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

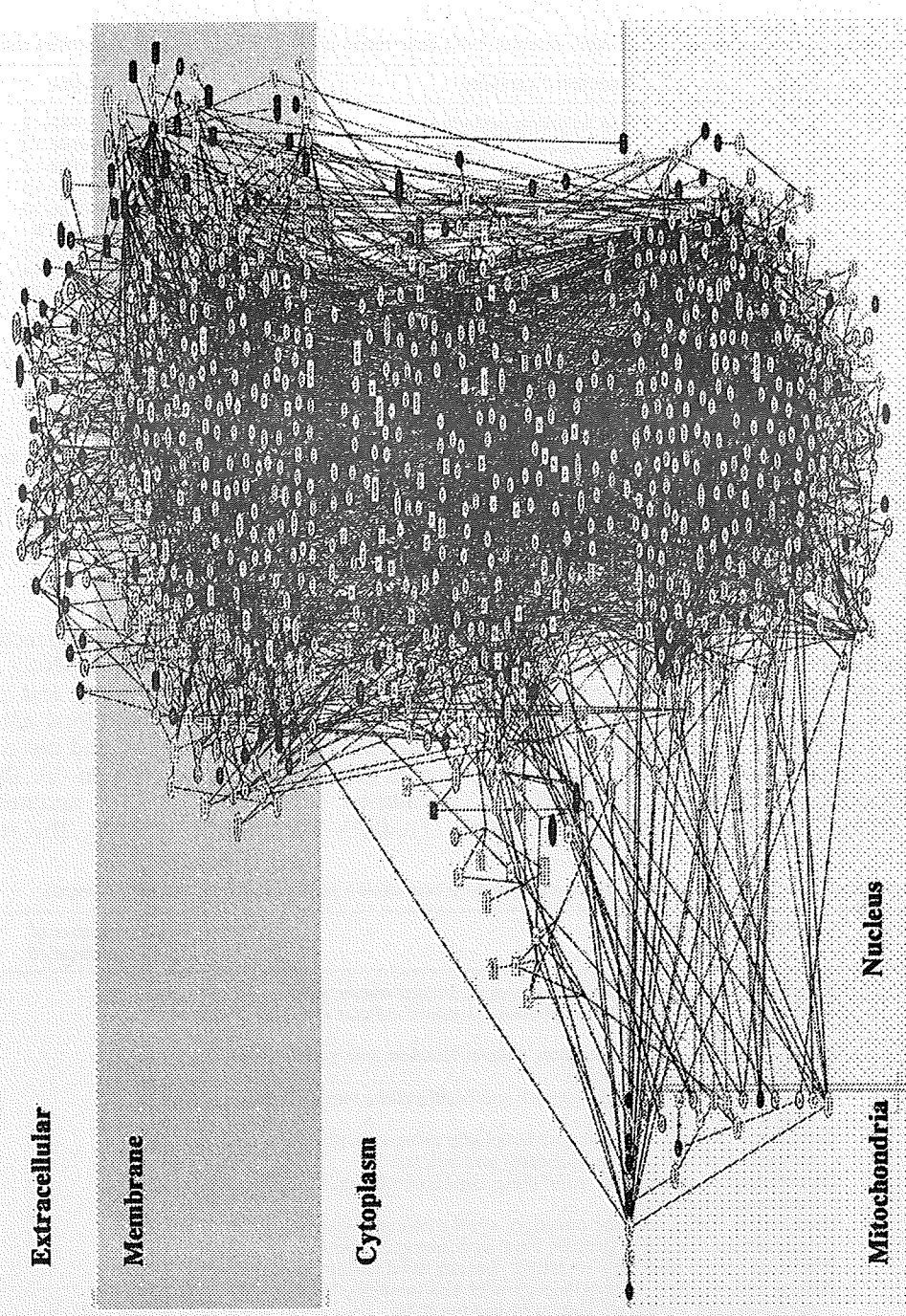

Figure 2 The molecular network of the CAP proteome suggested by KeyMolnet. The list of Entrez Gene IDs of CAP-specific proteome was uploaded onto the 'N-points to N-points search' tool of KeyMolnet. This generated a complex network composed of 1,120 fundamental nodes with 2,772 molecular relations, constructed 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.

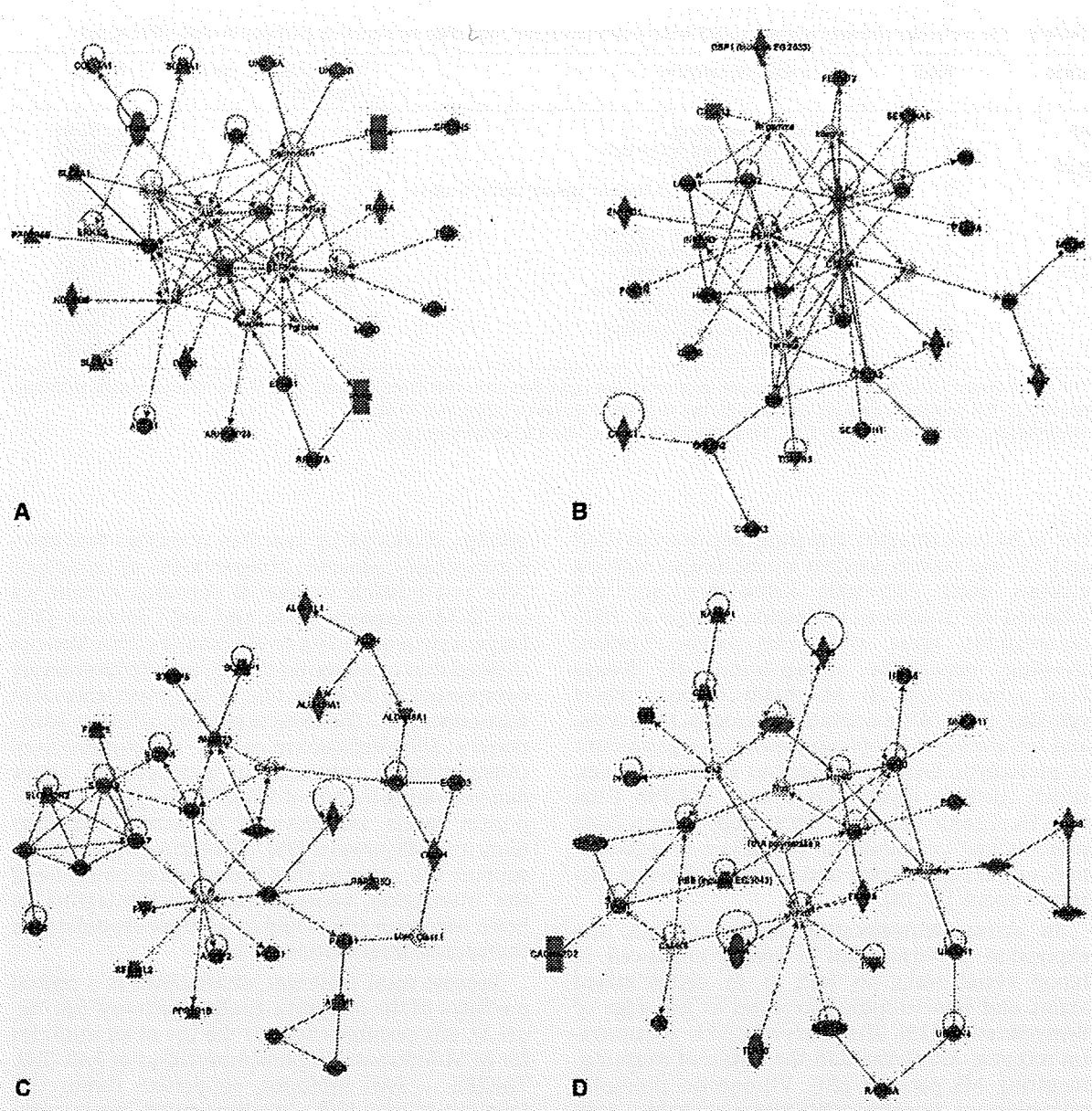

Figure 3 The molecular network of the AP, CAP, and CP proteome suggested by IPA. The list of Entrez Gene IDs of the MS lesion–specific proteome was uploaded onto the 'Core Analysis' tool of Ingenuity pathway analysis (IPA). Molecular networks most relevant to the AP (A), CAP (B and C), or CP (D) proteome 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

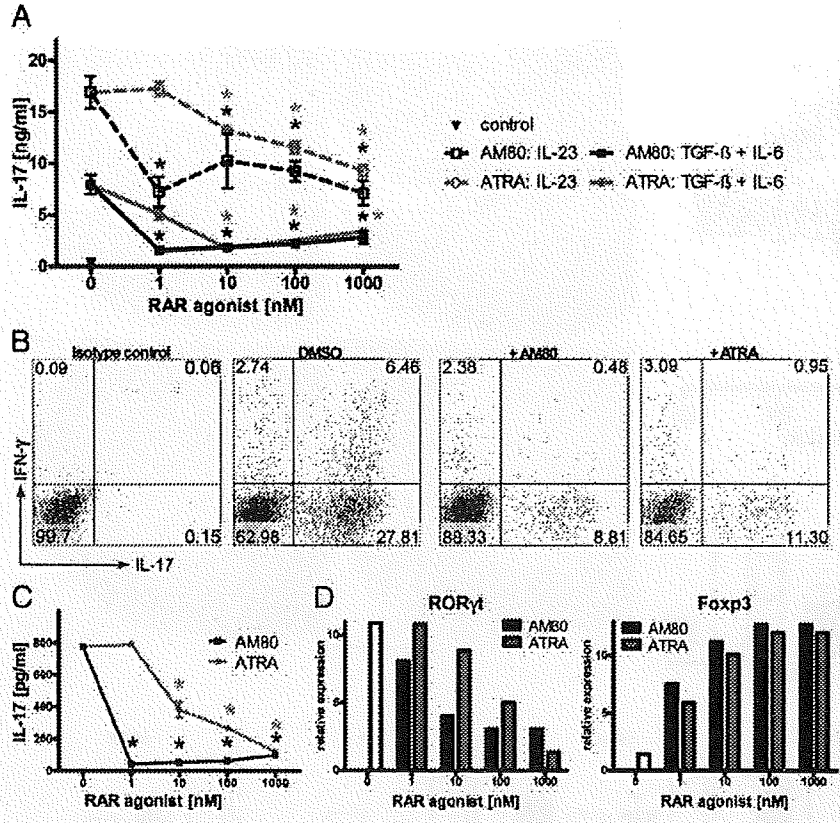

Figure 1. AM80 is a potent inhibitor for Th17 cell differentiation *in vitro*. Whole splenocytes were stimulated with soluble anti-CD3 with retinoids added at a range of concentrations. **A:** Cells were cultured in the presence of IL-23 (broken line), TGF-β and IL-6 (solid line), or without the addition 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

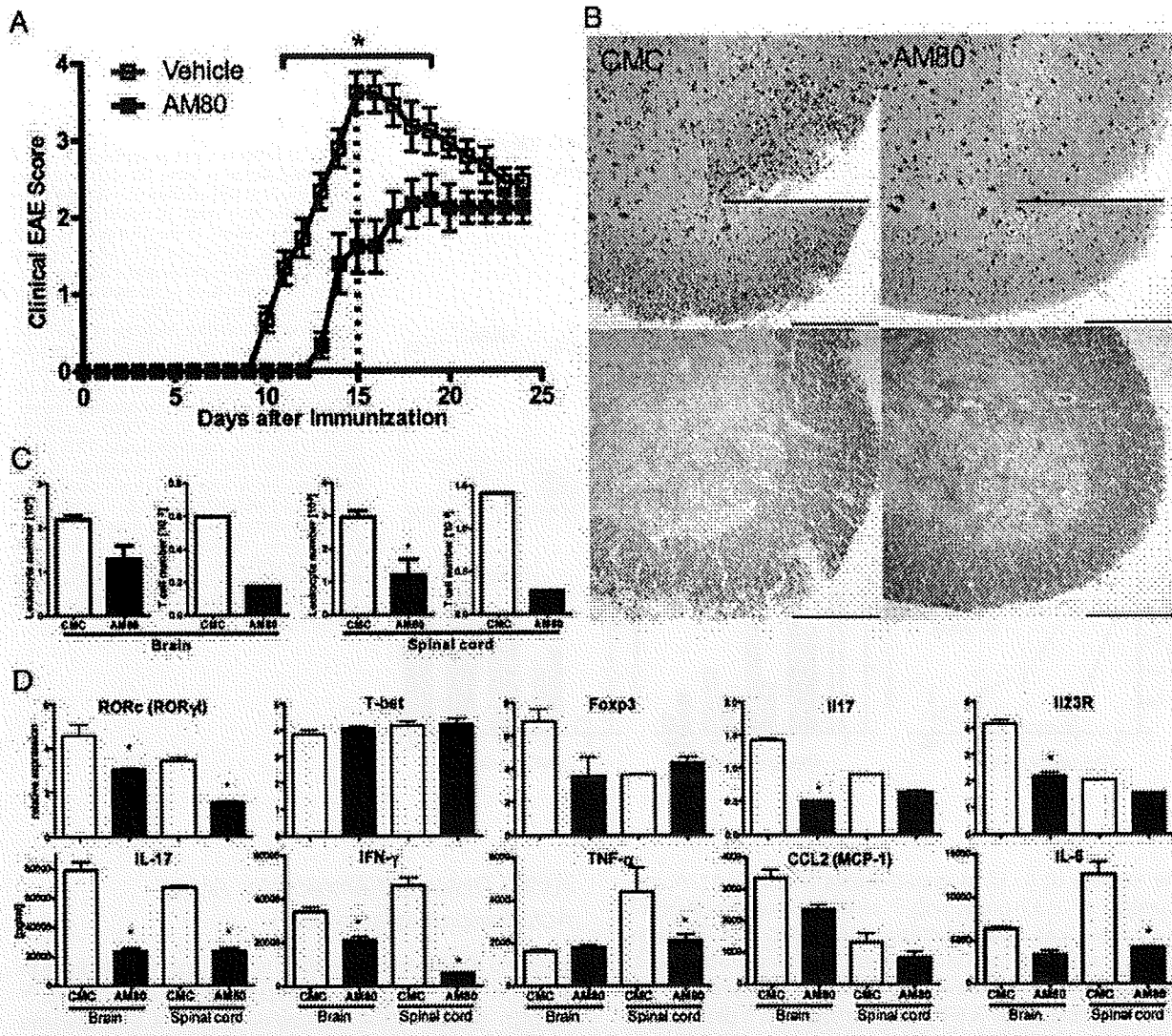

Figure 2. AM80 treatment ameliorates EAE with reduction of IL-17 production *in vivo*. EAE was induced in B6 mice by immunization with MOG_{35-55}. Groups of mice received either CMC or 3 mg/kg AM80 in CMC orally every other day from day 0. Mice were scored daily for clinical disease (**A**) analysis 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.

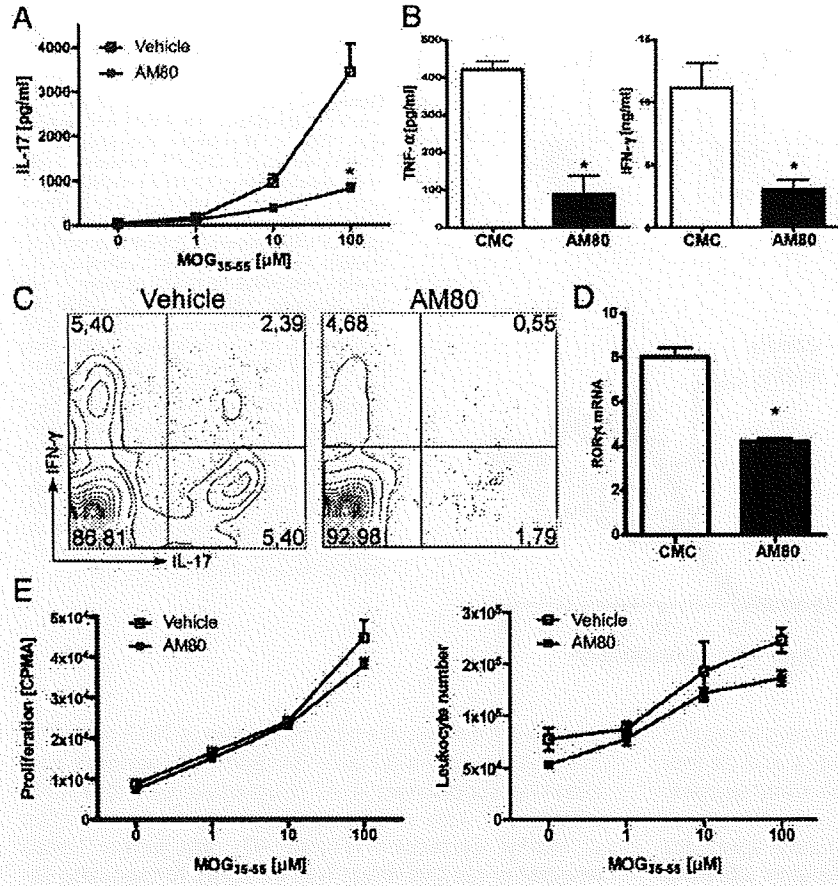

Figure 3. RAR agonist AM80 suppresses Th17 differentiation without inhibiting typical proliferative responses *in vivo*. B6 mice were immunized with MOG_{35-55} in CFA and vehicle (CMC) or AM80 (3 mg/kg in CMC) were administered orally every other day starting from day 0 until day 8. Draining lymph node 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 $IL\text{-}17^+$ and $IL\text{-}17^+/IFN\text{-}\gamma^+$ 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-

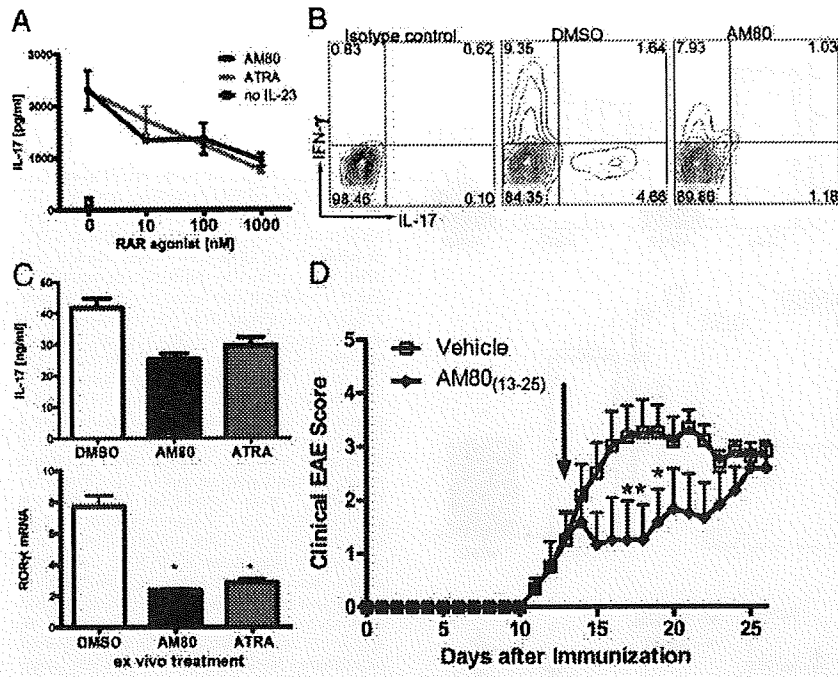

Figure 4. AM80 suppresses IL-17 production by differentiated Th17 cells and ameliorates EAE in therapeutic intervention. **A:** $CD4^+CD44^+CD25^-CD62L^{low}$ memory T cells were stimulated by plate-bound anti-CD3 antibody for 4 days in the presence of IL-23. Dose-dependent decrease of IL-17 production by RAR agonists was shown in left panel. Intracellular cytokine staining of TCR^+CD4^+ cells shows decreased percentage of $IL\text{-}17^+$ memory T cells after treatment with 100 nmol/L AM80 (**B**). Graphs are representative of two independent experiments. **C:** CNS-infiltrating T cells from mice with severe EAE (score 3–4) were isolated and restimulated with immobilized anti-CD3 in the presence of RAR agonists (100 nmol/L) for 3 days. Supernatants were analyzed for IL-17 production by ELISA and cells were subjected to quantitative RT-PCR for RORγt expression. Data are a representative of four similar experiments. **D:** Clinical EAE scores of animals treated daily from day 13 on (**arrow**) either with vehicle (CMC) or AM80. Displayed scores are representative of two experiments with $n = 6$.

rescence-activated cell sorting following re-stimulation with antigen did not differ between treated and control groups (Figure 3E). Collectively, these data suggest that rather acting as a systemic immunosuppressive agent, AM80 acts as an immunomodulary agent for antigen-specific T cell responses, primarily affecting Th17 cell function *in vivo*.

AM80 Treatment Ameliorates Ongoing Inflammatory Responses by Suppressing IL-17 Production from Differentiated Th17 Effector Cells

As we wished to evaluate the therapeutic potential of AM80 for MS treatment, we tested whether or not AM80 had an inhibitory effect on Th17 effector function in addition to its effect on Th17 differentiation. To this end, we stimulated memory cells in the presence of IL-23, a cytokine that promotes Th17 memory cell function and survival,[9] and in the presence of increasing doses of AM80 or ATRA. AM80 and ATRA suppressed IL-17 production by memory T cells responding to anti-CD3 mAb activation (Figure 4A). Intracellular cytokine staining also demonstrated that those retinoids inhibit IL-17 production by differentiated Th17 cells (AM80, Figure 4B; ATRA, data not shown). Furthermore, we also confirmed that IL-17 production by T cells differentiated *in vitro* by a combination of TGF-β and IL-6, was effectively inhibited in the presence of AM80 after restimulation with immobilized anti-CD3 mAb (data not shown). As Th17 cells are shown to have an unstable phenotype when differentiated *ex vivo*,[43] we examined how retinoids affect the function of Th17 cells that have stably differentiated *in vivo*. We stimulated CNS-infiltrating T cells isolated from mice at peak EAE, which consist of a high proportion of Th17 cells,[42] in the presence of AM80 or ATRA. Both RAR agonists successfully suppressed IL-17 production by those CNS infiltrating T cells concomitant with significant reduction of RORγt expression (Figure 4C). We further tested whether or not this effect was sufficient to modulate established EAE. Rescue treatment with AM80 starting after the onset of disease significantly suppressed the increase of disease scores that was observed in control vehicle-treated EAE mice (Figure 4D). The maximal disease score was reduced from 3.4 ± 0.39 in untreated EAE mice to 2.58 ± 0.47 in AM80-treated animals. Taken together, these data indicate the retinoid treatment is effective at inhibiting the function of activated Th17 cells.

Continuous AM80 Treatment Alters Cytokine Profile in the Chronic Phase of Disease

Our data have demonstrated that treatment with AM80 can both protect and rescue from EAE, associated with a suppression of pathogenic Th17 cell differentiation and function. However, when observed at later time points after EAE induction, the disease scores of both groups became alike (Figures 2A and 4D). Accordingly at such time points, there is no clear differences between treatment groups in terms of cellular infiltrates in CNS tissue as observed by histology (Figure 5A) or flow cytometry (Figure 5B). We then investigated the effector properties of CNS-infiltrating T cells derived from either vehicle-treated or AM80-treated animals with equivalent disease scores at day 25 after EAE induction. CNS-infiltrating T cells derived from AM80-treated animals contained strongly decreased levels of mRNA transcripts for RORγt, IL-23 receptor and IL-17 (Figure 5C). In addition, CNS-

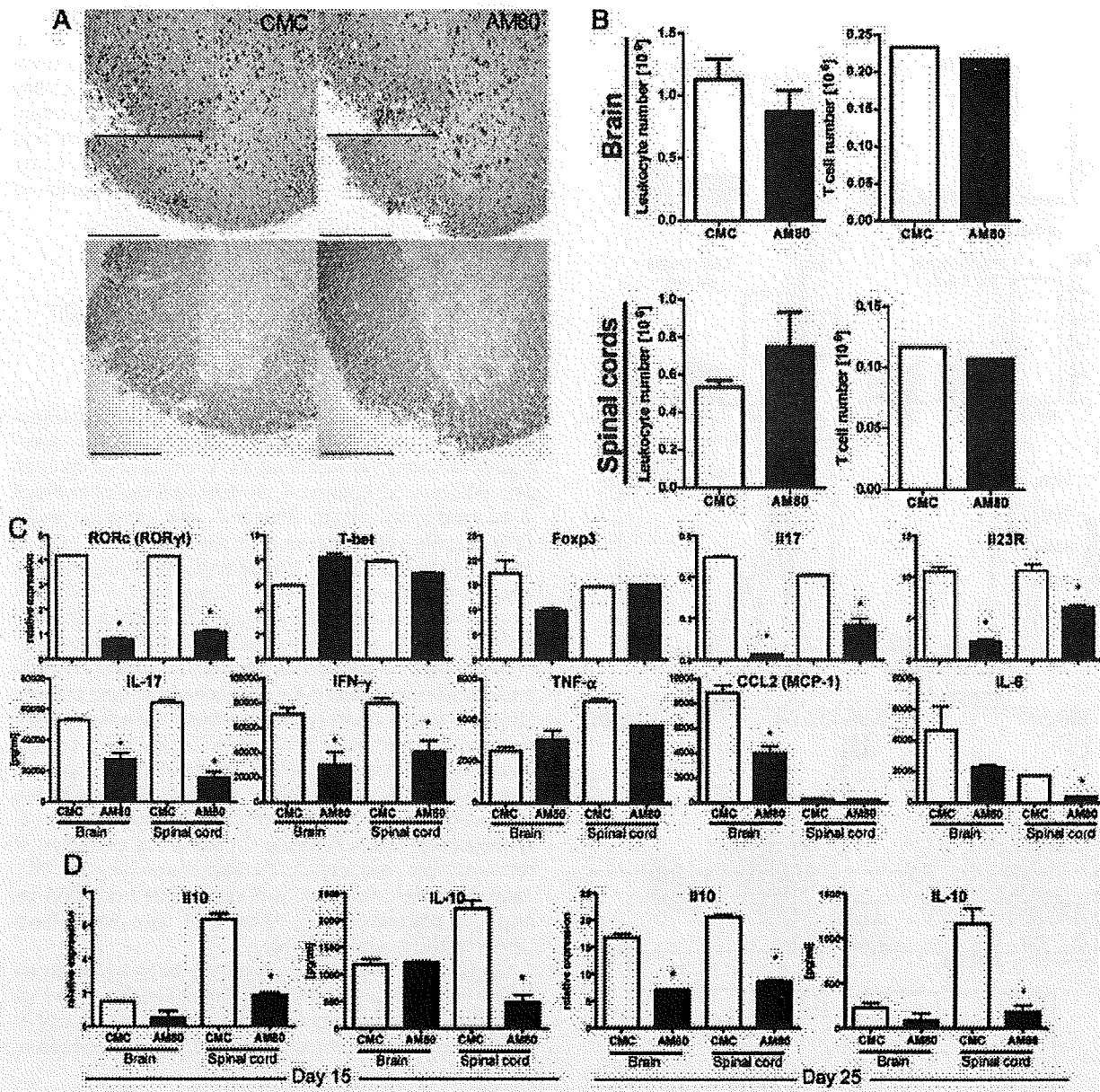

Figure 5. Continuous AM80 treatment is less effective on EAE suppression with a differentially modulated cytokine profile. **A:** The H&E-stained sections and the Luxol fast blue-stained sections in EAE mice treated with or without AM80 are shown. The control animal (score 2) and AM80-treated animal (score 2) at day 25 were subjected to histological examination. Scale bar = 200 μm. **B:** Total leukocyte numbers and isolated T cell numbers from spinal cords were evaluated in animals that had received CMC or AM80 at day 25 after immunization. The **upper row** of **C** depicts purified T cells from **B** that were subjected to quantitative RT-PCR. Error bars represent duplicated PCR of the same samples. In the **lower row** of **C**, CNS-infiltrating T cells were restimulated with immobilized anti-CD3 mAb and cytokine or chemokine production in culture supernatants were assessed by ELISA or CBA after 72 hours. Error bars represent measurements from duplicate wells (*$P < 0.001$). **D:** IL-10 production by stimulated CNS-infiltrating T cells derived from CMC-treated or AM80-treated animals were assessed by either quantitative RT-PCR or CBA. CNS-infiltrating T cells were isolated at day 15 or day 25 after EAE induction. **B** is a representative of four independent experiments; **A**, **C**, and **D** of two with six animals per group.

infiltrating T cells of AM80-treated animals produce significantly lower amounts of proinflammatory cytokines (IL-17, IFN-γ, and IL-6) and chemokines (CCL2) on restimulation *in vitro* (Figure 5C). Furthermore, although we have observed disease development until day 45, there were no further differences between treatment groups over this time period (data not shown). Collectively, these data suggest that inhibition of Th17 cell function alone is not sufficient to protect mice from later onset of the disease. Foxp3 expression in CNS infiltrating T by quantitative RT-PCR (Figure 5C) or intracellular Foxp3 staining (data not shown) showed no apparent differences between treatment groups, albeit a trend toward lower levels of Foxp3 expression in brain infiltrating T cells of AM80 treated animals could be observed. Next, we investigated several regulatory cytokines and found that levels of IL-10 were reduced in CNS-infiltrating T cell of animals that had received AM80 (Figure 5D). Interest-

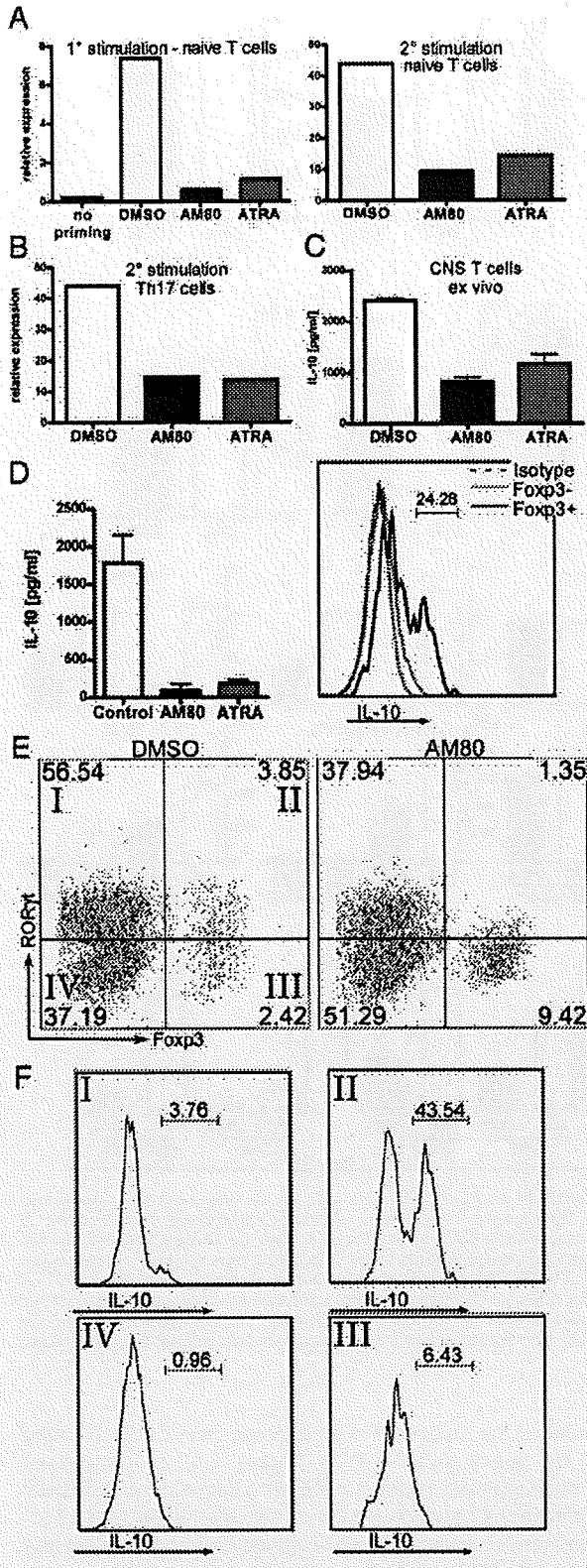

Figure 6. Continuous AM80 treatment impairs IL-10 production by T cells, which are identified as RORγt$^+$/FOXP3$^+$ population. **A:** Naïve T cells were cultured under neutral (no priming) or Th17-priming conditions in the presence of retinoids (100 nmol/L). After 96 hours, expression of IL-10 mRNA was assessed by quantitative RT-PCR. For further analysis, cells were rested for 48 hours and restimulated with anti-CD3 antibody for another 96 hours and examined for expression of IL-10 transcript. **B:** Naive T cells stimulated under Th17 priming conditions without RAR agonists were then restimulated as described above. RAR agonists (100 nmol/L) were added during the secondary stimulation and assessed for their expression of IL-10 mRNA by quantitative RT-PCR. Results are representative of two independent experiments. **C:** CNS-infiltrating T cells were isolated from EAE animals (score 3) and restimulated with immobilized anti-CD3 mAb in the presence of AM80 or ATRA (100 nmol/L) for 72 hours. The amount of IL-10 in culture supernatants was examined by ELISA. **D:** Whole splenocytes were cultured for 96 hours with TGF-β and IL-6 and RAR agonists (10 nmol/L) and CBA analysis were performed for analyzing the levels of IL-10 production in the culture supematants. Cells were then subjected to intracellular cytokine staining by fluorescence-activated cell sorting. The histogram gated on CD45^{high}TCR$^+$CD4$^+$ lymphocytes displays the comparative population of IL-10$^+$ cells by Foxp3$^+$ (black line) and Foxp3$^-$ (gray line) cell populations. Broken line represents the data stained with isotype control antibody. **E:** CD45^{high}TCR$^+$CD4$^+$ lymphocytes derived from **D** were analyzed for their expression of RORγt and Foxp3. Comparative dot plots were shown with or without AM80 treatment (10 nmol/L). Addition of AM80 decreased the number of RORγt$^+$ cells, including the RORγt$^+$FOXP3$^+$ cells. **F:** The levels of IL-10 producing cells from the quadrants labeled in **E** are shown.

ingly, we observed a more profound inhibitory effect of AM80 on IL-10 production by CNS-infiltrating T cells at the later time point after EAE induction (day 25) compared with those in earlier phases of the disease (day 15). Despite simultaneous suppression of IL-17 production, continuous AM80 treatment may deplete the host immune system of its intrinsic T cell production of IL-10, leading to a possible loss of the protective function of retinoids.

Continuous Treatment with RAR Agonists Abrogate IL-10 Production by T Cells, Which Are Identified To Be RORγtFoxp3 Double Positive

As continuous AM80 treatment suppressed the production of IL-10 by CNS-infiltrating T cells, we further investigated the effect of AM80 on the production of IL-10 *in vitro*. As shown in Figure 6A, we detected higher levels of IL-10 transcripts among effector T cells activated under Th17 priming conditions as compared with those primed under neutral conditions, supporting a previous study in which demonstrate increase IL-10 secretion in Th17 cultures on TGF-β and IL-6 signaling.[24] Addition of RAR agonists during the primary culture confers reduced expression of IL-10 transcripts, even when cells underwent a secondary stimulation in the absence of retinoids, pointing toward a stable phenotype. To assess the effect of retinoids on differentiated Th17 cells, T cells were primed under Th17 priming conditions without retinoids, but restimulation in the presence of retinoids decreased the expression of IL-10 (Figure 6B). In addition, *ex vivo* restimulation of CNS-infiltrating T cells in the presence of retinoids also reduced IL-10 expression (Figure 6C). Taken together, treatment with either AM80 or ATRA inhibits the production of IL-10 by Th17 cells, which have differentiated *in vitro* or *in vivo*.

Regulatory T cells have previously been indicated as main source of IL-10-producing T cell subsets.[44] In Figure 6D, we confirm that Foxp3$^+$ cells are the source of IL-10 in whole splenocyte stimulated in the presence of

TGF-β and IL-6. Interestingly, addition of retinoids expanded the number of Foxp3$^+$ cells (data not shown), but abolished their production of IL-10 (Figure 6D). Recently, a further subset of IL-10 producing regulatory T cells that express of RORγt and Foxp3 simultaneously have been highlighted.[25] Therefore, we hypothesized that such cells represent the major population of IL-10-secreting T cells in Th17 differentiation cultures and thus-it is these cells on which retinoids act to abolish IL-10 production. In fact, Foxp3$^+$ cells could be successfully divided into RORγt$^+$ and RORγt$^-$ populations (Figure 6E). Addition of AM80 to cultures reduced the proportion of RORγt$^+$ and RORγt$^+$Foxp3$^+$ double positive cells, but increased the proportion of Foxp3$^+$RORγt$^-$ cells (Figure 6E), at the same time as abolishing IL-10 production (Figure 6D). Intracellular IL-10 staining revealed that the RORγt$^+$Foxp3$^+$ double positive population was the predominant source of IL-10 (Figure 6F). Taken together, these data suggests that retinoid treatment reduces the production of IL-10 by inhibiting the effector function of a distinct RORγt$^+$Foxp3$^+$ population, which might serve as a regulatory T cell subset.

Discussion

ATRA, the physiologically active metabolite of vitamin A, inhibits the differentiation of Th17 cells and reciprocally promotes the generation of Treg cells *in vitro*.[27,29] In this study, we demonstrate for the first time that the synthetic RAR agonist AM80 inhibits the differentiation and effector function of Th17 cells more effectively than ATRA. Oral administration of AM80 attenuates antigen-specific Th17 cell differentiation, thus such treatment is able to reduce disease in the acute phase of EAE.

ATRA suppresses Th17 differentiation and effector function via RARα signaling,[27,28] but ATRA can also bind to RARβ and RARγ, which can form a variety of homo- and heterodimers with three RXR receptors.[26,34] Non-selective receptor binding is thought to be a major cause of the side effects associated with the administration of ATRA and other pan-RAR agonists in the clinic. AM80 is a synthetic RAR agonist that has high affinity to the RARα/β and is currently available as medication for human diseases such as APL and psoriasis.[35–37,45] In addition to greater specificity for RARα, AM80 offers several other advantages over ATRA as a therapeutic agent: it has a lower toxicity, higher stability, fewer potential side effects, and a superior bioavailability. Also, we demonstrate that a lower dose of AM80 is required to inhibit IL-17 production by T cells than similar treatment with ATRA. Interestingly, at very low doses, AM80 treatment reduced IL-17 production dramatically, while RORγt expression was only slightly reduced. Although RORγt expression is a pre-requisite for Th17 development,[17] recent studies have demonstrated that Foxp3 can physically interact with RORγt inhibiting IL-17 production when both are co-expressed.[25,46,47] It is conceivable that the disconnection between RORγt expression and IL-17 secretion following low dose AM80 treatment we observed may result from concomitant Foxp3 up-regulation. However, at higher doses of AM80, the inhibition of IL-17 production was associated with a reduction in RORγt expression; thus there may be multiple mechanisms of action in operation.

A recent study demonstrated that intraperitoneal injection of high doses of ATRA protected animals from EAE induction and that this protection was associated with reduced IL-17 and IFN-γ production.[30] However, such treatment was also found to reduce proliferative T cells responses following antigen restimulation *ex vivo*,[30] suggesting that, in this study, ATRA may ameliorate EAE by generating systemic immune-suppression. We have observed similar immunosuppression by ATRA and other retinoids at a high dose (data not shown). In our study, we treated with AM80 at a dose 5–10 times lower than the dose that ATRA has been previously tested at and we were able to administer the retinoid orally. Our treatment regimen also suppressed Th17 cell differentiation and IL-17 production, but antigen-specific T cell proliferation was not altered. Thus, we were able to target pathogenic Th17 cells specifically, without inducing general immunosuppression.

We and others have found that there is no induction of regulatory T cells when treating inflammatory diseases with RAR agonists,[29,30] and it has been speculated that this may be due to a lack of TGF-β *in vivo*.[29] An alternative hypothesis is that Treg generation is inhibited by the strong induction of inflammatory cytokines, including IL-6, TNF-α, and IL-1.[30] Thus, under both of these suggestions, it is likely that AM80 suppresses EAE by inhibiting the generation and activity of Th17 cells.

AM80 treatment inhibited acute EAE in mice, but continuous administration of AM80 did not suppress chronic inflammation. Interestingly, T cells isolated from the CNS tissue of AM80-treated mice during the chronic phase of the disease continued to express only low levels of RORγt, IL-23 receptor, and IL-17. This was in marked contrast to vehicle-treated control mice, which, despite analogous clinical disease scores, had CNS-infiltrating T cells that expressed high levels of Th17-related factors. Thus, we suggest that inhibition of Th17 cell function alone is not sufficient to protect mice from chronic CNS inflammation.

In fact, we found that treatment with RAR-agonists suppressed T cell production of IL-10 at late stage disease. Recently, a unique T cell subset that co-expresses RORγt and Foxp3, and predominantly secretes regulatory IL-10 has been identified *in vivo*.[23] Intriguingly, we show that RAR agonists not only suppress pathogenic Th17 cells, but also suppress IL-10 production by these RORγt+Foxp3+ T cells. Korn *et al* propose a model of sequential infiltration by different subsets of differentiated CD4$^+$ T cells during organ-specific autoimmunities such as EAE. In this model, Th17 cells mediate the acute phase of disease, while Th1 cells are more prominent in the chronic phase, and at much later phase of disease, there is a moderate up-regulation of IL-10 production.[48,49] In addition, previous studies indicate that IL-10 is a key cytokine for the suppression of T cell-mediated autoimmune inflammation in the CNS.[23,49] Therefore, the residual expression of IL-17 or IFN-γ at later time points

with continuous AM80 treatment may cause moderate progression of EAE development under the condition with reduced IL-10 production *in vivo*. We have little information about how those two subsets of IL-17-producing inflammatory RORγt+ T cells and IL-10-producing immunoregulatory RORγt+Foxp3+ T cells developed during immunological responses. Although we demonstrate here that the suppressive effect of AM80 on EAE is most likely through modulation of T cell production of both IL-17 and IL-10 *in vivo*, we don't exclude the possible involvement other factors, as EAE/MS are complex diseases that do not depend only on a IL-17 versus IL-10 dichotomy. For example, ongoing neurodegeneration[50] may also contribute significantly to the observed phenotype especially at the later phase of disease. As Th1 cells also play a significant role in the induction or EAE/MS, we should also point out that retinoic acid has been previously shown to exert direct effects on T cells, suppressing Th1 development and enhancing Th2 development via retinoic acid receptors.[51] This indicates that AM80 has multiple beneficial effects for disease protection through oral administration. The immunomodulatory effect of RAR agonists on those Th17-like regulatory T cells in inflammatory autoimmune diseases is to be considered if conducting *in vivo* attenuation of Th17 cells for treatment of autoimmune diseases with retinoids. At this point in time, we advocate that the use of AM80 on inflammatory autoimmune diseases should target the acute phases of Th17-mediated pathogenesis.

In addition, we found that T cells that had infiltrated the spinal cord produced much lower amounts of CCL2 (MCP-1) as compared with brain-infiltrating T cells. CCL2 plays a crucial role in the progression of EAE[52,53] and previously we have shown that human Th17 cells express the corresponding receptor, CCR2.[54] Our data showing reductions in CCL2 in the spinal cord may represent previously reported differences in inflammatory cell populations at different CNS sites.[55]

In summary, we demonstrate that oral treatment with AM80 effectively inhibits IL-17 production without generating systemic immunosuppression. In addition, AM80 treatment protected mice from the development of acute EAE and rescued mice with established EAE from acute autoimmune inflammation. Collectively, these data advocate AM80 as a potent therapeutic agent against acute Th17-mediated autoimmune diseases including MS.

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Suppression of Experimental Autoimmune Encephalomyelitis by Ghrelin[1]

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Ghrelin is a recently identified gastric hormone that displays strong growth hormone-releasing activity mediated by the growth hormone secretagogue receptor. While this unique endogenous peptide participates in the regulation of energy homeostasis, increases food intake, and decreases energy expenditure, its ability to inhibit the production of proinflammatory cytokines in vitro indicates its role in the regulation of inflammatory process in vivo. Here we examine the effect of exogenous ghrelin on the development of experimental autoimmune encephalomyelitis (EAE), a representative model of multiple sclerosis. In the C57BL/6 mouse model of EAE induced by sensitization to myelin oligodendrocyte glycoprotein 35–55 peptide, we found that alternate-day s.c. injections of ghrelin (5 μg/kg/day) from day 1 to 35 significantly reduced the clinical severity of EAE. The suppression of EAE was accompanied by reduced mRNA levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the spinal cord cellular infiltrates and microglia from ghrelin-treated mice at the peak of disease, suggesting the role of ghrelin as an antiinflammatory hormone. Consistently, ghrelin significantly suppressed the production of proinflammatory cytokines in LPS-stimulated microglia in vitro. These results shed light on the new role of ghrelin in the regulation of inflammation with possible implications for management of human diseases. *The Journal of Immunology,* **2009, 183: 2859–2866.**

Small synthetic compounds, referred to as growth hormone (GH)[3] secretagogues (GHS), have been known to stimulate GH release, working through a G protein-coupled receptor called GHS receptor (GHS-R) (1–3). It is now established that a new endogenous peptide, ghrelin, discovered in rat gastric extracts, is an endogenous ligand for GHS-R and is involved in the regulation of GH release. Ghrelin is a 28-aa polypeptide with an essential *n*-octanoyl modification on serine at position 3 (4). Although ghrelin is predominantly secreted from mucosal endocrine cells of stomach, it is widely distributed in various organs, including lymphoid tissues (5, 6). Furthermore, it is measurable in the systemic circulation, indicating its hormonal nature (7).

Ghrelin does not only stimulate GH release, but it also increases food intake, regulates energy homeostasis, and decreases energy expenditure by lowering the catabolism of fat (4, 8, 9). Because of its orexigenic and adipogenic character, ghrelin may be potentially useful for the treatment of anorexia and cachexia (10, 11). Although the precise mechanisms remain to be clarified, the orexigenic activities of ghrelin may be mediated by another feeding regulatory hormone neuropeptide Y (NPY) via stimulation of Y1 and Y5 receptors (12). Furthermore, the antagonistic effect of ghrelin on leptin-induced decrease of food intake seems to be mediated by ghrelin-induced release of NPY and subsequent stimulation of the Y1 receptor (13).

Ghrelin has been shown to exhibit antiinflammatory functions against T cells and macrophages in vitro (14–16). The potential activity of ghrelin as antiinflammatory reagent in vivo was shown in several animal models, including bowel disease (17), arthritis (16, 18), sepsis, and endotoxemia (16, 19, 20). Here we report that s.c. injections of ghrelin could significantly attenuate the clinical severity of the representative model of experimental autoimmune encephalomyelitis (EAE) induced in C57BL/6 (B6) mice by sensitization against myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide. Furthermore, we demonstrate that in vivo treatment with ghrelin significantly suppressed the mRNA levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in microglia and infiltrating T cells derived from the spinal cords of ghrelin-treated mice. Finally, we confirm that LPS-stimulated microglia and monocytes produced lower amounts of proinflammatory cytokines when they were pretreated with ghrelin in vitro. In conclusion, the present study indicates the potential use of ghrelin as an antiinflammatory drug to control human CNS pathology.

Materials and Methods

Mice and reagents

We used female B6 mice (CLEA Japan) between 6 and 10 wk of age in specific pathogen-free conditions. Animal care and use were in accordance

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[3] Abbreviations used in this paper: GH, growth hormone; EAE, experimental autoimmune encephalomyelitis; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; NPY, neuropeptide Y.

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Table I. *Amino acid sequence of mouse ghrelin and des-acyl ghrelin*

Peptide	Amino Acid Sequence[a]	Ser^3 acylation	Reference
Ghrelin	GS<u>S</u>FLSPEHQKAQQRKESKKPPAKLQPR	*n*-Octanoicacid	(4)
Des-acyl ghrelin	GS<u>S</u>FLSPEHQKAQQRKESKKPPAKLQPR		(7)

[a] The underlined letter S represents the third serine (Ser^3).

with institutional guidelines. Animal experiments were approved by our institutional review committee. Rat MOG_{35-55} (amino acid sequence MEVGWYRSPFSRVVHLYRNGK) was synthesized at Toray Research Center (Tokyo, Japan). Ghrelin and des-acyl ghrelin (Table I) were synthesized as previously described (4, 7).

Immunization and clinical assessment of EAE

We immunized mice (n = 5–15 per group) s.c. in the tail base with 100 μg of MOG_{35-55}-peptide dissolved in 0.1 ml of PBS and 0.1 ml of CFA containing 1 mg of *M. tuberculosis* H37Ra (Difco). Shortly after immunization and 48 h later, the mice were injected i.p. with 200 ng of pertussis toxin (List Biological Laboratories). Clinical scores of EAE were daily assigned as follows: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, forelimb paralysis or moribund; 6, death. The cumulative scores were calculated for individual mice by summing up the daily scores.

Administration of ghrelin and des-acyl ghrelin

For EAE treatment, we s.c. injected ghrelin and des-acyl ghrelin diluted in 0.9% saline. In the first series of experiments, mice were injected with ghrelin or des-acyl ghrelin at doses of 0.5, 5, or 50 μg/kg every other day for 35 days. Sham-treated animals were injected with 0.9% saline (standard protocol). In the next experiment, we injected the mice with 5 μg/kg ghrelin every day from day 1 to 10 (induction phase treatment) or from day 11 to 20 (effector phase treatment) and in-between with 0.9% saline. The controls were injected every day from day 1 to 20 with 0.9% saline (alternative protocol).

Assessment of histological EAE

To evaluate the histological manifestations of EAE, we treated mice with 5 μg/kg ghrelin or 0.9% saline following the standard protocol and sacrificed them on day 17 postimmunization. The spinal cords were removed and fixed in buffered formalin. They were embedded in paraffin, sectioned, and stained with H&E and Luxol fast blue for histopathological analysis.

Flow cytometry and isolation of mononuclear cells from the CNS

B6 mice were challenged for EAE, treated following the standard protocol with 5 μg/kg ghrelin or 0.9% saline and sacrificed on day 17 postimmunization. We removed spleen, lymph nodes (LN), and thymus as well as spinal cord from the ghrelin- and saline-treated mice for flow cytometer analysis. Single-cell suspensions were prepared according to standard methods. The spinal cord cell suspensions were centrifuged at 200 × *g* for 10 min and resuspended in 4 ml of 70% isotonic Percoll (Amersham Biosciences)/PBS and overlaid by equal volumes of 37% and 30% isotonic Percoll. The gradient was centrifuged at 500 × *g* for 15 min and the mononuclear cells were harvested from the 37%–70% interface, washed, and counted. The cells were stained for 5 min with anti-FcRγ III/II mAb (BD Pharmingen), washed, and labeled with the following mAbs for surface phenotype analysis: FITC-CD4 mAb, FITC-CD19 mAb, PE-CD8a mAb, PE-NK1.1 mAb, PE-CD25 mAb, allophycocyanin-FOXP3, and PerCP-Cy5.5-CD3e mAb (BD Pharmingen) and FITC-F4/80 mAb (Dainihon Seiyaku). The cytofluorometric analysis was performed using a FACSCalibur operated by CellQuest software (BD Biosciences).

Cytokine and cell proliferation assay

MOG_{35-55}-immunized B6 mice were treated s.c. with 5 μg/kg/day of ghrelin or 0.9% saline every day from day 1 to 10. The LN cells were collected on day 11 after immunization and suspended in our standard lymphocyte culture medium (RPMI 1640 supplemented with 5 × 10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin) added with 1% syngeneic mouse serum. The cells were cultured in 96-well round-bottom plates at 1 × 10^6/well for 72 h in the presence of 100 μg/ml MOG_{35-55}. Levels of IFN-γ, IL-17, and IL-4 in the supernatant were determined by using a sandwich ELISA. Proliferative responses were measured using a Beta-1205 counter (Pharmacia) to detect the incorporation of [^{3}H]thymidine (1 μCi/well) for the final 16 h of culture.

Evaluation of encephalitogenic T cell induction in B6 mice treated with ghrelin

To evaluate whether in vivo ghrelin treatment may affect the induction of encephalitogenic T cells after immunization with MOG_{35-55}, we evaluated the ability of the lymphoid cells from ghrelin- or saline-treated mice to passively transfer EAE into naive recipients. Donor B6 mice were immunized with MOG_{35-55} and treated every day from day 1 to 10 with 5 μg/kg/day of ghrelin or 0.9% saline. We removed spleens and LN from the donor mice on day 11 and prepared lymphoid cell suspensions. The lymphoid cells were stimulated with MOG_{35-55} (33 μg/ml) in the standard medium added with FCS (10%) for 96 h and then we isolated the $CD4^+$ T cells for cell transfer by depletion of $CD8^+$, $CD19^+$, and $NK1.1^+$ cells. In brief, the MOG_{35-55}-stimulated total lymphoid cells were labeled with PE-CD8a mAb, PE-NK1.1 mAb, and PE-CD19 mAb (BD Pharmingen) for 30 min, washed, and incubated with anti-PE microbeads (Miltenyi Biotec) for 15 min. Using autoMACS (Miltenyi Biotec), we isolated $CD4^+$ T cells ($CD8^-$, $CD19^-$, and $NK1.1^-$ fraction) as a pass-through and suspended the cells in PBS. We injected 1.0 × 10^7 of the cells into the peritoneal cavity of syngeneic recipient mice that had been X-irradiated (550 rad) shortly before. We also injected 200 ng of pertussis toxin i.p. on the same day and 48 h later.

Reverse transcription and real-time PCR

To analyze the mechanism of ghrelin effects in vivo, we extracted total RNA from spinal cord, spleen, thymus, and LN samples using the RNeasy Mini Kit (Qiagen). The RNA was subjected to reverse transcription with the Advantage RT-for-PCR kit (BD Biosciences). Real-time PCR was conducted in the LightCycler quantitative PCR system (Roche Molecular Biochemicals) by using the LightCycler-FastStart DNS Master SYBR Green I kit (Roche Molecular Biochemicals). We followed the manufacturer's specification using 4 mM $MgCl_2$ and 1 pM primers. The primers used are as follows: TNF-α, CTGTGAAGGGAATGGGTGTT (sense) and GGT CACTGTCCCAGCATCTT (antisense); IL-1β, TGAAATGCCACCTTT TGACA (sense) and GTAGCTGCCACAGCTTCTCC (antisense); IL-6, TTCCATCCAGTTGCCTT–CTT (sense) and CAGAATTGCCATTGC ACAAC (antisense); TGF-β, TGCGCTTGCAGA–GATTAAAA (sense) and GCTGAATCGAAAGCCCTGTA (antisense); and HPRT, GTTGGA–TACAGGCCAGACTTTGTTG (sense) and GAGGGTAGGCTGGCCT ATAGGCT (antisense). Values are presented as the relative amount of transcript of each sample normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

In vitro effect of ghrelin on RAW 264.7 monocytes treated with LPS

To examine the effect of ghrelin on monocytes, RAW 264.7 monocytes (American Type Culture Collection) were suspended in the standard culture medium supplemented with 10% FCS and cultured in 96-well flat bottom plates at 1 × 10^5/well overnight. Various concentrations of ghrelin (10^{-6} M, 10^{-8} M, 10^{-10} M) were added to the culture and 1 h later the cells were stimulated with LPS (Sigma-Aldrich) at various doses (0.1, 1, 10 μg/ml). After 2 h of incubation at 37°C, supernatants were collected and the levels of TNF-α and IL-6 were detected by using a sandwich ELISA.

Isolation of microglial cells from the CNS

The spinal cords were incubated with 35 mg/ml Liberase Blendzyme 3 (Roche Molecular Biochemicals) and 0.1 mg/ml DNaseI (Roche Molecular Biochemicals) in RPMI 1640 medium at 37°C for 30 min. Mononuclear cells were isolated on 30%–80% discontinuous Percoll gradients and were stained with FITC-CD11b mAb, PE-CD45 mAb, and allophycocyanin-CD3 mAb (BD Pharmingen). $CD11b^{high}CD45^{high}$ macrophage cells, $CD11b^{int}CD45^{int}$ microglial cells, and $CD3^+$ T cells were isolated using