a light microscope (Eclipse E800M, Nikon, Japan).

Cell Isolation and Purification

CNS-infiltrating lymphocytes were isolated from spinal cords and brains as previously described.[39] Briefly, tissue was cut into small pieces and digested for 40 minutes at 37°C in media (GIBCO, Auckland, New Zealand) supplemented with 1.4 mg/ml collagenase H (Roche, Mannheim, Germany) and 100 μg/ml DNase I (Roche). Resulting tissue homogenates were forced through a 70-μm cell strainer and leukocytes were enriched using a discontinuous 40%/80% Percoll density gradient centrifugation. Leukocytes were collected from the interface and where indicated cell numbers for leukocytes and/or T cell subsets per mouse were counted with an improved

Neubauer counting chamber and via flow cytometry with reference to a cell number curve as previously described.[41] T cells were purified from splenocytes, draining lymph nodes and CNS infiltrates using a pan T cell MACS isolation kit with an AutoMACS separator according to manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Where required, naïve $CD4^+CD44^-CD25^-CD62L^{high}$ T cells or memory $CD4^+CD44^+CD25^-CD62L^{low}$ T cells were further sorted using a fluorescence-activated cell sorter ARIA (BD Cytometry System, Franklin Lakes, NJ).

Cell Culture

RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/ml penicillin-streptomycin, and 50 μmol/L 2-mercaptoethanol (Invitrogen, Carlsbad, CA) was used for all cultures. Cells were activated with immobilized anti-CD3 monoclonal antibody (mAb) (2C-11; 2 μg/ml) and soluble anti-CD28 mAb (BD PharMingen) or, when measuring recall responses of secondary lymphoid tissue 10 days after immunization, with MOG_{35-55} peptide (0–100 μmol/L). Where indicated, cells were cultured under Th17 polarizing conditions: 2 ng/ml TGF-β, 20 ng/ml IL-6 (PeproTech, London, UK), anti-IFN-γ mAb (HB170; 10 μg/ml), and anti-IL-4 mAb (HB188; 10 μg/ml). Into some cultures ATRA (Sigma-Aldrich, Steinheim, Germany) or AM80 dissolved in dimethyl sulfoxide (Sigma) was added at the required concentrations. Supernatants were harvested at 72 or 96 hours for cytokine measurement and proliferation was determined by incubating with [^{3}H]thymidine (1 μCi/well) for the final 12 hours of culture assessing incorporation of radioactivity with a beta 1205 counter (Pharmacia Biotech, Freiburg, Germany). In some experiments, to measure differentiation of T cells, after 96 hours of activation cells were rested for 48 hours before restimulation with anti-CD3 for a further 96 hours.

Cytokine Measurement

IL-17 was assessed via a mouse IL-17 DuoSet (R&D Systems). All other cytokines were assessed by cytometric bead array using a mouse inflammation kit or a mouse Th1/Th2 cytokine kit (BD Biosciences).

Intracellular Staining for Cytokines and Transcription Factors

To stain cells for production of cytokines, cells were restimulated with 50 ng/ml phorbol 12-myristate 13-acetate and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 to 6 hours before surface staining in the presence of monensin-containing GolgiPlug (BD Biosciences). A LIVE/DEAD fixable dead cell stain kit (Invitrogen) was used to exclude dead cells. Intracellular staining was then performed using a Cytofix/cytoperm kit (BD PharMingen), according to manufacturer's instructions. To stain intracellular transcription factors, we used an anti-mouse/rat Foxp3 staining set (eBioscience, San Diego, CA) with anti-RORγt antibody (BioLegend, San Diego, CA) and visualized with a secondary PE-conjugated goat anti-rabbit antibody (Invitrogen).

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cell populations using an RNeasy Mini Kit (Qiagen, Maryland) according to the manufacturer's instructions. cDNA was prepared using a first-strand cDNA Kit (Takara, Otsu, Shiga, Japan). Quantitative real-time PCR was performed using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) with a LightCycler real-time PCR machine (Roche). Gene expression values were normalized to the expression of the GAPDH housekeeping gene. Primers used in this study were GAPDH fw: 5′-AACGACCCCTTCATTGAC-3′ rv: 5′-TCCACATACTCAGCAC-3′, RORc fw: 5′-TGTCCTGGGCTACCCTACTG-3′ rv: 5′-GTGCAGGAGTAGGCCACATT-3′, mFOXP3 fw: 5′-TTCTCACAACAAGGCCACTTG-3′ rv: 5′-CCCAGGAAAGACAGCAACCCT-3′, mT-bet fw: 5′-GCCAGGGAACCGCTTATATG-3′ rv: 5′-GACGATCATCTGGGTCACATTGT-3′, mIL-10 fw: 5′-CATGGGTCTTGGGAAGAGAA-3′ rv: 5′-CATTCCCAGAGGAATTGCAT-3′.

Statistics

EAE clinical scores for groups of mice are presented as the mean group clinical score ± SEM, and statistical differences were analyzed by two-way analysis of variance (analysis of variance) for repeated measures and significance calculated with a Bonferroni post-test. Cytokine secretion data were analyzed with a two-tailed Student's *t*-test or with one-way analysis of variance with Bonferroni's multiple comparison test. Unless otherwise stated, $P < 0.05$ was considered significant and indicated on plots by asterisks.

Results

RAR Agonists AM80 Inhibit Th17 Cell Differentiation in Vitro

The synthetic retinoid AM80 is already available as medication for human diseases such as APL and psoriasis and characterized by superior pharmacological properties compared with ATRA. As previous studies demonstrated that Th17 cells are the dominant pathogenic cellular component in autoimmune inflammatory diseases, such as MS, and ATRA modulates Th17 differentiation,[27–29] we examined whether AM80 could be used to treat autoimmune diseases. To this end, whole splenocytes or purified naive T cells were stimulated under a range of Th17-inducing conditions (ie, with either TGF-β plus IL-6, or IL-23) with or without either AM80 or ATRA and were assessed for their IL-17 production. Addition of retinoids to the splenocyte culture significantly reduced

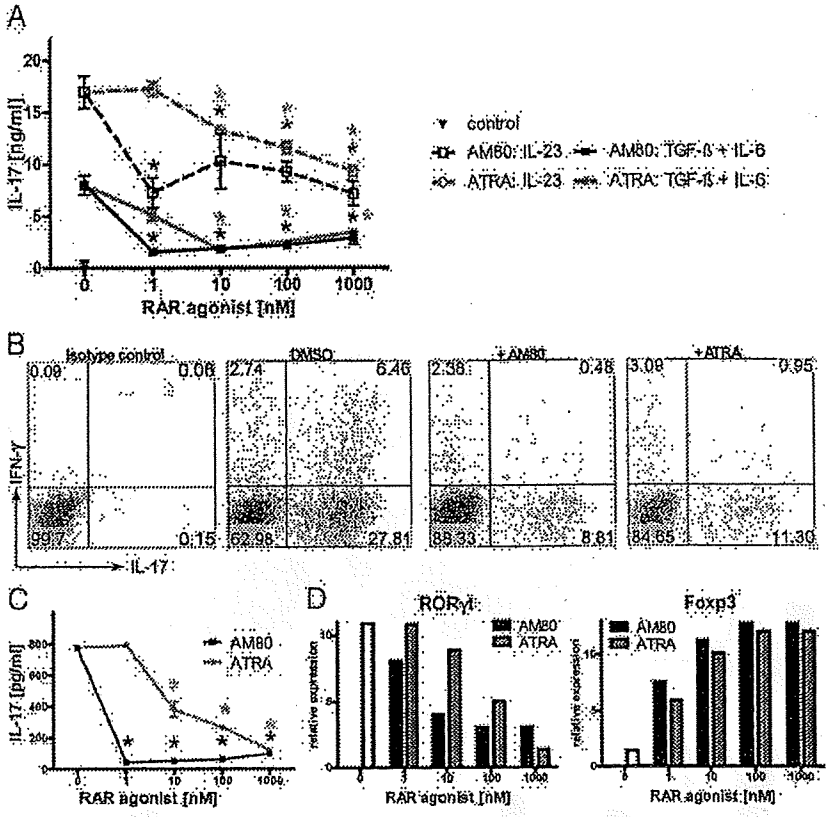

Figure 1. AM80 is a potent inhibitor for Th17 cell differentiation *in vitro*. Whole splenocytes were stimulated with soluble anti-CD3 with retinoids added at a range of concentrations. A: Cells were cultured in the presence of IL-23 (broken line), TGF-β and IL-6 (solid line), or without the addition of cytokines (closed triangle) for 3 days and IL-17 production assessed by enzyme-linked immunosorbent assay (ELISA). B: Intracellular cytokine staining of IL-17 and IFN-γ among TcR^+CD4^+ cells with or without 10 nmol/L retinoid treatment assessed after 3 days of culture. Data depicted in A and B are representative of three independent experiments. In C, $CD4^+CD44^-CD25^-CD62L^{high}$ naïve T cells were stimulated under Th17-priming conditions with retinoids at a range of concentrations for 96 hours. Cells were rested for 48 hours before restimulation and IL-17 production was measured in culture supernatants after further 96 hours of stimulation. $^*P < 0.001$. RNA from these cells was analyzed by quantitative RT-PCR for the transcription factors RORγt and Foxp3 (D). Data depicted in C and D are representative of four similar experiments.

the amount of IL-17 in supernatants (Figure 1A). Accumulation of IL-17-producing T cells after restimulation of the culture was also suppressed by AM80 or ATRA with minimal effect on the development of IFN-γ-producing T cells (Figure 1B). Interestingly, AM80 appeared to be more effective than ATRA at inhibiting IL-17 production especially at low doses. Furthermore, both AM80 and ATRA inhibited Th17 cell differentiation of naive T cells, as revealed by the reductions in IL-17 secretion (Figure 1C). Importantly, the effect of retinoid treatment on naïve T cell differentiation is not merely due to a suppression of T cell activation or an increase in cell death, as such treatment did not reduce proliferation, or total live cell number in the cultures (data not shown). Also, treatment with AM80 or ATRA led to reduced expression of RORγt, a key Th17 cell-specific transcription factor, as compared with untreated controls (Figure 1D). Such reductions in Th17 phenotype were accompanied by the reciprocal increase of Foxp3 expression, indicative of a regulatory T cell phenotype (Figure 1D). AM80 was also more effective at modulating the transcription factors specific for Th17 cells as compared with ATRA (Figure 1D). In addition, no up-regulation in T-bet and GATA-3 was observed, indicating that the inhibition of Th17 differentiation by retinoid treatment was not a result of an altered Th1/Th2 phenotype. These results suggest that AM80 is a superior modulator of *in vitro* Th17 differentiation as compared with ATRA.

AM80 Treatment Ameliorates Acute Autoimmune Inflammation

EAE, the murine model of MS,[3] is an autoimmune disease in which Th17 cells play an important pathogenic role.[42] At high doses ATRA can delay onset of this disease putatively via mechanisms that affect the development of Th17 cell function.[30] As AM80 is more effective in inhibiting Th17 development *in vitro*, we tested whether or not administration of this compound could modulate EAE. Thus, EAE was induced in B6 mice, and some groups of mice received AM80 orally every other day from the day of immunization. The onset of clinical disease was delayed and maximal clinical score was significantly reduced in animals treated with AM80 as compared with control mice treated with vehicle alone (Figure 2A). Histological examination of spinal cords at the peak of clinical disease (day 15) showed that AM80 treatment led to a marked reduction in cellular infiltrates into spinal cord and maintained normal myelin structure (Figure 2B). Flow cytometric analysis confirmed that AM80 treatment led to reduced numbers of infiltrating cells in the brains and spinal cords and this difference was particularly apparent when T cell numbers were compared (Figure 2C). As some T cell infiltration was still observed in the CNS of AM80-treated mice, we examined the functional properties of such cells to ascertain whether or not AM80 modulated T cell differentiation *in vivo*. The expression of

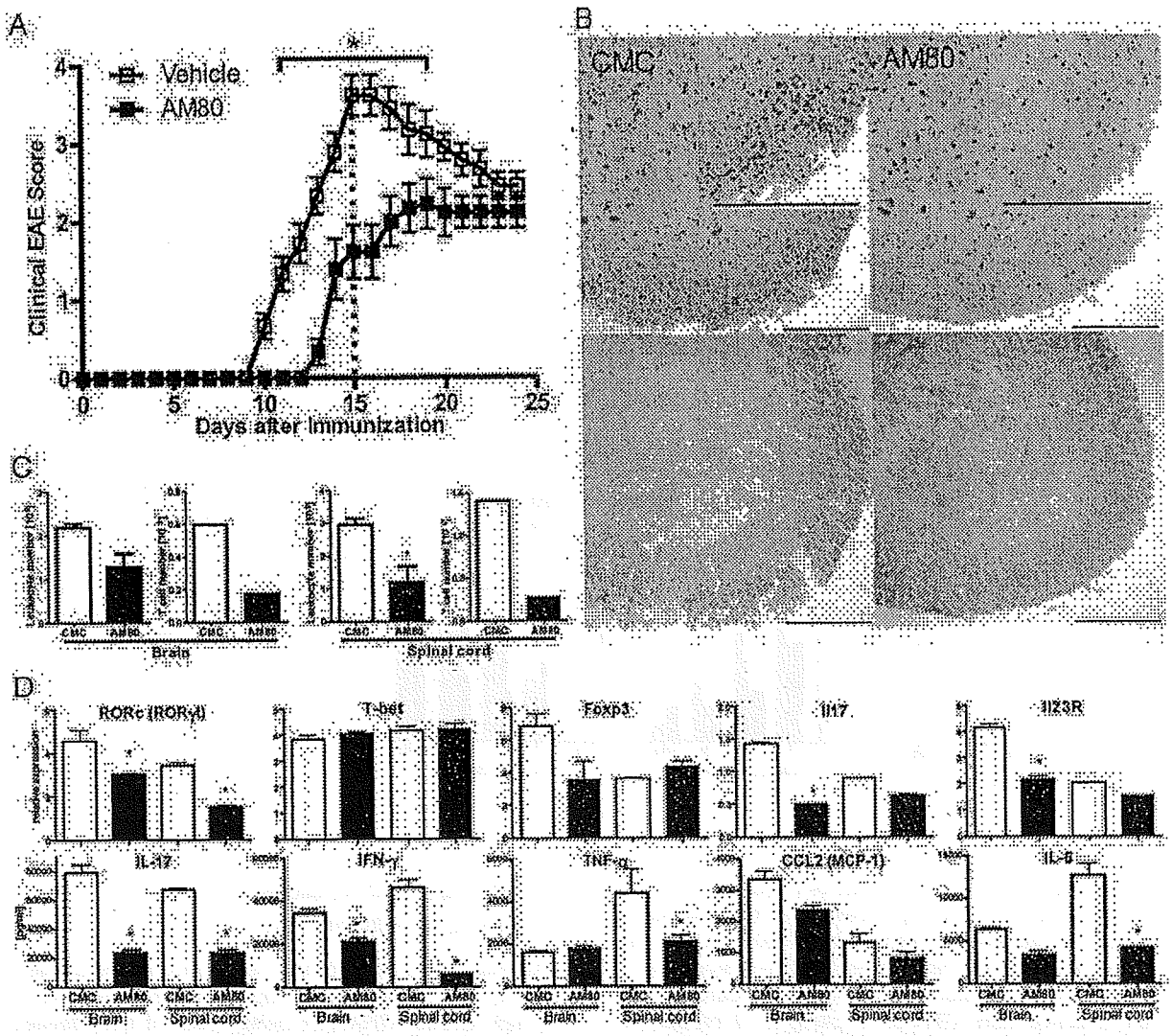

Figure 2. AM80 treatment ameliorates EAE with reduction of IL-17 production *in vivo*. EAE was induced in B6 mice by immunization with MOG_{35-55}. Groups of mice received either CMC or 3 mg/kg AM80 in CMC orally every other day from day 0. Mice were scored daily for clinical disease (A) analysis of variance for repeated measures shows significant differences from day 11 to 18 ($^*P < 0.001$). On day 15 after immunization, groups of mice were sacrificed and spinal cords were examined histologically. Representative sections are shown in B. H&E staining (**upper panels**), Luxol fast blue staining (**lower panels**). Scale bar = 200 μm. Leukocytes and T cells were purified from the CNS at day 15 after immunization and cell numbers evaluated by hemocytometer (C). Quantitative RT-PCR was used to measure an array of transcription factors and cytokines (D, **upper row**) in which error bars represent duplicated PCR of the same samples. In the **lower row**, CNS-infiltrating T cells were restimulated with immobilized anti-CD3 antibody and supernatants were measured after 72 hours for the presence of a range of cytokines and chemokines using ELISA or CBA. Error bars represent measurements from duplicate wells. Data are representative of at least two independent experiments.

Th17-specific genes including RORγt, IL-17 and IL-23 receptor in T cells isolated from CNS was reduced in the group treated with AM80 (Figure 2D). In contrast, the expression of Foxp3 and T-bet (specific for Treg cells and Th1 cells respectively) were not elevated by AM80 treatment. Flow cytometric analysis confirmed that fewer IL-17-producing cells but similar numbers of Foxp3-positive cells were present among the CNS infiltrating T cells in AM80-treated mice as compared with vehicle treated controls (data not shown). Also, we note that AM80 treatment did not affect the expression of activation markers (including CD62L, CD44 and CD25) by CNS-infiltrating T cells (data not shown). Furthermore, on anti-CD3 mAb stimulation, CNS-infiltrating T cells isolated from animals treated with AM80 produced significantly reduced levels of pro-inflammatory cytokines and chemokines (Figure 2D). Myeloid cell and T cell phenotyping via flow cytometric analysis revealed no significant differences between treatment groups (data not shown). Taken together, these results indicate that AM80 treatment decreases the number of T cells infiltrating the CNS during EAE and also lowers their IL-17 producing capacity.

We have performed adoptive transfer experiments to determine whether AM80 ameliorates EAE through a direct effect on T cells or not. Draining lymph node cells isolated from immunized mice with oral administration of AM80 or vehicle were restimulated with MOG peptide and cultured for one week without retinoid treatment.

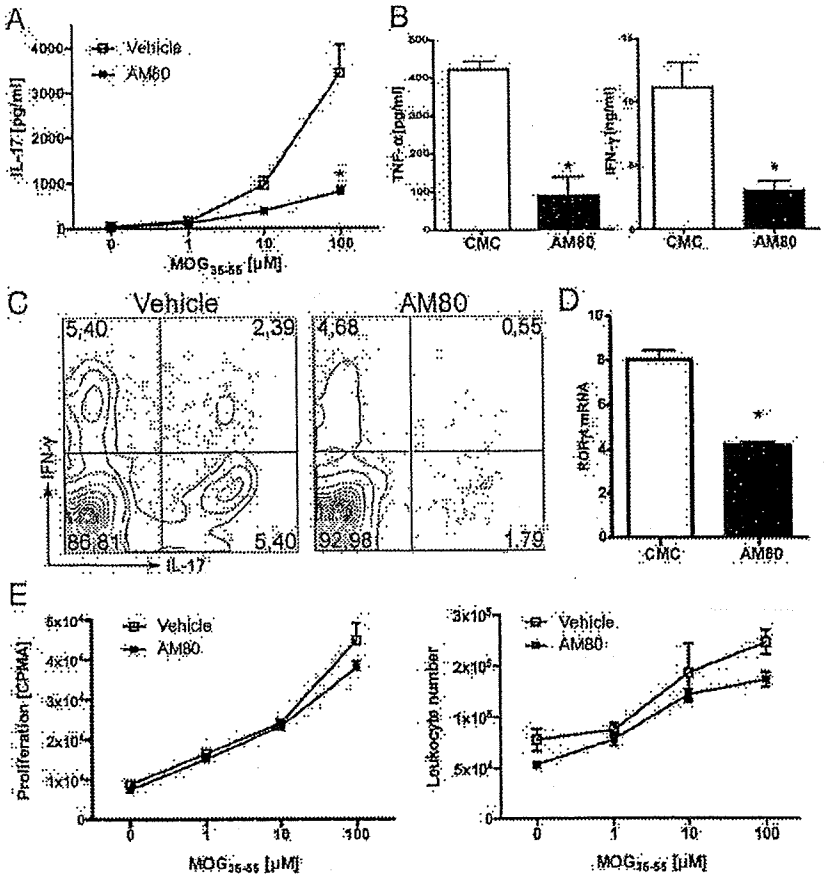

Figure 3. RAR agonist AM80 suppresses Th17 differentiation without inhibiting typical proliferative responses *in vivo*. B6 mice were immunized with MOG_{35-55} in CFA and vehicle (CMC) or AM80 (3 mg/kg in CMC) were administered orally every other day starting from day 0 until day 8. Draining lymph node cells were isolated at day 10 and restimulated with MOG peptide at various concentrations. After 72 hours, antigen-specific IL-17 production was assessed by ELISA (A). In B, TNF- α and IFN-γ production after restimulation with 100 μmol/L MOG were assessed by CBA. C: Intracellular cytokine staining of draining lymph node cells in the presence of 10 μmol/L MOG_{35-55} after 96 hours of culture shows reduced percentages of IL-17$^+$ and IL-17$^+$/IFN-γ^+ double producing cells. Plots are gated on TCR$^+$CD4$^+$ lymphocytes. D: Expression of RORγt in T cells obtained from C was assessed by quantitative RT-PCR. E: Cell proliferation was assessed either by [^{3}H]thymidine incorporation or cell number evaluation by fluorescence-activated cell sorting. Data in A, B, and E are representative of four independent experiments with three to five mice per group and C and D depict results from two experiments with three mice per group.

After transfer of MOG-reactive T cell blasts into naive mice, AM80-treated T cells caused only minimal disease compared with a significant disease development after transfer of vehicle-treated T cells (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). In addition, when encephalitogenic T cells isolated from vehicle-treated animals were incubated *ex vivo* with AM80 for 2 hours before adoptive transfer into naïve mice, no disease developed (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). Taken together, the suppressive capacity of AM80 is attributed, at least in part, to a direct inhibitory effect on encephalitogenic T cells *in vivo*.

AM80 Suppresses Antigen-Specific IL-17 Production of T Cells

Since AM80 treatment suppressed the onset of clinical EAE and also inhibited inflammatory cellular responses in the target organ, we set out to elucidate the cellular and molecular mechanism by which AM80 suppresses EAE development by examining antigen-specific effector T cells responses with or without AM80 treatment. MOG-specific production of pro-inflammatory IL-17 by draining lymph node cells was dramatically reduced after *in vivo* treatment with AM80 (Figure 3A). In addition, production of other proinflammatory cytokines such as IFN-γ and TNF-α was also significantly reduced (Figure 3B). The reduction in IL-17 secretion following retinoid treatment could result from decreased frequency of IL-17 producing T cells, or decreased production of cytokines by each T cell. Therefore, we examined cytokine production among draining lymph node T cells on a per cell basis using flow cytometric intracellular cytokine staining. Also, Th17 differentiation was estimated by quantifying RORγt expression among draining lymph node, using quantitative RT-PCR. As shown in Figure 3C, the IL-17-positive population in TCR$^+$CD4$^+$ subset was reduced after treatment with AM80, indicating that the treatment resulted in a lower frequency of IL-17 producing T cells developing in draining lymph node. In line with these findings, isolated T cells from animals that had received AM80 treatment expressed lower levels of RORγt (Figure 3D). Interestingly, unlike the effect of AM80 *in vitro*, such reductions in RORγt expression were not associated with an increase in Foxp3 expression (data not shown).

It is conceivable that AM80 may protect from EAE by generating systemic immunosuppression. Indeed, a previous study using relatively high doses of the broad spectrum RAR agonist ATRA to treat EAE was unable to rule out this mechanism of action as administration of ATRA resulted in a suppression of peripheral proliferative T cell responses.[30] With our treatment approach, however, cellular proliferation as measured by [^{3}H]thymidine incorporation and increase in cell numbers determined by fluo-