

Interleukin-17 in transverse myelitis and multiple sclerosis

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Abstract

CSF IL-6 is elevated in transverse myelitis (TM) and predicts disability. Since IL-17 regulates cytokines (TNF α , IL-1 β and IL-6) known to stimulate IL-6 production by astrocytes, we sought to determine whether IL-17 was increased in TM and MS compared to healthy controls (HC) and other neurologic diseases (OND). IL-17 and IL-6 levels were measured in stimulated peripheral blood mononuclear cell (PBMC) supernatants from HC, MS, TM and OND. IL-17 was increased in TM compared to HC, MS, and OND (mean pg/ml \pm standard error; HC: 36.1 \pm 11.7, MS: 89.4 \pm 23.3, TM: 302.6 \pm 152.5, OND: 41.2 \pm 13.0, $p=0.01$). IL-6 was increased in TM relative to MS and HC (HC: 2624 pg/ml \pm 641, MS: 6129 \pm 982, TM: 12,536 \pm 2657, OND: 6920 \pm 1801, $p<0.002$). MS patients with early disease (<2 years) also had increased levels of IL-17 ($p<0.04$) and IL-6 ($p<0.05$). Cytokine neutralization experiments demonstrated that IL-6 was the main inducer of astrocyte IL-6 production. We conclude that IL-17 and IL-6 production from PBMC in TM and early MS are increased and induce astrocyte IL-6 production through IL-6.

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1. Introduction

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system (CNS) characterized by recurring episodes of inflammatory demyelinating lesions with prominent astrogliosis. IL-17 producing cells (Th17), distinct from Th1 or Th2 cells, play a critical role in disease induction in experimental autoimmune encephalomyelitis (EAE) (Frohman et al., Greter et al., 2005; Heppner et al., 2005; Reder et al., 1998; Hollifield et al., 2003; Langrish et al., 2005; Harrington et al., 2005; Park et al., 2005). IL-17 levels in CSF are elevated in the optico-spinal form of MS in Japanese populations, which may have under-

lying pathogenic differences from conventional MS (Ishizu et al., 2005).

Idiopathic transverse myelitis (TM) is a rare disorder characterized by severe monofocal inflammatory lesions of the spinal cord with simultaneous motor, sensory and autonomic dysfunction. The underlying immunopathology of TM bears many similarities to MS but clinical course differs with a very low rate of subsequent conversion to MS or Devic's neuromyelitis optica (NMO) (Transverse Myelitis Consortium Working Group, 2002; Kerr and Ayetey, 2002; de Seze et al., 2005). We recently showed that IL-6 is increased in the CSF of TM patients and CSF IL-6 levels correlate with both acute and long term disability (Kaplin et al., 2005). IL-6 levels are also increased in both CSF and serum in MS though less than what we found in TM (Miljkovic et al., 2002). Astrocytes have been shown to produce IL-6 in the CNS in response to TNF α and IL-1 β , both of which are increased in the presence of IL-17 (Van Wagoner and Benveniste, 1999; Jovanovic et al., 1998). TNF α and IL-1 β interact synergistically to stimulate levels of IL-6 production by astro-

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cytes greater than either stimulates alone (Van Wagoner and Benveniste, 1999). In addition, IL-6 itself can stimulate increased IL-6 production by astrocytes via gp130 mediated “trans” signaling when soluble IL-6 receptor (sIL-6R) is present, as has been shown in MS patients’ CSF (Michalopoulou et al., 2004; Van Wagoner et al., 1999).

IL-17 is primarily made by a subset of activated memory CD4+ T cells distinct from Th1 or Th2 cells, called Th17. These IL-17 producing T cells arise from naïve precursors when TGF- β and IL-6 are present but Th1 and Th2 inducing cytokines (IFN γ and IL-4) are absent (Mangan et al., 2006). IL-23 produced by macrophages and dendritic cells appears to play a supporting role in the continued stimulation and survival of Th17 cells (Harrington et al., 2005; Park et al., 2005; Mangan et al., 2006; Bettelli et al., 2006; Veldhoen et al., 2006; Harrington et al., 2006). IL-23 and IL-12 have antagonistic properties, as IL-23 supports IL-17 production, whereas IL-12 inhibits it (Langrish et al., 2005; Harrington et al., 2006; Gran et al., 2004). IL-17 in turn stimulates macrophage production of IL-6, TNF α and IL-1 β , all of which have been implicated in MS pathology and astrocyte IL-6 production (Jovanovic et al., 1998; Van Wagoner et al., 1999). IL-17 mRNA expression is increased in acute MS lesions and in CSF and peripheral mononuclear cells during relapse (Lock et al., 2002; Matusевичius et al., 1999). CSF IL-17 levels in Japanese optico-spinal MS correlate with the size of spinal cord lesions, which is one of the main distinguishing features between optico-spinal MS and conventional MS (Ishizu et al., 2005). In EAE, Th17 cells have been shown to develop in response to IL-23 after myelin protein immunization and invade the CNS to initiate inflammation (Langrish et al., 2005; Park et al., 2005).

To investigate the role of IL-17 and its possible link with IL-6 in autoimmune inflammation of the CNS, we examined IL-17 and associated cytokine levels in ex vivo stimulated cell supernatants from peripheral blood mononuclear cells (PBMC) of healthy controls (HC) and patients with MS and TM. We found that IL-17 and IL-6 production was increased in PBMC supernatants from patients with TM and in the subset of MS patients with early disease. Furthermore, we found that IL-6, but not IL-17, could induce IL-6 production by astrocytes.

2. Methods

2.1. Patients

Patients with clinically definite relapsing-remitting multiple sclerosis (MS $n=37$) and idiopathic transverse myelitis (TM $n=13$) were recruited from the Johns Hopkins Medical Institutions according to previously published diagnostic criteria (Transverse Myelitis Consortium Working Group, 2002). None of these patients had longitudinally extensive TM lesions (≥ 3 vertebral segments in length) typical of Devic’s neuromyelitis optica (NMO). When any clinical suspicion for disease beyond a focal TM lesion existed, further testing was performed. Two patients had visual evoked potentials, both of which were normal. Four TM patients were tested for the NMO-IgG antibody against aquaporin-4, and all were negative (Lennon et al., 2004).

Healthy control (HC) volunteers with no history of inflammatory neurologic disease were also recruited ($n=16$). Ten patients with other neurological diseases (OND) including Alzheimer’s disease ($n=4$), neurosarcoid ($n=2$), compressive myelopathy ($n=2$), spinal cavernoma ($n=1$), and acute disseminated encephalomyelitis ($n=1$) were also recruited. Within the MS group, one patient each was taking Avonex, Rebif, Betaseron and Copaxone at the time of sampling and one patient had stopped Copaxone two weeks prior to sampling. In the TM group one patient was taking Avonex. The remaining MS, TM and OND patients had not received any steroids or other immunomodulatory drugs within the month prior to sampling. None of our MS or TM patients have subsequently manifested a clinical course consistent with primary progressive MS or NMO. All patients gave written, informed consent for participation in the study.

2.2. Cell culture

Peripheral blood mononuclear cells (PBMC) were obtained by venous puncture and separation of whole blood over Ficoll gradients. Cells were stored at $-80\text{ }^{\circ}\text{C}$ in whole T cell media (IMDM) with 10% DMSO. For culture, PBMC were thawed in 5% human serum (Sigma) and PBS, counted using a hemacytometer, and plated at 2×10^6 cells per well in a 24 well culture plate and incubated in T cell media at $37\text{ }^{\circ}\text{C}$ for 48 h. Cells were activated using soluble anti-CD3 and anti-CD28 at $1\text{ }\mu\text{g/ml}$ (Becton-Dickinson). Supernatants were harvested and stored at $-20\text{ }^{\circ}\text{C}$ until assay. For sorting experiments, cells were sorted using negative selection with MACS magnetic beads to obtain untouched CD4+ or CD8+ T cells (Miltenyi Biotech). CSF cells from MS and TM patients were obtained by sterile lumbar puncture and centrifugation of CSF, and stimulated as described above for 7 days in T cell media at $37\text{ }^{\circ}\text{C}$.

2.3. Cytokine assays and recombinant protein

Assays for IL-17 (R&D Systems), IL-6, IL-1 β , TNF α , IFN γ and sIL6R (Biosource International) were performed in duplicate according to manufacturer’s instructions. Recombinant human IL-23, IL-17, IL-12, IL-18, IL-1 β (R&D Systems), IFN γ and TNF α (Peprotech, Inc.) were added to astrocytes as indicated. Neutralizing antibodies to IL-23 (polyclonal goat antibody against IL23 p19), IL-17, IL-12, IFN γ (R&D Systems), IL-6, IL-18 (Biosource International), IL-1 β (Sigma, Inc.), and TNF α (BD Pharmingen) were used at neutralizing doses according to the manufacturer’s instructions and confirmed by our own ELISAs.

2.4. Astrocyte culture and staining

Both a human astrocyte-like cell line (SVGA) and primary human astrocytes were studied under a Johns Hopkins University IRB approved protocol (Chauhan et al., 2003). SVGA cells were maintained in DMEM media with 5% fetal serum albumin in an incubator at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 . For some experiments, human astrocytes were used. Briefly, human fetal brain specimens of 12–17 weeks gestation were obtained in accor-

dance with NIH guidelines. The tissues were then triturated after removing the meninges. Cells were then cultured in T75 flasks in opti-MEM (Invitrogen) with 5% fetal bovine serum (FBS, Invitrogen), 0.5% N2 supplement (Invitrogen) and 1% antibiotics for at least one month. After shaking at 180 rpm for 1 h, cells were separated with trypsin/EDTA. Cells were then seeded at 1×10^5 /ml on coverslips for another three weeks before treatment with Poly-IC (25 μ g/ml) and IFN γ (500 U/ml) for positive controls or T cell supernatants at 1:10 dilution, in Locke's Buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl $_2$ ·2H $_2$ O, 1.0 mM MgCl $_2$ ·6H $_2$ O, 3.6 mM NaHCO $_3$, 5 mM HEPES, 1.8 mg/ml glucose at pH 7.2) for 48 h. Golgi Stop (BD Pharmingen) was added for the final 6 h of culture at the concentration specified by the vendor. Cells were fixed with 4% Paraformaldehyde for 30 min and then blocked in 5% BSA with 0.005% Saponin, for 30 min. Rabbit anti-GFAP (Chemicon) at a dilution of 1:200 was added and incubated for 1 h at room temperature. Cells were washed with PBS and 0.005% Saponin, donkey anti-rabbit ALEXA 594 (7 μ g/ml) (Molecular Probe) and FITC conjugated anti-IL-6 (1:10) (BD Pharmingen) were added. Cells were incubated at room temperature for 45 min and then washed and counter stained with DAPI (1:500) (Molecular Probe) and mounted with GEL mount (BioMed cat # M01). To immunodeplete IL-6 from stimulated PBMC supernatants, 1 μ g of anti-IL-6 (Biosource Cat # AHC0562) was added to supernatants and incubated overnight at 4 °C with end-over-end shaking of the tubes. Thirty μ l/ml of protein A/G beads was then added (Pierce cat # 53133) and incubated for 1.5 h at 4 °C with end-over-end shaking. Samples were centrifuged for 2 min at 2000 rpm and tested by ELISA (Biosource Cat # CHC1263) for complete depletion of IL-6 or use in astrocyte culture. All astrocyte experiments were performed in triplicate.

2.5. Statistical analysis

Statistical analysis was performed using PRISM version 3.0. Differences between more than two groups were tested using one-way ANOVA and significant results ($p < 0.05$) were verified with post-hoc Bonferroni correction for multiple comparisons. Differences between two groups were evaluated using *t*-tests for Gaussian values (with Welch's correction for unequal variances) or Mann–Whitney tests for non-Gaussian distributions. Because of the small sample size of the groups, any significant findings from *t*-tests were confirmed by post-hoc Mann–Whitney. Binomial data were analyzed using Fisher's exact test. Two-tailed *p*-values less than 0.05 were considered significant.

3. Results

3.1. Patient demographics

There was no significant difference in the ages of the HC, MS or TM groups (31.9 \pm 1.6 years, 39.3 \pm 1.9 years and 35.1 \pm 4.1 years, respectively). There was no significant difference in the gender ratios of the MS and TM groups (24% and 31% male, respectively).

3.2. IL-17 and IL-6 are increased and TNF α production is decreased in stimulated PBMC from transverse myelitis patients as compared to healthy controls

Stimulated PBMC from patients with TM produced significantly higher levels of IL-17 than those from HC, MS or OND (mean pg/ml \pm standard error (SE) HC: 36.1 \pm 11.7 ($n=16$), MS: 89.4 \pm 23.3 ($n=32$), TM: 302.6 \pm 152.5 ($n=10$), OND: 41.2 \pm 13.0 ($n=10$), $p=0.01$ by ANOVA with post-hoc Bonferroni $p < 0.05$ for TM versus HC, MS and OND) (see Fig. 1A). TM PBMC also produced higher levels of IL-6 than HC and MS patients (mean pg/ml \pm standard error (SE) HC: 2624 \pm 641 ($n=13$), MS: 6129 \pm 982 ($n=36$), TM: 12,536 \pm 2657 ($n=10$), OND: 6920 \pm 1801 ($n=9$), $p < 0.002$ by ANOVA with post-hoc Bonferroni p -value < 0.05 for TM versus HC and MS) (see Fig. 1B). Increased levels of IL-1 β were also found in TM supernatants, but this difference was not significant (mean pg/ml \pm standard error (SE) HC: 308 pg/ml \pm 110 ($n=14$), MS: 416 \pm 116 ($n=32$), TM: 1114 \pm 604 ($n=12$), OND: 347 \pm 71 ($n=10$), $p=0.12$ by ANOVA). Interestingly, levels of TNF α were decreased in MS and TM supernatants as compared to HC though this finding did not reach statistical significance (mean pg/ml \pm standard error (SE) HC: 12,915 ng/ml \pm 1754 ($n=14$), MS: 8079 \pm 1098 ($n=32$), TM: 7466 \pm 1815 ($n=12$), OND: 9770 \pm 2111 ($n=10$), $p=0.09$ by ANOVA). Levels of IFN γ and soluble IL-6 receptor were not significantly different between the four groups (data not shown).

3.3. IL-17 producing cells are present in CSF

Levels of IL-17 were undetectable in CSF from TM patients ($n=7$). Leukocytes obtained from the CSF of MS and TM patients were cultured in T cell media for 7 days in the presence of anti-CD3 and anti-CD28. Detectable levels of IL-17 were found in 1 of 8 MS CSF cell supernatants and 3 of 6 TM CSF cell supernatants (mean pg/ml \pm SE; MS – 0.94 \pm 0.9, TM – 30.0 \pm 16.8, $p=0.1812$, Mann–Whitney). We set a cutoff value of 15 pg/ml for high versus low levels of IL-17 production in CSF cell supernatants and reanalyzed the binomial data, which revealed a borderline significant difference between TM and MS ($p=0.055$, Fisher's exact test), indicating that IL-17-producing leukocytes are present in the CSF and may be increased in TM as compared to MS.

3.4. IL-17 levels are increased in early MS as compared to established MS

We subdivided the group of MS patients into those with early disease (defined as patients with onset of disease less than two years previously) versus established disease (diagnosed more than two years before sampling). PBMC from patients with early MS produced significantly higher levels of IL-17 than patients with established MS (mean pg/ml \pm SE; early MS: 142 \pm 42.1 ($n=15$), established MS: 37.1 \pm 17.7 ($n=15$), $p < 0.04$, *t*-test with Welch's correction, confirmed by post-hoc Mann–Whitney $p=0.005$ (see Fig. 1C)). When the patients currently on immunomodulatory drugs were removed from this analysis, the fin-

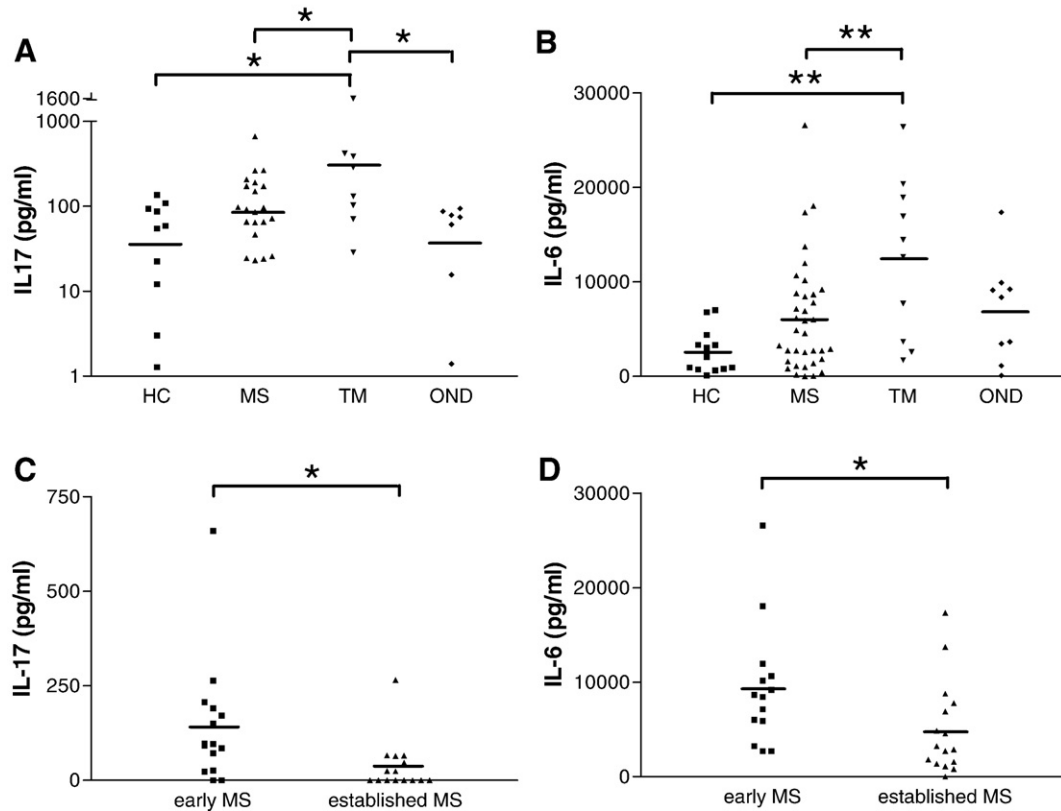


Fig. 1. (A) Stimulated PBMC from patients with TM produced higher levels of IL-17 than those from HC, MS or OND (mean pg/ml \pm standard error (SE) HC: 36.1 ± 11.7 ($n=16$), MS: 89.4 ± 23.3 ($n=32$), TM: 302.6 ± 152.5 ($n=10$), OND: 41.2 ± 13.0 ($n=10$), $p < 0.05$ by ANOVA) (note log scale). (B) TM PBMC produced higher levels of IL-6 than HC and MS patients (mean pg/ml \pm standard error (SE) HC: 2624 ± 641 ($n=13$), MS: 6129 ± 982 ($n=36$), TM: $12,536 \pm 2657$ ($n=10$), OND: 6920 ± 1801 ($n=9$), $p < 0.01$ by ANOVA). (C) PBMC from early MS (<2 years disease duration) produced higher levels of IL-17 than patients with established MS (mean pg/ml \pm SE; early MS: 142 ± 42.1 ($n=15$), established MS: 37.1 ± 17.7 ($n=15$), $p < 0.05$, t -test with Welch's correction). (D) PBMC from early MS also produced higher levels of IL-6 than established MS (mean pg/ml \pm SE; early MS: 9386 ± 1718 ($n=14$), established MS: 4965 ± 1225 ($n=16$), $p < 0.05$, t -test with Welch's correction) (bars indicate mean levels, *= $p < 0.05$, **= $p < 0.01$).

dings were still significant (mean pg/ml \pm SE; early MS: 150 ± 44.4 ($n=14$), established MS: 46.3 ± 21.5 ($n=12$), $p < 0.05$, t -test with Welch's correction, confirmed by post-hoc Mann–Whitney $p=0.01$). PBMC from patients with early MS also produced significantly higher levels of IL-6 than patients with established MS (mean pg/ml \pm SE; early MS: 9386 ± 1718 ($n=14$), established MS: 4965 ± 1225 ($n=16$), $p < 0.05$, t -test with Welch's correction, confirmed by post-hoc Mann–Whitney $p=0.02$ (see Fig. 1D)). When the patients currently on immunomodulatory therapy were removed from this analysis, the finding was no longer significant ($p=0.10$). There appeared to be higher levels of TNF α in early as compared to established MS, but this result did not reach significance (early MS: $10,083 \pm 1519$ ($n=15$) established MS: 6417 ± 1512 ($n=16$), $p=0.10$ by t -test with Welch's correction, Mann–Whitney=0.09). There were no significant differences in IL-1 β or IFN γ comparing early to established MS.

3.5. IL-23 increases PBMC production of IL-17 and IL-17 increases IL-6 in both TM and HC

Using both HC ($n=3$) and TM patients ($n=4$), we verified that stimulation of PBMC with anti-CD3 and anti-CD28 in the

presence of IL-23 increases IL-17 in a dose-dependent fashion (see Fig. 2A). There was significant variability between different patient and control samples, but trends within samples were consistent. We also examined IL-1 β , TNF α and IFN γ levels in a subset of IL-23 stimulated supernatants from TM and HC and found that IL-23 stimulation increased IFN γ and IL-1 β in a dose-dependent fashion while simultaneously having modest suppressive effects on TNF α secretion (data not shown). Therefore, IL-23 stimulation of PBMC simulated the pattern of cytokine secretion seen in TM as compared to HC. Using both HC ($n=2$) and TM patients ($n=3$) we verified that stimulation of PBMC with anti-CD3 and anti-CD28 in the presence of IL-17 stimulates IL-6 production in a dose-dependent fashion (Fig. 2B).

3.6. Stimulated PBMC supernatants induce IL-6 production in cultured human astrocytes

Primary human astrocytes or SVGA cells stimulated with activated PBMC supernatants from HC produced high levels of IL-6 (3960 pg/ml) (see Fig. 3A, B). We then incubated astrocytes with stimulated PBMC supernatants after selecting the adherent (primarily macrophages) and non-adherent (primarily T, B and

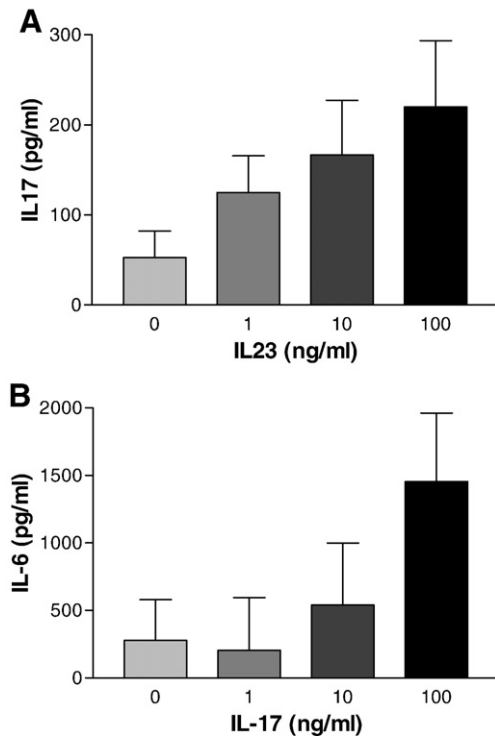


Fig. 2. IL-23 increases stimulated peripheral blood leukocyte production of IL-17 (A) in a dose-dependent fashion in both healthy controls ($n=3$) and patients with TM ($n=4$). Grouped data are shown. PBMC were stimulated with anti-CD3/anti-CD28 and IL-23 for six days in culture, mean levels of IL-17 in supernatants of cultured PBMC were 52.5 pg/ml \pm 78.0 SD without IL-23, 125.0 pg/ml \pm 107.3 SD with 1 ng/ml IL-23, 166.7 pg/ml \pm 160.9 SD with 10 ng/ml IL-23 and 220.2 pg/ml \pm 194.4 SD with 100 ng/ml IL-23. IL-17 increases stimulated peripheral blood leukocyte production of IL-6 in a dose-dependent fashion (B). Using both HC ($n=2$) and TM patients ($n=3$) we verified that stimulation of PBMC with anti-CD3 and anti-CD28 in the presence of IL-17 stimulates IL-6 production in a dose-dependent fashion (Fig. 2B). After 48 h in culture, mean levels of IL-6 in supernatants of cultured PBMC were 279.2 pg/ml \pm 242.3 SD without IL-17, 206.5 pg/ml \pm 156.5 SD with 1 ng/ml IL-17, 540.3 pg/ml \pm 369.7 SD with 10 ng/ml IL-17, and 1453.5 pg/ml \pm 408.8 SD with 100 ng/ml IL-17.

NK) cell subsets. Stimulated non-adherent cell supernatants did not stimulate significant astrocyte IL-6 production by themselves, while the stimulated supernatants of adherent cells (macrophages) recreated the IL-6 induction seen with whole PBMC supernatants (see Fig. 3C). When supernatants from adherent and non-adherent PBMC were combined, a synergistic effect was seen. This suggested that astrocyte activating factors are derived from adherent cells (most likely macrophages) and focused our further studies on macrophage derived cytokines. It is important to note that while macrophages produced high levels of IL-6, we diluted the PBMC samples 1:10 before adding the supernatants to the astrocytes, therefore we were not just measuring the added macrophage produced IL-6.

Selective addition of TNF α and IL-1 β alone to astrocyte cultures stimulated IL-6 production at lower levels than the stimulated PBMC supernatants (Fig. 3A,B), and neutralization of TNF α , IFN γ and IL-1 β only partially suppressed the induction of IL-6 by PBMC supernatants (Fig. 3D). We therefore suspected that it was IL-6 itself that was inducing astrocyte IL-6 production as has been described previously (Michalopoulos

et al., 2004; Van Wagoner et al., 1999). Since neutralization of IL-6 with an antibody would interfere with our ability to measure IL-6 produced by astrocytes we immunodepleted IL-6 in supernatants using beads coated with anti-IL-6, which specifically eliminated IL-6, but not other cytokines. We then incubated astrocytes with the IL-6 immunodepleted PBMC supernatants and found a dramatic reduction of IL-6, which was further lowered by adding IL-6 immunodepleted supernatant and a combination of blocking antibodies to IL-12, IFN γ , IL-1 β and TNF α (Fig. 3D, E, and F).

To verify that the IL-6 produced in our astrocyte cultures was derived from astrocytes themselves and not a contaminating cell type, we co-stained for GFAP (a specific marker of astrocytes) and IL-6 and found that the two co-localized in both PBMC or poly-IC stimulated astrocytes proving that the astrocytes in our cultures were producing IL-6 (Fig. 4A–C).

4. Discussion

We have shown that IL-17 and IL-6 production are increased in ex vivo stimulated PBMC of patients with TM compared to HC and MS. This provides further evidence that Th17 cells may be playing a critical role in autoimmune diseases. While it has previously been shown that IL-1 β , TNF α and IL-6 itself stimulate astrocyte IL-6 production, we delineate a pathway whereby Th17 cells may initiate astrocyte IL-6 production in TM and MS. IL-17 has already been shown to play a role in infectious CNS inflammation (Infante-Duarte et al., 2000; Duc Dodon et al., 2004). IL-17 and IL-6, produced by activated memory CD4 $^{+}$ T cells in response to TGF- β , IL-6 and IL-23 in the absence of Th1- or Th2-inducing IFN γ or IL-4, stimulate IL-6, TNF α and IL-1 β from macrophages (Harrington et al., 2005; Park et al., 2005; Harrington et al., 2006; Jovanovic et al., 1998). In addition, astrocytes themselves produce IL-6 and IL-23, which would allow them to participate in an autocrine loop of stimulation (Constantinescu et al., 2005).

IL-1 β is produced by monocytes, macrophages, microglia and astrocytes and can induce oligodendrocyte damage via cytokine production (including IL-6 and TNF α), nitric oxide, matrix metalloproteases, adhesion molecules and astrocyte-mediated glutamate excitotoxicity, all of which have been demonstrated to be involved in MS (Takahashi et al., 2003; John et al., 2005). IL-1 β production by PBMC is increased during relapse and in MS lesions (Hollifield et al., 2003; Hofman et al., 1986). One of the major actions of IL-1 β in the brain is the induction of a reactive astrogliosis with astrocyte activation and IL-6 production, an effect that is strongly augmented in the presence of TNF α , IFN γ , IL-6 and soluble IL-6 receptor (Van Wagoner and Benveniste, 1999; John et al., 2005). In our patients, IL-6 levels were increased and we found that soluble IL-6 receptor and IFN γ were present in sufficient levels to interact with IL-1 β .

TNF α is a product of activated macrophages and dendritic cells with multiple pro-inflammatory properties, many of which overlap or synergize with IL-1 β , including astrocyte activation, IL-6 production and glutamate excitotoxicity (Reder et al., 1998; Takahashi et al., 2003; John et al., 2005). TNF α levels are

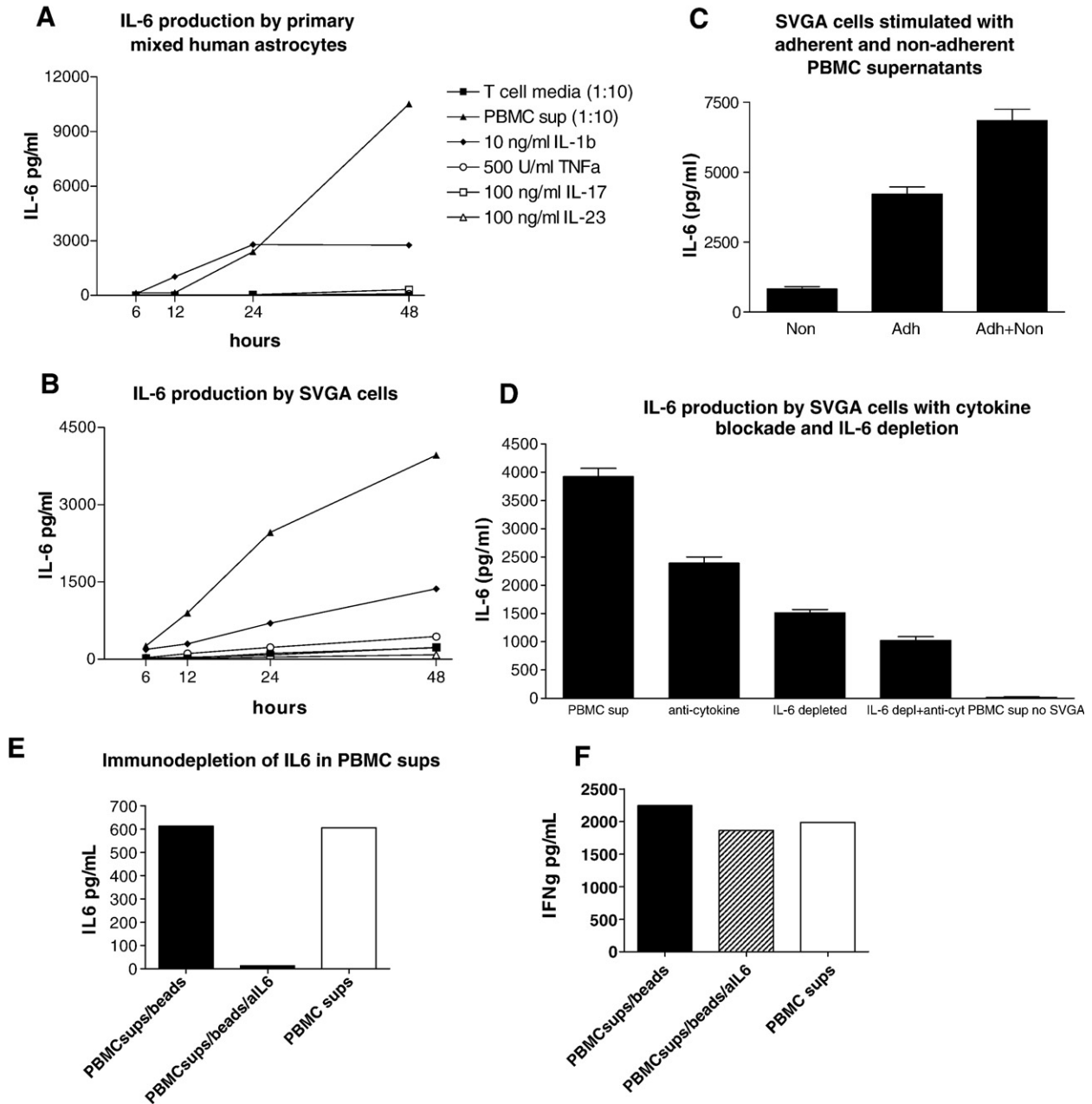


Fig. 3. IL-6 production from human astrocytes (A) and SVGA cells (B) cultured for 48 h produced higher levels of IL-6 in the presence of activated PBMC supernatants than those stimulated with IL-1 β or TNF α . IL-17, IL-23 and control T cell media did not directly stimulate IL-6 production by astrocytes. Supernatants of adherent PBMC stimulated astrocyte IL-6 production, while non-adherent PBMC supernatants had minimal effect. When non-adherent PBMC supernatants were recombined with adherent PBMC supernatants, a synergistic effect on astrocyte IL-6 production was seen (C). Astrocytes incubated with IL-6 depleted (IL-6 depl) supernatants produced less IL-6, and a cocktail of anti-cytokine antibodies (anti-IL-12, anti-TNF α , anti-IL1 β , anti-IFN γ (anti-cyt)) added to this effect without completely suppressing IL-6 production to basal levels (D). IL-6 in stimulated PBMC supernatants (PBMCsups) was selectively depleted using A/G beads and anti-IL-6 (α IL6) (E and F).

increased in CSF and peripheral mononuclear cells during MS relapse and correlate with a worse prognosis (Hollifield et al., 2003; Van Wagoner and Benveniste, 1999; Sharief and Henges, 1991). However, the role of TNF α in MS remains controversial, since TNF α blocking therapy has been reported to worsen or induce MS (Wiendl and Hohlfeld, 2002; Van Oosten et al., 1996; Hyrich et al., 2004). In our study, there was a trend towards decreased TNF α levels in stimulated PBMC of TM and MS patients compared to HC, despite increased IL-17 levels. This

finding may be explained by the known suppressive effects of IL-6 on TNF α (Xing et al., 1998).

We have confirmed prior studies that IL-23 stimulates IL-17 production in activated PBMC. IL-23 is primarily a product of activated macrophages, especially those of the M1 subset (as opposed to IL-12 producing M0 macrophages). The relative susceptibility of different murine subspecies to EAE has been shown to be associated with the endogenous ability of their macrophages to produce IL-23 instead of IL-12 (Andersson

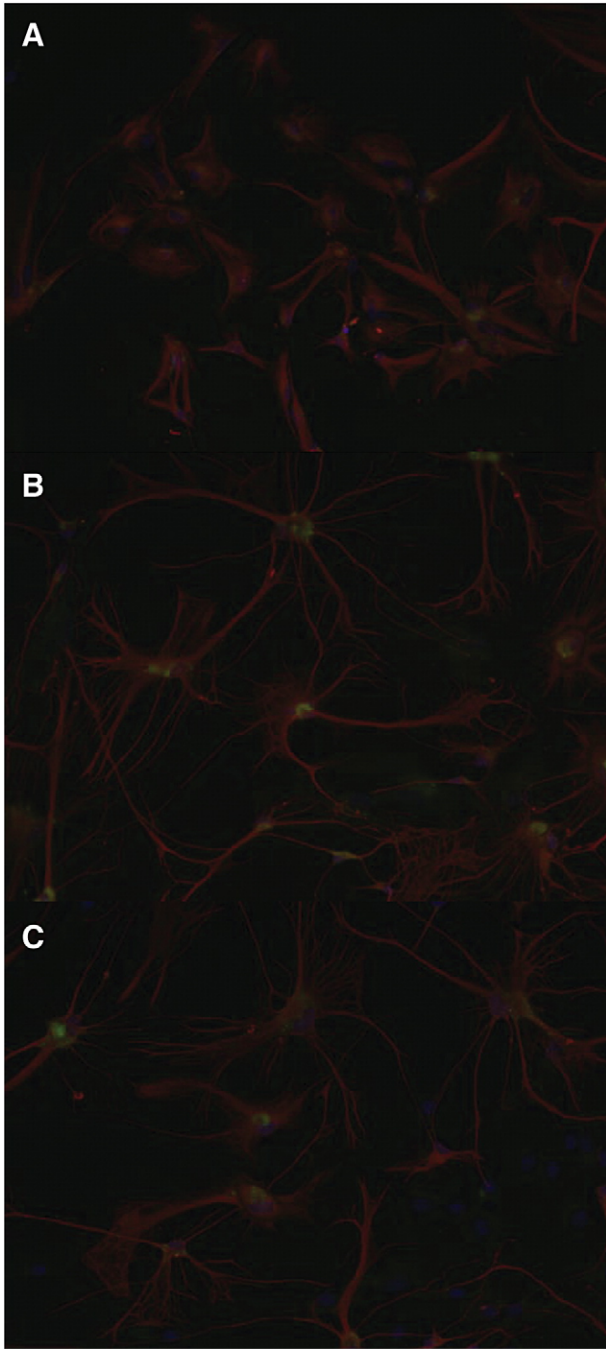


Fig. 4. Human fetal astrocytes stained with GFAP (red), IL-6 (green) and nuclear DAPI stain (blue) after 48 h in culture and treated with Locke's Buffer (negative control) (A), Poly(I:C) and IFN γ (B), or supernatants from PBMC stimulated with anti-CD3/anti-CD28 antibodies (C).

et al., 2004). IL-23 has been shown to support the persistence of Th17 cells that produce IL-17, TNF α and IL-6, but low levels of IFN γ (Cua et al., 2003; Aggarwal et al., 2003; Langrish et al., 2005). Blockade of IL-17 in EAE confers only partial protection from disease induction (Langrish et al., 2005; Park et al., 2005; Komiyama et al., 2006).

IL-6 within the CNS is a product of activated astrocytes and microglia with both neurotrophic and destructive properties. IL-6 production by astrocytes has been shown to be induced by

TNF α , IL-1 β , IL-6 itself and by autoreactive T cells (Van Wagoner and Benveniste, 1999; Van Wagoner et al., 1999; Colombatti et al., 2001). TNF α , IL-1 β and IL-6 interact synergistically to stimulate higher levels of IL-6 production by astrocytes than what they stimulate separately (Van Wagoner and Benveniste, 1999; Van Wagoner et al., 1999). IL-6 has previously been shown to be increased in both CSF and peripheral leukocytes in MS and in the CSF of TM patients, correlating with both acute and long term disability (Kaplin et al., 2005; Miljkovic et al., 2002). Our findings extend this to the peripheral immune compartment in TM. We have confirmed prior reports that TNF α , IL-1 β , IFN γ , and IL-6 all stimulate astrocyte IL-6 production, and furthermore demonstrate that the most substantial effect seems to be derived from IL-6 itself. We also provide evidence linking Th17 cells with astrocyte IL-6 production. However, immunodepletion of IL-6 and antibody blockade of TNF α , IFN γ , and IL-1 β did not completely suppress astrocyte IL-6 production, suggesting that other soluble factors may also be involved. While IL-6 can mediate neuronal and glial survival, it can also induce harmful nitric oxide metabolites and CNS damage. In murine models, IL-6 induces astrogliosis, microglial activation and potentiates neurologic degeneration (Campbell et al., 1993; Tilgner et al., 2001). Blockade or loss of IL-6 ameliorates EAE (Gijbels et al., 1995; Okuda et al., 1998). Interestingly, IL-6 has also been shown to alter dendritic cell maturation and peptide processing to increase the presentation of cryptic antigens, which could contribute to the phenomenon of epitope spreading (Drakesmith et al., 1998; Park et al., 2004). Soluble IL-6 receptor has been reported to be increased in the CSF of MS patients and can potentiate IL-6, TNF α and IL-1 β effects on astrocytes (Michalopoulou et al., 2004; Van Wagoner et al., 1999). We did not find differences in PBMC sIL-6R production comparing MS and TM to HC.

Limitations of this study include its modest sample size with a limited power to detect small differences between groups. In addition, the retrospective, cross-sectional nature of the study limits our ability to determine associations with disease activity and progression. The finding of similar IL-17 levels in TM and early MS raises the possibility that our TM cases are in fact early cases of MS that have not manifested lesions outside the spinal cord yet. However, episodes of complete TM are rare in MS and patients diagnosed with idiopathic TM according to published criteria rarely convert to MS over time (de Seze et al., 2005). None of our TM patients have subsequently converted to MS or Devic's neuromyelitis optica (NMO). It is also possible that IL-17 is an early triggering event in autoimmune CNS diseases. It has been shown that the regulatory cytokine TGF- β in conjunction with IL-6 drives the Th17 lineage (Mangan et al., 2006; Bettelli et al., 2006; Veldhoen et al., 2006). Therefore, one could speculate that excessive IL-6 production may break tolerance and perpetuate an autocrine loop in which Th17 cell lineages are expanded, migrate to the CNS, and induce more IL-6 with disastrous consequences. Future studies should address temporal relationships between the cytokines studied and disease activity, other characteristics of cellular subsets secreting IL-17, clinical and radiological findings associated with cytokine levels, and effects of therapeutic agents on IL-17 levels.

In summary, we have demonstrated that stimulated PBMC from TM patients produce increased levels of IL-17 and IL-6 as compared to healthy controls and MS patients. IL-17 and IL-6 levels are also increased in early as compared to established MS. IL-17 and its downstream cytokine effects (IL-6) act on numerous cell subsets (astrocytes, microglia, macrophages and T cells) so that even a small population of IL-17 producing cells invading the CNS could induce potent astrocyte activation with resultant toxic levels of IL-6, thereby accounting for the IL-6 mediated cascade leading to destructive and disabling pathology as we have described in TM (Kaplin et al., 2005). This report links Th17 cells to a CNS IL-6 cascade mediated via activated astrocytes.

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