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IL-11 Induces Th17 Cell Responses in Patients with Early Relapsing-Remitting Multiple Sclerosis

Xin Zhang,* Yazhong Tao,* Manisha Chopra,* Irena Dujmovic-Basuroski,† Jianping Jin,‡ Yunan Tang,* Jelena Drulovic,† and Silva Markovic-Plese*§

Clinically isolated syndrome (CIS) suggestive of multiple sclerosis (MS) is the earliest clinically evident phase of the disease, which may provide valuable insight into the molecular mechanisms of the initiation of the autoimmune response in MS. Our results introduce IL-11 as a new cytokine that may play a role in the autoimmune response in the early phase of the disease. IL-11 is the highest upregulated cytokine in the sera and cerebrospinal fluid from CIS patients, which is also increased in patients with clinically definitive relapsing-remitting MS in comparison with healthy control subjects. Serum IL-11 levels are significantly increased during clinical exacerbations in comparison with remissions in the same patients. CD4+ cells represent a predominant cell source of IL-11 in the peripheral circulation, and the percentage of IL-11+CD4+ cells is significantly increased in CIS patients in comparison with healthy control subjects. Furthermore, we have identified IL-11 as a new Th17-promoting cytokine, because it induces a differentiation of naive CD4+ T cells into Th17 cells, as well as expansion of Th17 memory cells. Because the Th17 cytokines IL-17F, IL-21 and TNF-α, and TGF-β induce differentiation of naive cells in the IL-11-secreting CD4+ cells, we propose that cross-talk between IL-11+CD4+ and Th17 cells may play a role in the inflammatory response in relapsing-remitting MS. *The Journal of Immunology, 2015, 194: 5139–5149.

Multiple sclerosis (MS), a CNS inflammatory demyelinating disease, is a leading cause of disability in the young adult, predominantly female population (1, 2). The autoimmune pathogenesis of the relapsing-remitting MS (RRMS) has been supported by reports on the increased frequency of activated myelin-reactive CD4+ cells in patients in comparison with healthy control subjects (HCs) (3), and the association of the myelin-reactive T cell expansion with the worsening of clinical disease activity (4). However, the character of the initial disease-triggering events remains poorly understood. Clinically isolated syndrome (CIS) suggestive of MS is the earliest clinically evident phase of the disease, which may provide valuable insight into the molecular mechanisms of the initiation of the autoimmune response in MS. Diagnosis of CIS is established upon the first clinical presentation and magnetic resonance imaging evidence of at least two CNS demyelinating lesions (5). Following positive results of several large, placebo-controlled clinical trials (6–8), in which immunomodulatory treatment of CIS patients postponed conversion to clinically definitive RRMS, the U.S. Food and Drug Administration has extended the indications for immunomodulatory therapies to this early phase of the disease. Additional biomarkers of the early inflammatory response are sought to more accurately identify patients amenable to disease-modifying therapy.

The involvement of Th17 cell lineage in the development of MS has been documented by several studies (9, 10). Human Th17 cells are functionally defined by the production of the effector cytokines IL-17A, IL-17F, IL-21, IL-22, and TNF-α (11, 12), which mediate the production of inflammatory cytokines, chemokines, and metalloproteinases, and promote recruitment of granulocytes and lymphocytes to the sites of inflammation (13). Earlier studies have demonstrated an elevated IL-17A gene and protein expression in active MS brain lesions compared with normal-appearing white matter (9, 10). IL-17A gene expression is significantly increased in the mononuclear cells derived from the blood and cerebrospinal fluid (cSF) of MS patients, and the number of IL-17A-expressing blood mononuclear cells was higher during disease exacerbations than during clinically silent periods (14). Durelli et al. (15) have reported that patients with active RRMS have about a 7-fold increase in the percentage of IL-17A–producing CD4+ cells in comparison with patients with inactive disease or HCs, and that the number of Th17 cells in their peripheral circulation positively correlates with the disease activity. Anti–IL-17A mAb treatment is currently tested in a phase II clinical trial, after an initial report that this treatment induced a significant decrease in the number of new inflammatory CNS lesions (16).

Human Th17 cell differentiation is orchestrated by IL-1β, IL-6, IL-21, and IL-23, which stimulate, and IFN-γ, IL-4, IL-12, IL-10, and IL-27, which inhibit the differentiation of this cell subset, whereas the effect of TGF-β is still controversial (17–19). IL-6 critically contributes to the expression of the Th17 transcription factor retinoic acid–related orphan nuclear hormone receptor (ROR)γ and STAT3, and to IL-17A production in humans (19, 20).

Our results introduce IL-11 as a new cytokine that may play a role in the development of the autoimmune response in the early phase of MS, because IL-11 was the highest upregulated cytokine in the cSF and serum derived from CIS patients in comparison with...
HCs. Furthermore, we have identified IL-11 as a new Th17-promoting cytokine. IL-11 is a pleiotropic cytokine (21–23) and a member of the IL-6 family, with which it shares a gp130 signaling pathway (24). IL-11 competes with IL-6 for binding to the same gp130 epitopes and induces the same early response genes (25). Similar to IL-6, IL-11 induces STAT3 phosphorylation in several noninflammatory cell subsets (26–29) and induces autoreactive Ab production by B cells (30). Several studies have reported on the IL-11 anti-inflammatory effects, including the inhibition of NF-κB–mediated cytokine secretion in macrophages (31, 32) and monocytes (22), and the induction of apoptosis in myeloid dendritic cells (23, 33). However, in the recent literature, IL-11 has been strongly implicated as a proinflammatory cytokine because of its induction by oxidative stress (34), its production by synoviocytes in rheumatoid arthritis (35), and by eosinophils in atopic dermatitis (36). IL-11 induces bronchial inflammation (35, 36), and it is induced by respiratory viral infections (37, 38). Parallel increases in the IL-11 and IL-17 gene expression have been demonstrated in the animal model of rheumatoid arthritis (39), in inflammatory periodontal disease (40), and in the gastrointestinal mucosal Th17 cell inflammatory infiltrate associated with IL-11/STAT3 overexpression (29). The chromosomal region containing the IL-11 gene (19q13) has been associated with susceptibility to MS (41); however, its role in the development of the inflammatory response in MS has not been elucidated. Zhang et al. (41) have reported that IL-11 expression in MS brain lesions is primarily localized to activated astrocytes, and IL-11Rα is expressed on oligodendrocytes, but the infiltrating inflammatory cells were not studied. In vitro studies have demonstrated that IL-11Rα signaling increased oligodendrocyte survival and proliferation via STAT3 phosphorylation (33). The same group has subsequently reported a significant increase in chronic progressive experimental autoimmune encephalomyelitis activity in IL-11R knockout mice and decreased disease activity upon administration of IL-11. However, the examined disease model is a chronic progressive experimental autoimmune encephalomyelitis, and the study primarily reported decreased oligodendrocyte and neuronal numbers in the absence of IL-11 signaling, whereas the effect on the inflammatory response was mediated by the IL-11–inhibited proliferation of CD11c+ APCs (23).

Our study has demonstrated that the production of IL-11 is significantly increased in the serum and csf samples derived from CIS and RRMS patients in comparison with HCs. Moreover, IL-11 serum levels were significantly increased during clinical relapses of the disease. CD4+ cells are the main mononuclear cell source of IL-11 in the peripheral circulation, and their percentage is increased in CIS patients in comparison with HCs. We have demonstrated that in CIS patients, IL-11 induces Th17 cell differentiation and expansion of Th17 memory cells, which may contribute to the autoimmune response in MS.

Materials and Methods

Study subjects

A total of 118 CIS patients, 59 RRMS patients, and 78 control subjects was enrolled in the study upon signing an Institutional Review Board–approved informed consent form. All CIS patients had experienced their first clinical presentation consistent with demyelinating disease within a year from blood sample collection, had at least two magnetic resonance imaging lesions consistent with MS (5), and had not received immunomodulatory therapy before the blood or csf sample collection. Control subjects that have provided csf samples had the following diagnoses: headache (8), stroke (3), mental status change (3), normal pressure hydrocephalus (2), and other noninflammatory neurologic conditions (7). Demographic data of all study subjects enrolled in the study are presented in Table I. Untreated RRMS patients who have provided samples during the clinical attacks were enrolled in the study within 30 d of the onset of the new clinical symptoms, whereas the samples in remission were obtained at least 30 d after the last clinical exacerbation.

ELISA

Serum and csf samples and supernatants (SNs) from the cultured CD4+ naive and memory T cells were collected and stored at −80°C until the cytokine measurements by ELISA. The production of IL-17A, IL-17F, IL-21, IL-22, IL-9 (eBioscience), IFN-γ, IL-4, IL-10, TGF-β1 (BD Biosciences), and IL-11 (R&D Systems) was measured following the manufacturer’s recommendations. For the cytokine measurements in the serum and csf samples, the incubation was extended to 24 h at 4°C, and the detection Ab incubation was prolonged to 2 h at room temperature. The results are expressed for each subject as the cytokine concentration (in pg/ml).

Where indicated, CD4+ naive and memory T cells were isolated using a Naive and Memory CD4+ T Cell Isolation Kit (Miltenyi Biotec) from freshly PBMCs derived from six CIS patients. Naive cells were differentiated and memory cells were expanded in the presence of anti-CD3 (1 μg/ml) and anti-CD28 mAb (5 μg/ml), anti–IFN-γ and anti–IL-4 mAb (10 μg/ml), and IL-11 (10, 50, and 100 ng/ml) and anti–IL-11 (10 μg/ml) (both from R&D Systems) for 12 d before the SN collection for the indicated cytokine measurements by ELISA.

In experiments that identified cytokines that induced IL-11+CD4+ differentiation and expansion, T cells were isolated from six CIS patients. Naive cells were differentiated and memory cells were expanded in the presence of anti-CD3 and anti-CD28 mAbs, anti–IFN-γ and anti–IL-4 mAbs, and individual cytokines previously reported to induce IL-11 secretion in other cell types (42–46), including IL-17A, IL-17F, IL-21, IL-22, TNF-α, TGF-β1, IL-1β, IL-4, IL-6, IL-11, IL-12, and IL-13 for 12 d before the SN collection. The secretion of IL-11 was measured using ELISA.

The effect of IL-11 on the monocytes’ cytokine secretion was examined in three CIS patients using ELISA for the cytokine measurements in the SNs of the separated CD14+ monocytes (106 cells/condition) cultured for 48 h.

Affymetrix gene arrays

Differential gene expression between the CIS patients and HCs was tested using Affymetrix Human Gene U133 (HG-U133) arrays (Affymetrix). PBMCs were separated from 15 CIS patients and 7 HCs, and the total RNA was extracted using an RNEasy kit (Qiagen). The arrays were hybridized for 16 h at 45°C in a GeneChip Hybridization Oven 640 (Affymetrix), washed, and stained with R-PE streptavidin in a GeneChip Fluidics Station 400 (Affymetrix). The arrays were scanned with a Hewlett Packard GeneArray Scanner. Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning, and basic analysis. To detect differential gene expression profiles between the CIS patients and HCs, we used a two-class paired test of significance analysis. Differentially expressed genes were determined using a Welch two-sample t test. A p value <0.05 and >1.5-fold change was considered significant.

RT-PCR

CD4+ and CD8+ T cells, CD19+ B cells, and CD14+ monocytes were separated from the PBMCs of nine CIS patients and nine age-, sex-, and race-matched HCs using CD4+, CD8+, CD19-MicroBeads (Miltenyi Biotec) and an EasySep Negative Selection Monocyte Enrichment Kit (Stem Cell Technologies). The purity of separated cells (>98%) was confirmed by FACS. The total RNA was extracted and cDNA was synthesized using a High Capacity cDNA Archive Kit (Applied Biosystems). The primers for IL-1R1, IL-11Rα, and 18S mRNA were purchased from Applied Biosystems, and gene expression was measured by RT-PCR using TaqMan Gene Expression Assays (Applied Biosystems) in triplicates. The results are expressed as the average relative gene expression and normalized against the 18S mRNA expression for each subject.

Flow cytometry

For the intracellular cytokine staining, the fresh separated PBMCs from nine CIS patients and nine age-, sex-, and race-matched HCs were incubated in serum-free medium and stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) for 2 h and with BFA (1:1000 dilution; Invitrogen) for an additional 3 h, as previously reported (47). The cells were harvested, fixed, permeabilized, and stained with FITC-conjugated IL-17A (eBioscience) (when co-stained with IL-21, IL-22, and IL-11), IL-17F (R&D Systems), and IFN-γ (eBioscience); PE-conjugated IL-11 (R&D Systems), IL-17A (when co-stained with all cytokines except IL-21, IL-22, and IL-11), and IL-21 (eBioscience); and allophycocyanin-conjugated IL-4, IL-10, IL-9, and IL-22 (eBioscience) mAbs, as well as PE-Cy5.5–conjugated CD4 mAb for gating. For IL-11 containing with IL-21 and IL-22, cells were stained by primary Abs against human IL-11, followed by FITC-conjugated secondary Abs. Isotype controls were used for determining the background. The percentages of the cells expressing
IL-11 is significantly increased in the serum and csf samples from CIS patients in comparison with HCs

Studies of the serum samples from 17 CIS patients and 28 HCs (Table I) revealed the most significantly increased levels of IL-9 (13.7-fold) and IL-11 (7.5-fold) in the CIS patients. The Th17 cytokines IL-17A, IL-17F, IL-21, IL-22, and the Th1 cytokine IFN-γ were also increased, whereas IL-4 levels were decreased in the CIS patients (Fig. 1A). Linear correlation analysis revealed a positive correlation between IL-11 and IL-22 in CIS patients \( (r = 0.6881, p = 0.0023) \) and in HCs \( (r = 0.4677, p = 0.0121) \). Similarly, csf samples from 14 CIS patients and 23 control subjects with no evidence of CNS inflammatory disease revealed the most significant upregulation of IL-11 (26.7-fold) in CIS patients. Th17 cytokines IL-17A, IL-17F, and IL-21, the Th1 cytokine IFN-γ, and Th9 cytokine IL-9 were also increased, whereas IL-10 was significantly downregulated in comparison with the control subjects (Fig. 1B).

To examine whether increased serum and csf levels of IL-11 persist in the later phase of the disease, we measured IL-11 concentrations in the serum of 37 RRMS patients and in the csf samples of 8 RRMS patients. IL-11 measurements in both compartments showed significantly increased concentrations in comparison with control subjects, suggesting that findings on the role of IL-11 in the early phase of the disease (CIS) are also applicable to the patients with clinically definitive RRMS (Fig. 1C).

To demonstrate the in vivo function of IL-11 in the active phase of the disease, we measured the IL-11 in the serum samples from 12 RRMS patients during their clinical relapses and remissions. We found that the concentration of IL-11 and the pathogenic IL-17A cytokine are significantly increased during relapses compared with remissions (Fig. 1D), suggesting that IL-11 plays a role in the inflammatory response in MS.

Gene expression profiling of the PBMCs reveals upregulation of IL-11Ra in T cells from CIS patients

To capture complex gene expression changes in CIS patients, we used Affymetrix Human Gene array U133 (HG-U133) study with 45,000 probe sets representing ~33,000 human genes. The results of gene expression profiling in PBMCs derived from 15 CIS patients and 7 HCs revealed 26 differentially expressed \( (p < 0.05, >1.5\)-fold) immune response genes (Fig. 2A), among which cytokine/chemokine receptors CCR1, IL-1R1, IL-11Ra, CCR6, and IL-21R were significantly upregulated in CIS patients. To uncover which cell subsets contribute to the upregulated gene expression of these cytokine and chemokine receptors, we measured their expression in CD4+ and CD8+ T cells, CD19+ B cells, and CD14+ monocytes. Studies of these magnetic bead-separated cell subsets were performed in an independent cohort of nine CIS patients and nine age-, sex-, and race-matched HCs using RT-PCR. IL-11Ra was the only cytokine receptor with a significantly higher gene expression in CD4+ and CD8+ T cells in CIS patients when compared with HCs (Fig. 2B). The increased IL-11Ra expression on CD4+ cells from CIS patients was confirmed at the protein level by the FACS staining in nine CIS patients and matched HCs (Fig. 2C). Similarly, the gene expression of IL-11, a cytokine that binds and signals through IL-11Ra, was predominantly expressed by CD4+ T cells derived from CIS patients in comparison with CD4+ T cells from HCs (8.3-fold; Fig. 2B, lower panel).

Among the inflammatory cytokine-producing CD4+ cells, IL-11+CD4+ cells are most significantly increased in CIS patients

To determine the cytokine secretion profile in CD4+ cells from CIS patients, we performed intracellular cytokine staining in PBMCs from the same nine CIS patients and nine matched HCs as in Fig. 2C. The percentage of IL-11–producing CD4+ T cells was the most significantly increased in the CIS patients in comparison with the HCs. We also detected a significantly higher percentage of the CD4+ cell–producing Th17 cytokines IL-17A, IL-17F, IL-21 and IL-22, IL-9, and Th1 cytokine IFN-γ–producing CD4+ cells in the CIS patients. The percentages of the Th2 cytokine IL-4– and IL-10–producing cells were significantly lower in the CD4+ T cells derived from CIS patients than from their matched HCs (Fig. 3A, 3B). Linear correlation analysis revealed a positive correlation between IL-11– and IL-17F–producing cells in both CIS patients \( (r = 0.8777, p = 0.0019) \) and HCs \( (r = 0.6766, p = 0.0453) \).

Furthermore, intracellular IL-11–staining of the PBMCs derived from four CIS patients revealed that CD4+ lymphocytes constitute the predominant cell source of IL-11 in the peripheral circulation, representing 68.4% of the IL-11–producing cells. This was significantly higher in comparison with the numbers of IL-11–producing CD8+ lymphocytes, CD19+ B cells, γδ T cells, and CD56+ NK cells \( (p < 0.001; \text{Fig. 4A, 4B}) \).

We next measured the coexpression of IL-11 with T cell subsets prototypical cytokines in PBMCs from six CIS patients. Surprisingly, only a small percentage of IFN-γ–, IL-4–, and IL-17–expressing cells produced IL-11 (Fig. 4C). In contrast, only a small percentage of IL-11+CD4+ T cells produced IFN-γ, IL-17A, IL-17F, IL-9, IL-4, and IL-10 (Fig. 4D). Similar to the intracellular cytokine staining results, only a small percentage of IL-11+CD4+ cells expressed Th1, Th2, Th17, and Treg transcription factors (Fig. 4E). These results indicated that IL-11–producing CD4+ T cells might represent a distinct T cell subset, whose phenotype and function have not been previously characterized.
Thus, we examined the surface phenotype of IL-11+CD4+ cells, particularly the expression of cytokine and chemokine receptors that were differentially expressed in the gene expression profiling study between CIS patients and HCs (Fig. 2A). IL-11+CD4+ T cells derived from eight CIS patients expressed significantly greater levels of IL-1R1, IL-21R, and CCR6, and lower levels of IL-11R in comparison with Th1, Th2, and Th17 cells (Fig. 4F).

**IL-11 induces Th17 cell differentiation**

Because IL-11Rα is expressed in a high percentage of CD4+ cells (76 ± 12% in CIS patients; Fig. 2C), we examined the effect of IL-11 on naïve CD4+ T cell differentiation. In our preliminary study, cultures of resting naïve CD45RA+ and memory CD45RO+CD4+ cells in the presence of IL-11 revealed significantly increased percentages of IL-17F+CD4+ cells in naïve CD4+ cells, and of IL-17A+ cells in memory CD4+ cells, and induced production of IL-17F, IL-21, and IL-22 in naïve and IL-17A in memory cells. In contrast, IL-11 did not induce an increase in the percentage of IFN-γ– and IL-4–secreting cells, nor secretion of Th1 or Th2 cytokines (Supplemental Fig. 1A, 1B). Moreover, cultures of anti-CD3 + anti-CD28 mAb–stimulated naïve and memory CD4+ cells in the presence of IL-11 induced differentiation of IL-17A+CD4+ cells and the

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**FIGURE 1.** IL-11 is significantly increased in the sera and csf from CIS patients in comparison with HCs. (A) Serum samples from 28 HCs and 17 CIS patients and (B) csf samples from 23 control subjects and 14 CIS patients were collected, and the production of indicated cytokines was measured using ELISA. Statistical analysis was performed using a nonparametric Mann–Whitney U test. Each symbol represents one subject. (C) Serum samples from 37 RRMS patients and csf from 8 RRMS patients were collected, and the production of IL-11 was measured using ELISA. Statistical analysis was performed using a repeated-measures ANOVA, *p < 0.05, **p < 0.001. (D) Serum samples from 12 RRMS patients collected during clinical relapse (within 30 d of the onset of new symptoms) and remission (sample collected >1 mo after the last relapse) were used for IL-11 and IL-17A measurements using ELISA. Statistical analysis was performed using a paired \( t \) test.
expansion of IL-17A- and IFN-γ-producing memory CD4+ cells, but not the differentiation of Th1/Th2 cells (Supplemental Fig. 1C).

These preliminary data set the stage for the following experiments, where we examined in greater detail a dose-titration effect of IL-11.
on the naive CD4 cell differentiation using the experimental approach established for the other Th17-polarizing cytokines (19).

CD4+CD45RA+ naive T cells isolated from six CIS patients and differentiated in the presence of anti-CD3 and anti-CD28 mAb, anti–IFN-γ and anti–IL-4 mAb, and IL-11 for 12 d showed a significant dose-dependent increase in the percentage of IL-17A– and IL-21–secreting CD4+ T cells (Fig. 5A, 5C). This effect was reversed after blocking with anti–IL-11 mAb, but not anti–IL-6 mAb (Fig. 5B).

**Figure 4.** IL-11+CD4+ cells are predominant IL-11–producing mononuclear cells in the peripheral circulation. (A) Fresh PBMCs derived from four CIS patients were stimulated by PMA and ionomycin. The percentage of IL-11+ cells was determined in gated CD4+ and CD8+ T cells, CD19+ B cells, CD56+ NK cells, and γδ TCR+ T cells. Statistical analysis was performed using a repeated-measures ANOVA. ***p < 0.001. (B) Representative staining from one CIS patient. (C) Fresh PBMCs derived from six CIS patients were stained with the indicated fluorescently-labeled Abs. The percentages of IL-11+ cells were determined in gated IFN-γ–, IL-4–, and IL-17A–producing CD4+ T cells. Panels present representative staining from one CIS patient. (D) Fresh PBMCs derived from six CIS patients were stained with indicated fluorescently labeled Abs. The percentages of the cells expressing each cytokine were determined in gated IL-11+CD4+ T cells. Panels present representative staining from one CIS patient. (E) PBMCs derived from six CIS patients were stimulated with PMA and ionomycin and stained for the indicated lineage-specific transcription factors. Each symbol represents one subject. (F) Fresh PBMCs derived from eight CIS patients were stained with indicated fluorescently labeled Abs. The percentages of IL-1R1+, IL-21R+, CCR6+, and IL-11Rα+ cells were determined in gated Th1 (IFN-γ+), Th2 (IL-4+), Th17 (IL-17A+), and IL-11+CD4+ T cells. Statistical analysis was performed using a repeated-measures ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001.

Naive CD4+ T cells differentiated in the presence of IL-11 expressed RORc (Fig. 5D), the transcription factor for Th17 cells, which was abrogated in the presence of anti–IL-11 mAb (Fig. 5E). In addition, the cells differentiated in the presence of IL-11 secreted increased levels of IL-17A, IL-17F, IL-21, IL-22, and IFN-γ (Fig. 5F), whereas the secretion of IL-4, IL-9, TGF-β1, and IL-10 was not induced (data not shown). Anti–IL-11 mAb abolished the induction of IL-17A, IL-17F, IL-21, and IL-22, but not IFN-γ (Fig. 5G), suggesting that the induction of
FIGURE 5. IL-11 induces Th17 cell differentiation. (A) CD4^+CD45RA^+ naive T cells were separated from six CIS patients. A total of 2 × 10^6 cells per condition was stimulated by plate-immobilized anti-CD3 (1 μg/ml), anti-CD28 (5 μg/ml) mAb, anti–IFN-γ and anti–IL-4 (10 μg/ml) mAbs in the absence or presence of IL-11 (10, 50, 100 ng/ml), or (B) IL-11 (50 ng/ml) plus anti–IL-11 mAbs or anti–IL-6 mAbs (10 μg/ml). After 12 d of differentiation, the cells were restimulated by PMA and ionomycin, and stained with the indicated fluorescently labeled Abs. The percentage of cells expressing IL-17A and IL-21 was determined in gated CD4^+ T cells. (C) Representative staining from one experiment. (D and E) The percentage of RORc^+ cells was determined in gated CD4^+ T cells. (F and G) The production of the indicated cytokines was measured by ELISA in CD4^+ cell SNs after 12 d of culture in the absence or presence of anti–IL-11 mAbs. Statistical analysis was performed using a repeated-measures ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001.
IFN-γ secretion is not directly mediated via IL-11, but may represent a nonspecific IFN-γ induction upon anti-CD3/anti-CD28 mAb stimulation.

To determine to what extent the IL-11–induced Th17 cell differentiation represents an independent pathway, or enhances the effect of the established Th17-polarizing cytokines (IL-1β, IL-6, IL-23, IL-21).

**FIGURE 6.** IL-11 induces the expansion of Th17 memory CD4⁺ cells. (A and B) A total of 2 × 10⁶ CD4⁺CD45RO⁺ T cells per condition was separated from the same six CIS patients and cultured as in Fig. 5. (C) Representative staining from one CIS patient. (D and E) The percentage of RORc⁺ cells was determined in gated CD4⁺ T cells. (F and G) The production of the indicated cytokines was measured by ELISA in CD4⁺ cell SNs after 12 d culture. Statistical analysis was performed using a repeated-measures ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001.
IL-23), we induced the Th17 cell differentiation in the presence of IL-11, Th17-polarizing cytokines, or a combination of IL-11 and Th17-polarizing cytokines. Our results on T cells derived from six CIS patients indicate that IL-11 increased the percentage of Th17 cells 2.2-fold, Th17-polarizing cytokines increased it 2.5-fold, and IL-11 and the Th17-polarizing cytokines 2.9-fold, whereas those combinations of polarizing cytokines did not induce differentiation of IFN-γ and IL-4+ CD4+ cells (Supplemental Fig. 2).

**IL-11 induces expansion of Th17 memory CD4+ T cells**

Because Th17 memory cells are predominantly involved in the development of the autoimmune response (48), we examined the effect of IL-11 on the expansion of memory CD4+ cells. Studies of the CD45RO+ memory CD4+ cells derived from six CIS patients and expanded under the same conditions as in Fig. 5 revealed that IL-11 induces a significant dose-dependent increase in the percentages of IL-17A−, IL-21−, IL-22−, and IFN-γ−producing cells (Fig. 6A, 6C). Anti–IL-11 mAb abolished the induction of IL-17A and IL-22, but not IL-21 and IFN-γ, indicating that the expansion of the two latter CD4+ T cell populations was not directly induced by IL-11 (Fig. 6B). We have identified that an increased percentage of memory Th17 cells expanded in the presence of IL-11–expressed RORc (Fig. 6D), which was reversed by anti–IL-11 mAb (Fig. 6E). Similarly, IL-11 induced increased secretion of IL-17A, IL-17F, IL-21, IL-22, and IL-9 by memory cells (Fig. 6F), which was reversed for IL-17A, IL-17F, and IL-21 by anti–IL-11 mAb (Fig. 6G). In contrast, IL-11 failed to increase the expression of T-bet, GATA3, or FOXP3, or the secretion of IFN-γ, IL-4, TGF-β1, or IL-10 in memory CD4+ cells (data not shown).

The physiological relevance of the IL-11 concentrations measured in the serum and csf samples from CIS patients was demonstrated by the IL-11 dose titration from 35 to 1000 pg/ml, concentrations detected in the csf and serum samples from the CIS patients, respectively (Fig. 1). Because IL-11 at 1000 pg/ml induced naive CD4+ cell differentiation into Th17 cells (Supplemental Fig. 3A, 3B), and IL-11 at both 35 and 1000 pg/ml concentrations induced memory Th17 cell expansion (Supplemental Fig. 3C, 3D), we confirmed the biological relevance of the ex vivo–detected serum and csf IL-11 levels.

To measure the effect of IL-11 on the secretion of Th-polarizing cytokines by monocytes, we measured the cytokine secretion of separated CD14+ monocytes from three CIS patients. Our results revealed that IL-11 induced the secretion of Th17-polarizing cytokines IL-1β, TGF-β, IL-21, and IL-23 (Supplemental Fig. 4), although it did not change the secretion of IL-12, IL-10, and IL-27 (data not shown).

**Th17 cytokines induce differentiation of IL-11+CD4+ cells**

Because CD4+ cells represent the main source of IL-11 in the peripheral circulation, we next examined which cytokines induce IL-11+CD4+ cell differentiation. We induced differentiation of naive CD4+ T cells from six CIS patients in the absence or presence of IL-1β, IL-4, IL-6, IL-11, IL-12, IL-13, IL-17A, IL-17F, IL-21, IL-22, IL-23, TGF-β1, and TNF-α, cytokines reported to induce IL-11 secretion in the other cell types (42–45). Our data indicate that Th17 cytokines IL-17F, IL-21, TNF-α, as well as TGF-β1 significantly increased the percentage of IL-11+CD4+ T cells after 12 d of differentiation (Fig. 7A). In addition, IL-1β and TGF-β1 also sig-

**FIGURE 7.** Th17 cytokines induce differentiation of IL-11–producing cells. (A) CD4+CD45RA+ naive T cells were separated from six CIS patients. A total of 2 × 10^6 cells per condition was stimulated by plate-immobilized anti-CD3 (1 μg/ml), anti-CD28 (5 μg/ml) mAb, anti–IFN-γ, and anti–IL-4 (10 μg/ml) mAbs in the absence or presence of the indicated cytokines (50 ng/ml). After 12 d of differentiation, the cells were restimulated by PMA and ionomycin, and stained with the fluorescein-labeled Abs against IL-11 and CD4. The percentage of cells expressing IL-11 was determined in gated CD4+ T cells. Statistical analysis was performed using a paired t test. (B) The concentration of IL-11 in the SNs from the above cultures was measured using ELISA. (C) CD4+CD45RO+ memory T cells were separated from six CIS patients, and 2 × 10^6 cells per condition were cultured as indicated in (A). After 12 d, the SNs were collected, and the IL-11 concentration was measured by ELISA.
significantly induced IL-11 secretion by the naive CD4+ T cells during differentiation (Fig. 7B).

To characterize the expansion of IL-11+CD4+ memory cells, we activated memory CD4+ cells from the same six CIS patients in the absence or presence of the same cytokines as in Fig. 7A. We found that none of the tested cytokines induced the expansion of IL-11+CD4+ T memory cells (data not shown). However, IL-17F, TNF-α, IL-1β, TGF-β1, and IL-11 increased the production of IL-11 by memory CD4+ T cells (Fig. 7C).

Discussion
Cytokine profiling of serum and csf samples obtained at the time of CIS diagnosis revealed that IL-11 was the most significantly increased cytokine in comparison with control subjects, which suggests the potential involvement of IL-11 in the autoimmune response in early MS. Similar findings were documented in patients with clinically definitive RRMS, suggesting the proinflammatory role of IL-11, which was further confirmed by its significantly higher serum concentrations during the clinical relapses in comparison with the remissions of untreated RMS patients.

An independent Affymetrix gene array study of PBMCs derived from CIS patients has identified an increased expression of several cytokine and chemokine receptors, which may reflect patients’ constitutive susceptibility for an enhanced inflammatory response. Our results revealed that a high percentage of CD4+ T cells express IL-11Ra (44 ± 29% in HCs and 76 ± 22% in CIS patients), whereas only a low percentage of CD4+ T cells secrete IL-11 (1.3 ± 0.4% in HCs and 2.8 ± 1.1% in CIS patients). Those results are reflected in the gene array results from the bulk PBMCs. Confirmatory RT-PCR studies of the IL-11Ra expression in multiple cell subsets have identified its predominant expression in T cells, which was increased in CIS patients, accompanied by a similar expression pattern of its binding cytokine IL-11. CD4+ lymphocytes constitute the main cellular source of IL-11 in the peripheral circulation, representing 68.4% of the IL-11–producing PBMCs. Whereas the previous studies identified stromal cells, including synoviocytes (35), lung fibroblasts, epithelial cells (49), and eosinophils (36), as a source of IL-11, our findings demonstrated that IL-11 is also produced by multiple peripheral blood mononuclear, predominately CD4+ T cells. Furthermore, we identified a subset of CD4+ T cells that secrete IL-11, justifying their more detailed study in the pathogenesis of MS.

The surface marker phenotype of IL-11+CD4+ T cells was characterized by significantly higher IL-1R1, IL-21R, and CCR6 and lower IL-11Ra expression in comparison with the IFN-γ (Th1), IL-4 (Th2), and IL-17A+ (Th17) cells. Increased expression of IL-1R1 on IL-11+CD4+ T cells is consistent with the reported role of IL-1 in the induction of IL-11 secretion in fibroblasts (43, 45) and in CD4+ cells, as found in our study (Fig. 7B, 7C). IL-21 is an autocrine cytokine secreted by Th17 cells, whose binding to IL-21R promotes or maintains Th17 lineage commitment (50, 51) and also induces the differentiation of IL-11+CD4+ naive cells (Fig. 7A). CCR6 is a key chemokine receptor that mediates cell migration into the CNS (52). The high level of CCR6 expression on IL-11+CD4+ T cells may mediate their migration into the CNS, which is consistent with a significant increase of IL-11 in csf from CIS patients.

IL-11 binding to the IL-11Ra leads to the receptor complex formation, which involves a signal-transducing U, which is shared by multiple IL-6 family members. IL-11 binds to the gp130 complex and induces STAT3 phosphorylation in several noninflammatory cell subsets (26–28). Similar to IL-6, which has an established role in the induction of Th17 cell differentiation (53, 54), IL-11 selectively induced naive CD4+ T cells differentiation into Th17 cells, characterized by RORc expression and dose-dependent increase in IL-17A, IL-17F, IL-21, and IL-22 cytokine secretion. Although IL-11–induced Th17 differentiation was inhibited by anti–IL-11 mAb, it was not inhibited by anti–IL-6 mAb, indicating that IL-11–induced Th17 cell differentiation is independent of IL-6. Although IL-11 alone can induce Th17 cell differentiation, our experiments revealed that the combination of IL-11 and the Th17-polarizing cytokines (IL-1β, IL-6, and IL-23) most effectively induced Th17 cell differentiation. Therefore, IL-11 induces an independent, but also a complementary pathway to the other Th17-polarizing cytokines.

Given significantly higher numbers of memory Th17 cells (48) and their capacity to produce high amounts of cytokines in comparison with the naive cells, we examined the role of IL-11 in the expansion of memory Th17 cells in CIS patients. Our results revealed that the activation of CD45RO+ memory CD4+ cells in the presence of IL-11 induced expansion of the IL-17A–, IL-21–, and IL-22–producing RORc+ Th17 cells.

Whereas the average IL-11 concentration detected in the sera of CIS patients induced differentiation of naive Th17 cells and the expansion of memory Th17 cells, the IL-11 concentration detected in the csf was only able to induce the expansion of memory Th17 cells. A prominent response of memory Th17 cells to the low concentrations of IL-11 may play a role in the amplification of Th17-mediated autoimmune response in the CNS of CIS patients.

Regarding the regulation of IL-11 secretion, previous studies have reported that the IL-11 secretion was induced by IL-17F in bronchial epithelial cells (42), by IL-22 in myofibroblasts (46), by IL-1β in fibroblast and bone marrow stromal cells (43, 44), and by TGF-β in fibroblast (43). In this study, we found that Th17 cytokines IL-17F, IL-21, and TNF-α, as well as TGF-β1, induced the differentiation of IL-11+CD4+ T cells. Interestingly, although none of the tested cytokines induced the expansion of memory IL-11+ CD4+ T cells, IL-17F, TNF-α, TGF-β1, IL-1β, and IL-11 induced IL-11 secretion by memory CD4+ T cells. The induction of IL-11+ CD4+ T cell differentiation and IL-11 secretion in response to the above early proinflammatory cytokines may explain an increased IL-11 level in serum and csf samples from CIS patients. In addition to the IL-11–induced Th17 cell differentiation, the induction of IL-11+CD4+ T cell differentiation by Th17 cytokines inferred a positive feedback mechanism by which Th17 cells cross-talk with IL-11+CD4+ cells and may amplify the inflammatory response in the early stage of MS.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Fig. 1.

**A**

Supplementary Figure 1. IL-11 induces the differentiation of Th17 cells. 2x10^6 CD4^+ naïve and memory CD4^+ cells per condition were separated using magnetic beads from fresh PBMCs derived from 10 CIS patients, and cultured in serum-free medium in the absence or presence of IL-11 (50 ng/ml) for 48 h prior to intracellular cytokine staining and SN collection.

(A) For the intracellular cytokine staining, the cells were re-stimulated by PMA and Ionomycin, and stained with the indicated fluorescently-labeled antibodies. The percentage of cells expressing each molecule was determined in gated CD4^+ T cells.

(B) The production of the indicated cytokines was measured in SNs by ELISA. Statistical analysis was performed using a repeated measures ANOVA. * indicates p<0.05, ** p<0.01 and ***p<0.001.

(C) CD4^+ naïve and memory cells were isolated from 6 CIS patients. The cells were stimulated with plate-immobilized αCD3 (1 μg/ml) and αCD28 (5 μg/ml) mAbs, in the absence and presence of IL-11 (50 ng/ml) for 12 days prior to the intracellular cytokine staining. Statistical analysis was performed using a paired t-test.

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SUPPLEMENTARY FIGURE 2. IL-11 independently induces Th17 differentiation and enhances the Th17 cell differentiation mediated by established Th17-polarizing cytokines. CD4⁺ naïve T cells were isolated from 6 CIS patients and differentiated in the presence of αCD3 and αCD28 mAb, αIFN-γ and αIL-4 mAb and IL-11 only, or the Th17-polarizing cytokines IL-1β, IL-6 and IL-23, or a combination thereof for 12 days prior to intracellular cytokine staining. The percentage of cells expressing IL-17A, IFN-γ and IL-4 was determined in gated CD4⁺ T cells. Statistical analysis was performed using a repeated measures ANOVA; * indicates p<0.05, ** p<0.01.
SUPPLEMENTARY FIGURE 3. The IL-11 concentration detected in the CSF of CIS patients induces the expansion of memory Th17 cells, while the IL-11 concentration detected in the sera of CIS patients induces naïve cell differentiation and memory Th17 cell expansion. Naïve (A, B) and memory (C, D) CD4⁺ T cells were isolated from 6 CIS patients and cultured in the presence of plate-immobilized αCD3 (1 µg/ml), αCD28 (5 µg/ml) mAbs, αIFN-γ and αIL-4 (10 µg/ml) mAbs, and IL-11 (35, 1000 pg/ml) for 12 days prior to the intracellular cytokine staining. Statistical analysis was performed using a repeated measures ANOVA. * indicates p<0.05, ** p<0.01. Figures B and D show representative staining from one CIS patient.
SUPPLEMENTARY FIGURE 4. IL-11 induces the secretion of Th17-polarizing cytokines in monocytes. Separated CD14⁺ monocytes from 3 CIS patients were cultured in the absence or presence of IL-11 (50 ng/ml) for 48 h prior to SN collection. The secretion of the indicated cytokines was measured using ELISA. Statistical analysis was performed using a paired t-test.