A Paradoxical Effect of Systemic IL-23 in EAE – Limitation of Autoimmune Inflammatory Demyelination

Tarik Touil[1], Niansheng Chu[1], Ulrike Bohlmann[2], Patrick Bargsten[2], Yonghai Li[1], Denise Fitzgerald[1], Guang-Xian Zhang[1], Burkhard Becher*[,2], Abdolmohamad Rostami*[,1] and Bruno Gran*[,1,3]

[1]*Department of Neurology, Thomas Jefferson University, Philadelphia, PA, USA*

[2]*Inst. Experimental Immunology, University Hospital of Zurich, Switzerland*

[3]*Department of Neurology, University of Nottingham, UK*

Abstract: The heterodimeric cytokine IL-23 plays a non-redundant function in the development of cell-mediated, organ-specific autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE). To further characterize the mechanisms of action of IL-23 in autoimmune inflammation, we administered IL-23 systemically at different time points during both relapsing and chronic EAE. Surprisingly, we found suppression of disease in all treatment protocols. We observed a reduction in the number of activated macrophages and microglia in the CNS, while T cell infiltration was not significantly affected. Disease suppression correlated with reduced expansion of myelin-reactive T cells, loss of T-bet expression, loss of lymphoid structures, and increased production of IL-6 and IL-4. Here we describe an unexpected function of exogenous IL-23 in limiting the scope and extent of organ-specific autoimmunity.

Keywords: Autoimmunity, cytokines, T helper cells, animal models, EAE / multiple sclerosis.

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease that serves as a model for multiple sclerosis (MS), an inflammatory demyelinating disease of the CNS [1]. Adoptive transfer studies directly demonstrated the essential role of $CD4^+$ T cells in disease induction [2]. Since the T helper 1 (Th1) $CD4^+$ phenotype was associated with the encephalitogenic properties of myelin-reactive T cells, numerous studies have addressed the role of IL-12, the pivotal cytokine in Th1 differentiation, in EAE [3]. IL-12, a heterodimeric cytokine with a p40 and p35 subunit, was considered essential for EAE susceptibility based on disease resistance in IL-12p40-deficient mice [4]. However, the discovery of IL-23, a heterodimer of IL-12p40 and a novel IL-23p19 subunit related to IL-12p35 [5], offered new insight into the role of heterodimeric cytokines in autoimmune demyelination. EAE-resistant, IL-12p40-/- mice were in fact deficient for both IL-12 and IL-23. Our groups were the first to independently show that IL-12p35-/- mice, which only lack IL-12, are susceptible to severe EAE [6, 7]. It was then directly shown by Cua *et al*. [8] that IL-23p19-deficient mice are resistant to EAE, demonstrating that IL-23, rather than IL-12, is required for disease susceptibility. Consistent with these data, lack of IL-12 responsiveness in IL-12Rβ2-/- mice is associated with severe EAE [9], whereas loss of responsiveness to both IL-12 and IL-23 in IL-12Rβ1-/- mice protects from disease [10]. A divergent role for IL-12 and IL-23 was also recently demonstrated in the context of tumor rejection [11].

IL-23 was recently reported to promote the expansion of a novel population of $CD4^+$ T cells, which produce predominantly IL-17, IL-6, and TNF-α [12-14]. Adoptive transfer studies showed that induction of IL-17, a potent effector of inflammation, correlates with the encephalitogenic properties of effector T cells [15]. Also, IL-17 has been shown to alter the integrity of CNS endothelial cells conceivably altering the integrity of the blood-brain-barrier (Huppert and Prat, in press). Even though IL-23 is essential for the development of EAE, it remains unclear as to how it endows T cells with such pathogenic potential. Clearly, the fact that IL-23 promotes IL-17 secretion is not the underlying mechanism [16-18].

To date, no studies have addressed the role of systemically administered IL-23 in EAE pathogenesis. The central hypothesis for the studies presented here was that exogenous IL-23 would exacerbate disease. In two distinct EAE models, we report the unexpected finding that exogenous IL-23 markedly suppressed clinical and pathological signs of disease. In both relapsing and chronic EAE, IL-23 suppressed disease when administered during induction or at the onset of clinical signs. In relapsing EAE, IL-23 also suppressed subsequent clinical attacks. In IL-23-treated mice, there was reduced proliferation of autoreactive T cells, reduced levels of the Th1 transcription factor T-bet, and increased levels of IL-4 and IL-6. Our findings indicate that systemic IL-23 has

*Address correspondence to these authors at the Division of Clinical Neurology, University of Nottingham, C Floor, South Block, Nottingham University Hospitals, QMC Campus, Nottingham NG7 2UH, United Kingdom; Tel: +44 115 8231442; Fax: +44 115 970 9738;
E-mail: bruno.gran@nottingham.ac.uk
Department of Neurology, Thomas Jefferson University, Hospital for Neuroscience, Ste. 200; 900 Walnut Street, Philadelphia, PA 19107, USA; Tel: +1 215 955 8100; Fax: +1 215 503 5848;
E-mail: a.m.rostami@jefferson.edu
Division of Neuroimmunology, Neurology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland; Tel: +41 44-635-3701;
Fax: +41 44 635 6883; E-mail: burkhard.becher@neuroimm.unizh.ch

1876-8946/10 2010 Bentham Open

the potential to limit tissue damage in organ-specific inflammation.

MATERIALS AND METHODS

Mice and EAE Induction

Relapsing [19] and chronic EAE [7] were induced in eight week old female SJL and C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). Briefly, mice received a subcutaneous inoculation of the proteolipid protein peptide $PLP_{139-151}$ (100 μg/mouse; relapsing EAE) or myelin oligodendrocyte glycoprotein (MOG) peptide MOG_{35-55} (100 μg/mouse, chronic EAE) in CFA (containing 1 mg/ml *Mycobacterium tuberculosis* H37 Ra; Difco Laboratories, Detroit, MI) over two sites on the back (total injection volume, 200 μl). C57BL/6 mice also received pertussis toxin (PT, 200 ng in PBS) intraperitoneally (i.p.) on days 0 and 2 post-immunization (p.i.). Experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and the Swiss Veterinary Office. Mice were scored daily for clinical signs of EAE according to a published scale [7].

IL-23 Treatment

Recombinant IL-23 was purchased from R&D Systems (Minneapolis, MS). Levels of LPS were below 1.0 EU per 1 μg protein as determined by the LAL method (Associates of Cape Cod Inc., Falmouth, MA, USA). To increase stability and half-life in mice, in selected chronic EAE experiments we also utilized an IL-23-IgG3:Fc fusion protein (IL-23:Fc) [20]. IL-23-treated mice received either carrier-free IL-23 dissolved in PBS-0.1% BSA (abbreviated as IL-23 in the figures) or IL-23:Fc. Control mice received PBS containing 0.1% BSA (abbreviated as PBS in the figures) or a control mouse monoclonal IgG3 dissolved in PBS (abbreviated as PBS-IgG). Both IL-23-IgG3:Fc and IgG3 were a gift from Dr P. Puccetti, University of Perugia [20].

Histology and Immunohistochemistry

Mice sacrificed at different times p.i. were perfused transcardially with 0.1 M PBS (pH 7.4) and then with a solution of paraformaldehyde 4 % in 0.1 M PBS (pH 7.4) under deep anesthesia during termination. Cervical spinal cord sections were used for histological and immunohistochemical studies. Histological analysis for demyelination (Luxol fast bleu, LFB) and inflammatory infiltration (hematoxylin-eosin, HE) and immunohistochemistry for β-amyloid precursor protein (APP) (Chemicon International, Temecula, CA), nitrotyrosine (antibody from Upstate, Lake Placid, NY), iNOS (antibody from BD Transduction Laboratories, San Diego, CA) and GSI-B4 isolectin (Sigma, St. Louis, MO) were performed as described [21]. Severity of lesions was scored as previously reported [21]. Briefly, cervical spinal cord sections (approximately 300 mm apart) were used to assess inflammatory infiltrates. The average number of perivascular infiltrates was determined on at least four sections for each animal by arbitrary scoring: 0, no infiltrate; 1, a single infiltrate observed in three sections; 2, one infiltrate per section; 3, two or more infiltrates per section; 4, many infiltrates per section and at least one infiltrate per field view.

T-Cell Activation Assays and Detection of Cytokines

Proliferation of splenocytes was measured by incorporation of ^{3}H-thymidine after 72 h of culture in X-Vivo 15 medium [7]. The levels of IFN-γ, TNF- α, IL-6, IL-4, and IL-17A (kits purchased from PharMingen, San Jose, CA) were measured by ELISA in the supernatants of spleen cell cultures. Splenocytes were cultured in medium in the presence or absence of 60 μg/ml myelin peptides or 2.5 μg/ml Con A as a positive control. Supernatants were collected after 48 h for quantitative ELISA assays. Flow cytometric analysis and intracytoplasmic staining of splenocytes for detection of intracellular cytokine accumulation was performed as described [7]. Mononuclear cells were isolated from the CNS of immunized mice by 60/30% Percoll gradient centrifugation and analyzed by flow cytometry [7]. Data were acquired on a FACSAria flow sorter (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Real-Time PCR

Quantification of IL-4, IL-6, T-bet and GATA-3 was performed in pooled spleen cell samples from different treatment groups as described [22]. The internal control 18S rRNA was used as housekeeping gene. Change in expression was reported as $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT=\Delta CT$ samples-ΔCT controls.

Statistical Analysis

Non-parametric Mann-Whitney U test was used for clinical score comparisons and for the analysis of lesion numbers and indices.

RESULTS

IL-23 Suppresses Clinical Signs of Disease in Two Different EAE Models

To assess the effect of IL-23 on the clinical course of EAE, we used two different models of disease, namely, relapsing and chronic EAE. Relapsing EAE closely resembles the typical clinical course of early MS, with spontaneous exacerbations and remissions [19] and was induced in female SJL mice with $PLP_{139-151}$ (Fig. **1A-D**). Chronic EAE [7] reproduces pathophysiological aspects of more advanced MS and was induced in female C57BL/6 mice immunized with MOG_{35-55} (Fig. **1E-G**). C57BL/6 mice were treated with either IL-23:Fc or isotype matched control IgG.

IL-23 dissolved in PBS 0.1% BSA (IL-23) or the vehicle only (PBS 0.1% BSA) were administered i.p. at three different stages of relapsing EAE (induction phase - from day 5 to 10 post-immunization, p.i.; at disease onset - when mice reached a clinical score of 1; and at the end of the first clinical attack - when mice had recovered to a clinical score of 1 (Fig. **1**). IL-23:Fc or a control IgG (each dissolved in PBS) were administered at two stages of chronic EAE (induction phase and at disease onset). Clinical EAE was significantly suppressed in all treatment protocols (Fig. **1**). In chronic EAE, disease suppression was also observed with administration of recombinant IL-23, but to a lesser extent and only when IL-23 was given during the induction phase (Fig. **S1**). Cumulative score, maximal score, and average score were all significantly lower in IL-23-treated mice (all $p < 0.01$, Fig. **S1-2**). IL-23:Fc was not tested in relapsing EAE.

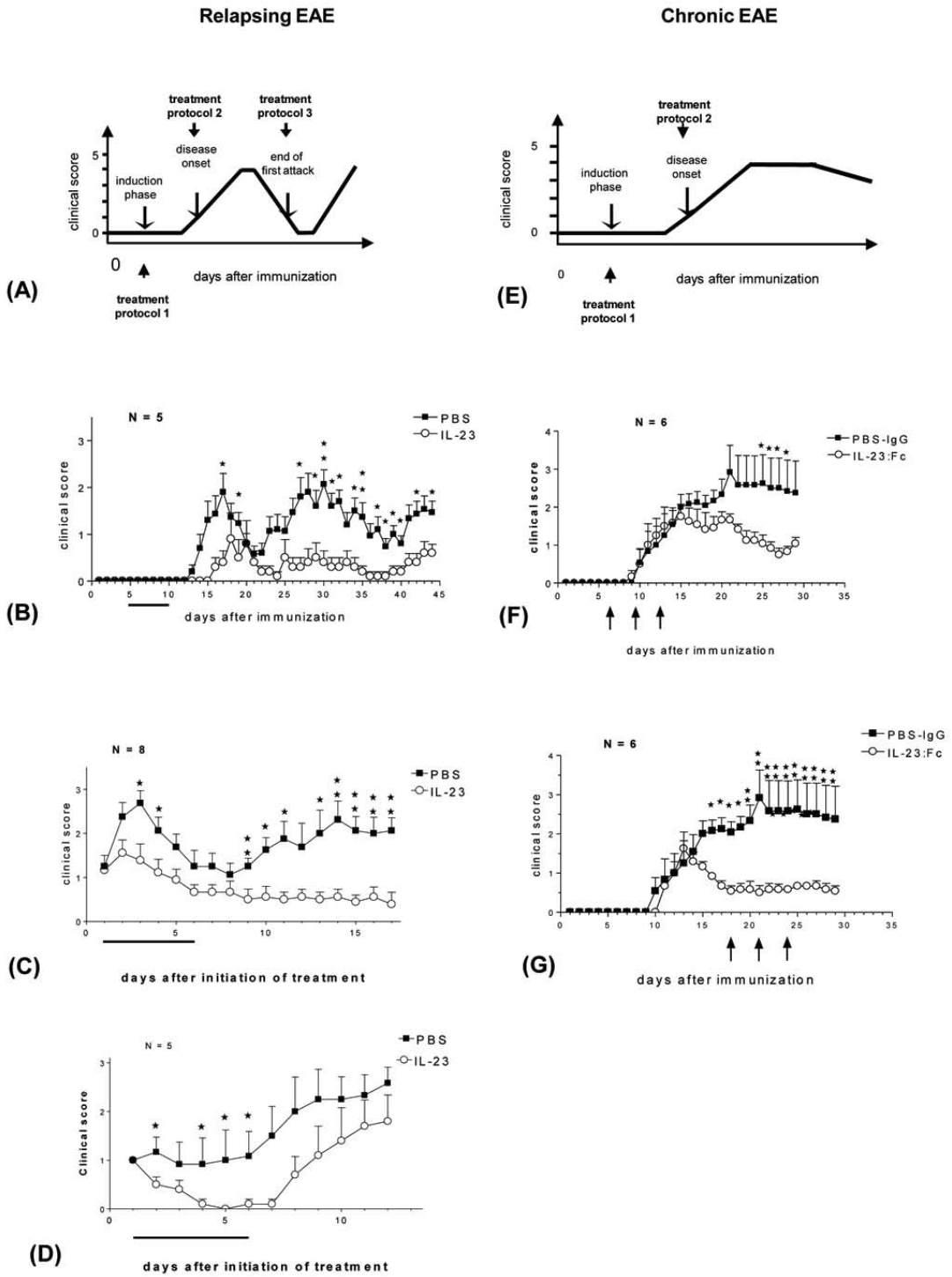


Fig. (1). Clinical effects of IL-23 administration at different time stages of relapsing (**A-D**) and chronic (**E-G**) EAE. Panel **A-D** represent the effects of IL-23 treatment of female SJL mice immunized with $PLP_{139-151}$ in CFA to induce relapsing EAE. Mice received six daily i.p. injections of 200 ng IL-23 dissolved in PBS 0.1% BSA or PBS 0.1% BSA starting at the indicated time points (panel A, disease induction, disease onset, and end of the first clinical attack; horizontal bars represent duration of treatment). **B.** Mice were treated from day 5 to day 10 p.i. **C.** Mice were treated starting on the day when they reached a clinical score of 1 and sacrificed 17 days after the onset of treatment. **D.** Mice were treated at the end of the first clinical attack, starting on the day they recovered to a clinical score of 1. Mice were then sacrificed 12 days after initiation of treatment. Data represent average clinical scores ± SE; * $p < 0.05$; ** $p < 0.01$. Panel **E-G** represent the effects of IL-23:Fc treatment of female C57BL/6 mice immunized with MOG_{35-55} in CFA (200 ng pertussis toxin given i.p. on day 0 and 2 p.i.) to induce chronic EAE. Mice received IL-23:Fc i.p. injections (2 μg/mouse, dissolved in 100 μl PBS; abbreviated as IL-23) at days 0, 3 and 6 p.i (panel E, F; arrows) or at disease onset and 3, 6 days later (panel **E**, **G**; arrows). Control mice received 2 μg IgG dissolved in PBS. Clinical EAE was scored daily according to a 0 to 5 severity scale. Data represent the mean clinical scores ± SE.

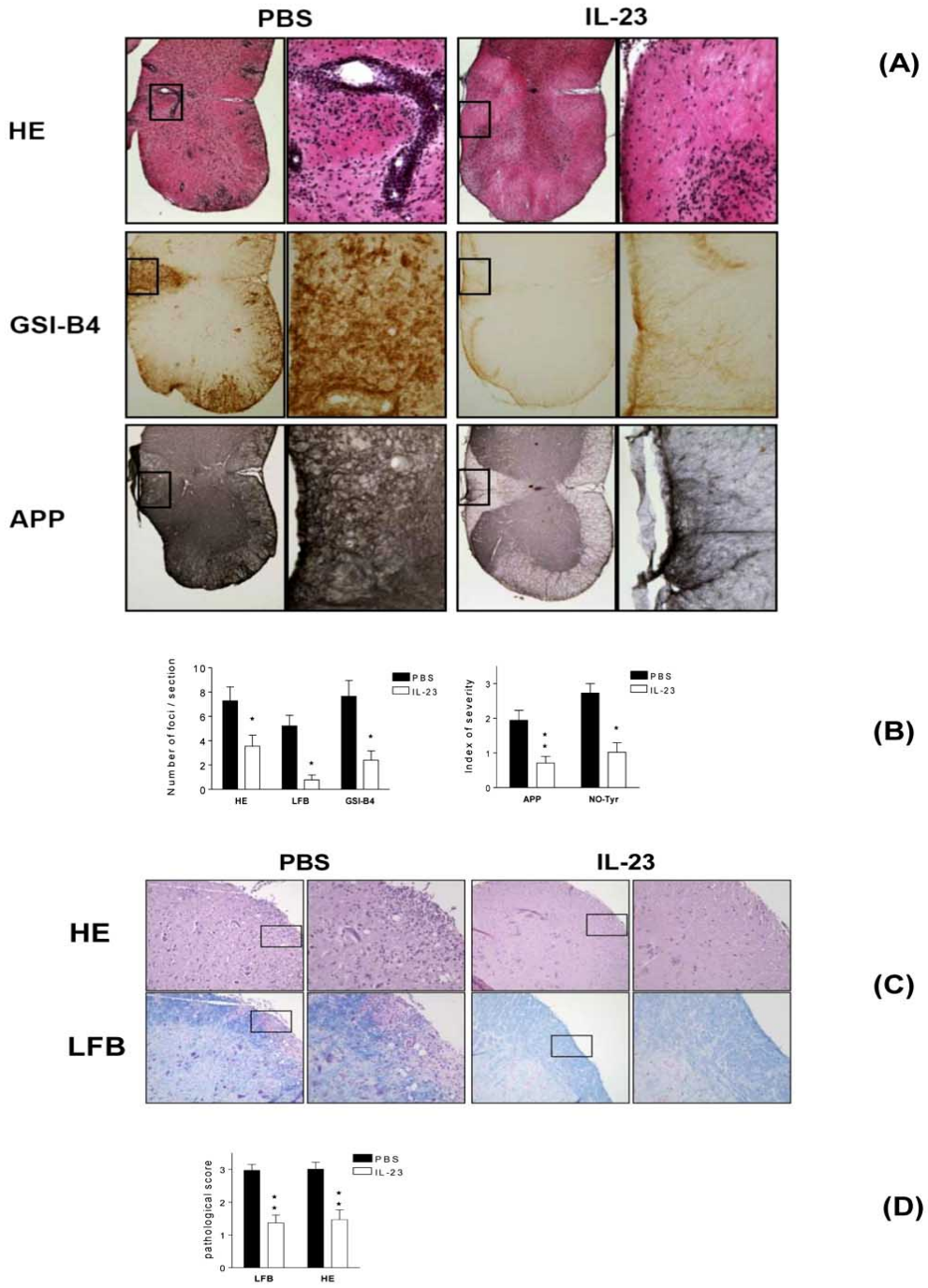
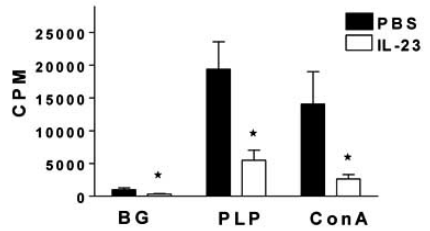


Fig. (2). Histological examination of spinal cords in IL-23 and PBS-treated mice. **A**. **Relapsing EAE**. Female SJL mice were immunized with $PLP_{139-151}$ in CFA, treated with IL-23 or PBS from day 5 to day 10 p.i., (see Fig. **1B**) and sacrificed 45 days p.i. after extensive perfusion. **B**. Quantitative histological analysis of spinal cords. Bars represent the number of foci of mononuclear cell infiltration (hematoxylin-eosin staining, HE), demyelination (Luxol Fast Blue staining, LFB), and activated microglia (GSI-B4 labeling) per spinal cord section. Bars represent the index of severity for axonal damage (accumulation of APP) and the production of peroxynitrite (immunolabeling of tyrosine residues of proteins, NO-tyr, a footprint of peroxynitrite production). Data represent average + SE; ** $p < 0.01$; * $p < 0.05$.

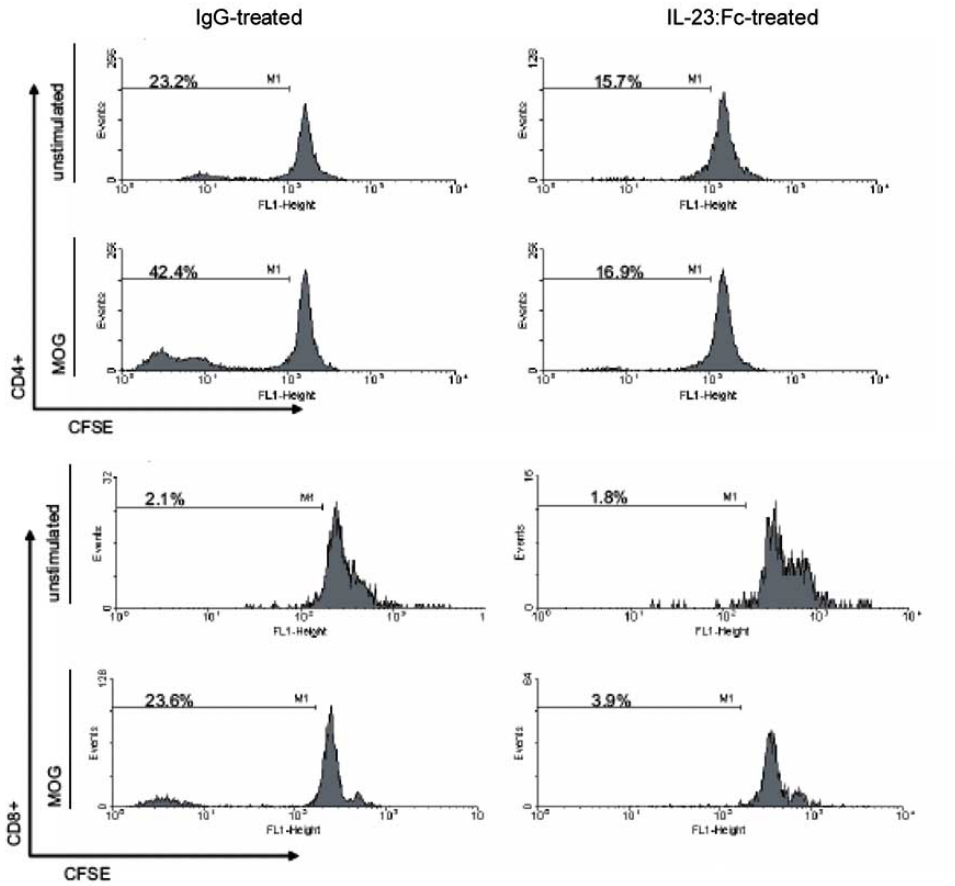
C. Chronic EAE. Female C57BL/6 mice were immunized with MOG_{35-55} in CFA, treated with IL-23 or PBS from day 0 to day 5 p.i., and sacrificed at day 21 p.i. Spinal cords were harvested after extensive perfusion. 30 µm sections were stained with H&E (inflammation) or LFB (myelin stain). Representative microphotographs for inflammation and demyelination are shown. Magnifications are 4x (first and third columns) and 20x (second and fourth columns, areas indicated in boxes). Panel **D** shows quantitative analysis of spinal cord inflammation (HE) and demyelination (LFB). Lesion areas were measured in three spinal cord sections per mouse. Values represent average + SE of normalized size of inflammatory or demyelinated lesions.

To determine the effect of systemic IL-23 on CNS inflammation and demyelination, we performed histological analysis of spinal cord sections of mice with relapsing (Fig. **2A-B**) and chronic EAE (Fig. **2C-D**) treated with IL-23 or vehicle. Both inflammatory infiltration (HE staining) and demyelination (LFB staining) were significantly inhibited in IL-23-treated mice (Fig. **2A-D**). In relapsing EAE, further immunohistochemical analysis showed a reduction in spinal cord areas containing activated microglial cells (GSI-B4 positive cells, p=0.047), the number of NO-Tyr positive cells (p=0.013), and areas of acute axonal damage (detected with APP staining; p=0.009; Fig. **2B**).

To determine the pattern of inflammatory cell composition in the CNS, we performed flow cytometric analysis on lymphocytes, monocytes, macrophages and microglia isolated from the CNS of relapsing EAE mice sacrificed 17 days after the initiation of treatment. The percentage of activated microglia / macrophages ($CD11b^{+}CD45^{hi}$) [7, 23] was significantly lower in IL-23-treated mice (N = 5 per group;



(A)



(B)

Fig. (3). Effect of IL-23 treatment on T-cell proliferation in EAE mice. **A**. Female SJL mice immunized with $PLP_{139\text{-}151}$ and received IL-23 or PBS i.p. from day 5 to 10 p.i. (Fig. **1B**). Splenocytes from sacrificed mice (2.5 x 10^5 cells/well in 96-well microtiter plates) were cultured in the presence or absence of 60 μg/ml $PLP_{139\text{-}151}$, or 2.5 μg/ml Con A. Proliferation was measured by addition of ^{3}H-thymidine during the final 12 h of incubation. Data represent average and SE of triplicate CPM values; ** $p < 0.01$. **B**. To study the early effects of IL-23 treatment on the expansion of myelin-reactive T cells, C57BL/6 mice immunized with $MOG_{35–55}$ were sacrificed 7 days p.i. Splenocytes were harvested, stained with CFSE and cultured for 5 days in the presence or absence of 50 μg/ml $MOG_{35–55}$ peptide. After staining for CD4 or CD8, splenocytes were fixed, and analyzed by flow cytometry. Numbers represent the percentage of proliferating $CD4^{+}$ or $CD8^{+}$ cells.

PBS, 15.04 ± 9.75; IL-23, 4.99 ± 2.11, $p < 0.05$). The percentages of resting resident microglia, $CD4^+$ T cells, and $CD8^+$ T cells did not significantly differ between the two groups ($CD11b^+CD45^{lo}$: PBS, 22.3 ± 4.25, IL-23, 26.33 ± 8.73; $CD4^+$: PBS, 15.83 ±3.24, IL-23, 32.75 ±16.55; $CD8^+$: PBS, 3.31 ± 0.56, IL-23, 3.29 ± 2.14).

Exogenous IL-23 Suppresses the Activation of Myelin Reactive T Cells *In Vivo*

To assess the effect of IL-23 on the activation of myelin-reactive T cells, we studied the proliferative responses of splenocytes obtained from mice sacrificed at different time points after immunization. As shown in Fig. (**3A**), the proliferative response of splenocytes obtained from SJL mice sacrificed 17 days after initiation of treatment and activated *in vitro* with $PLP_{139-151}$ or the mitogen Con A was significantly lower in IL-23-treated mice (no antigen, $p = 0.021$; $PLP_{139-151}$, $p = 0.015$; Con A, $p = 0.021$). Similar inhibition of activation was observed in splenocytes obtained from C57BL/6 mice treated with IL-23 or PBS, sacrificed 21 days p.i. and cultured *in vitro* with MOG_{35-55} or Con A ($n = 5$ mice per group; stimulation index, PBS, 20.47 ± 7.58; IL-23, 11.10 ± 5.14; $p < 0.05$; CPM in the absence of antigen, PBS, 674; IL-23, 3,682).

To further characterize early effects of exogenous IL-23, we also studied cell division in splenocytes obtained from C57BL/6 mice sacrificed 7 days p.i., stained with CFSE and cultured for 5 days in the presence or absence of 50 μg/ml MOG_{35-55}. Flow cytometric analysis showed that Ag-driven expansion of $CD4^+$ and $CD8^+$ T cells obtained from IL-23-treated mice was significantly reduced compared with control mice (Fig. **3B**). Consistent with reduced expansion of autoreactive T cells, the average number of splenocytes obtained from SJL mice sacrificed 17 days p.i. was lower in IL-23-treated ($N = 5$ mice per group; 33.6 ± 4.08 x 10^6 cells) compared with control mice (54.5 ± 12.67 x 10^6 cells; $p = 0.035$).

Exogenous IL-23 Disrupts Follicular Architecture in Secondary Lymphoid Structures

The fact that exogenous IL-23 limits rather than exacerbates EAE is surprising in light of the fact that mice lacking IL-23 are completely refractory to disease induction. To determine as to how exogenous IL-23 limits T cell priming, C57BL/6 mice were immunized with MOG_{35-55} and sacrificed 7 days p.i., when they were perfused and draining inguinal LNs and spleens were removed for immunohistochemical analysis. We consistently found lower numbers of $CD4^+$ T cells as well as $CD21^+$ B cells, and $CD11b^+$ APC in IL-23- as compared with PBS-treated mice (Fig. **4**). Overall, exogenous IL-23 appears to lead to the disintegration of follicular integrity in secondary lymphoid organs, which in turn likely contributes to the diminished expansion of myelin antigen-reactive T cells during the early induction phase of disease [24, 25]. In addition, IL-23 may also limit the expansion and mobilization of other immune cells, such as B cells and macrophages (Fig. **4**), which contribute to antigen presentation in EAE [26].

Exogenous IL-23 Blunts Effector Cytokine Expression

To determine how administered IL-23 impacts on T cell responses in regards to their expression of effector cytokines we cultured lymphocytes harvested from individual SJL mice in the presence or absence of 60 μg/ml $PLP_{139-151}$ or 5 μg/ml Con A and measured the production of IL-17A, IFN-γ, TNF-α, IL-4, and IL-6 by ELISA in supernatants collected after 48-h culture. Surprisingly, production of IL-17A, a proinflammatory cytokine induced in CD4+ T cells by IL-23, was not significantly different in the two groups of mice (Fig. **5A**). This was in contrast to induction of IL-17A by IL-23 added *in vitro* to spleen cell cultures (medium, 24.4 ± 8.3 pg/ml IL-17A; Con A, 303 ± 7.4 pg/ml; Con A + 50 ng/ml IL-23, 604 ± 7.9 pg/ml; $p < 0.001$) as previously reported [8, 12, 13].

The production of IFN-γ in response to $PLP_{139-151}$ and Con A was reduced, whereas production of IL-6 and IL-4 was significantly higher in IL-23-treated than in control mice (Fig. **5A**). Similar data were obtained in C57BL/6 mice immunized with MOG_{35-55}, and treated with rIL-23; however the reduction in IFN-γ was not statistically significant (Fig. **S3**). We cultured splenocytes from naïve SJL mice in the presence of absence of the T-cell mitogen Con A and 100 ng/ml IL-23 and found that the expression of mRNA for the Th1 transcription factor T-bet was strongly suppressed by IL-23 (Fig. **5B**). Consistent with reduced levels of T-bet, the expression of mRNA for the Th2 transcription factor Gata-3, and for the cytokines IL-6 and IL-4 was higher after exposure to IL-23 (Fig. **5B**). The expression of Gata-3 mRNA was also increased by 7.7 fold in pooled spleens of mice treated *in vivo* with IL-23 compared with control mice.

We then tested IL-4 as a possible mediator of the suppressive effect of IL-23 and found that IL-4 neutralization reversed IL-23-induced disease suppression in both relapsing and chronic EAE (Fig. **5C**). Anti-IL-4 treatment alone did not enhance EAE severity. These data suggest that the antibody blocked IL-4 produced in excess upon IL-23 injection and indicate that immune deviation to a Th2 phenotype contributes to the suppressive effect of IL-23.

DISCUSSION

Our observations provide evidence that IL-23 administered as an exogenous cytokine limits the expansion of myelin-reactive T cells. Clonal expansion of such cells is a key step in the induction phase of EAE and therapeutic manipulation during this phase accomplishes suppression of EAE by keeping autoreactive T cells below a pathogenic threshold [27]. In addition to anti-proliferative effects, the observed immune deviation suggests that exogenous IL-23 stimulates pathways that are distinct from those activated by endogenous IL-23. The latter mechanisms involve the induction of IL-17, as shown by experiments conducted in IL-23-deficient mice [8]. Adoptive transfer experiments, in which IL-23 was added *in vitro* to myelin reactive T cells, also showed that the pro-encephalitogenic activity of IL-23 is mediated by IL-17 [15]. As expected, we observed induction of IL-17 by IL-23 *in vitro*. However, we failed to observe a significant induction of IL-17 in splenocytes cultured *ex vivo* after i.p. injection of IL-23, suggesting that spatio-temporal effects markedly alter the function of IL-23 in inflammation. IL-23 has been recently shown to be dispensable for the *de novo* generation of IL-17-producing cells [28], which develop under the influence of TGF-β and IL-6 in the presence

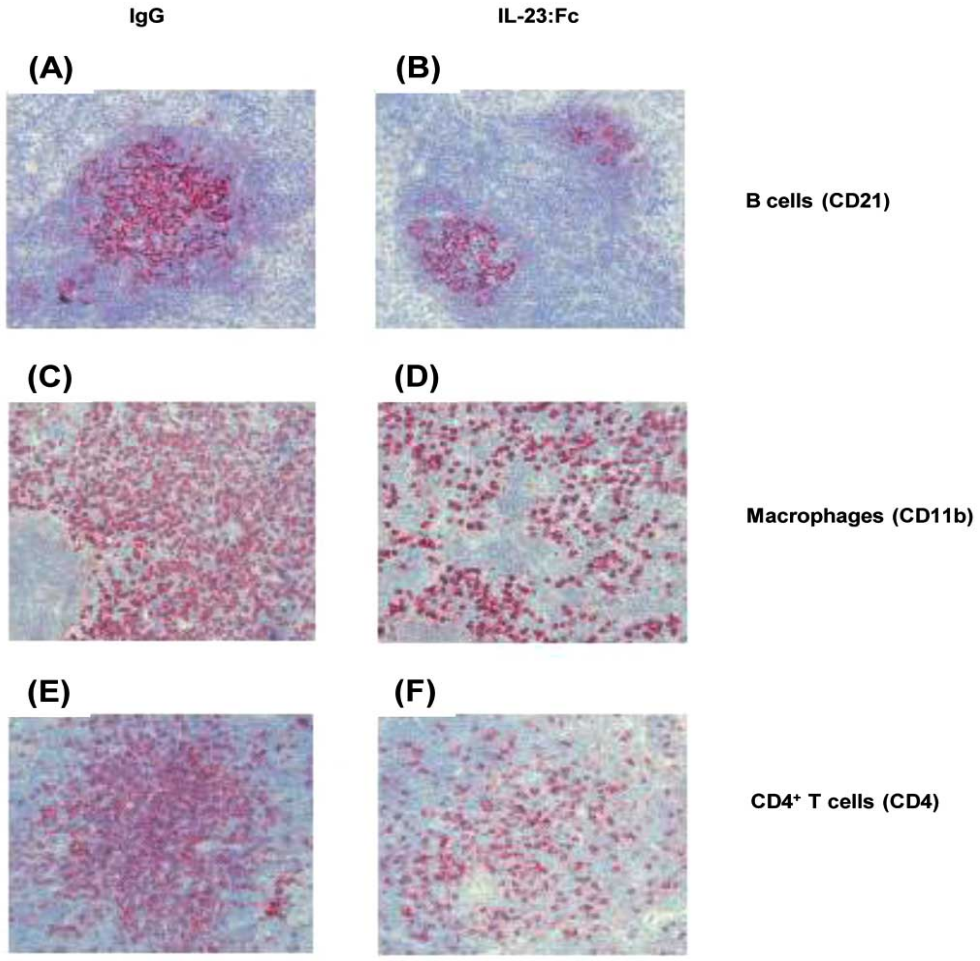


Fig. (4). Immunostaining of spleen tissue in $MOG_{35–55}$-immunized C57BL/6 mice treated with IL-23:Fc or IgG and sacrificed 7 days p.i. The figure compares splenic cell composition for B cells (CD21, panels **A** and **B**), macrophages and monocytes (CD11b, panels **C** and **D**) and $CD4^+$ T cells (CD4, panels **E** and **F**) of $MOG_{35–55}$-immunized C57BL/6 mice treated with a control IgG antibody (left column) and with IL-23:Fc (right column).

Panels **A-B** (CD21) show that although B-cell areas (lymphoid follicles of the white pulp) are not significantly affected in $MOG_{35–55}$-immunized mice by a control IgG (**A**), the administration of IL-23 leads to their disruption (**B**).

Panels **C-D** (CD11b) and **E-F** (CD4) show that macrophages (**C**, **D**) and $CD4^+$ T cells (**E**, **F**) in the red pulp are also disrupted by treatment with IL-23 (**D**, **F**) as compared with a control IgG (**C**, **E**).

of an inflammatory microenvironment [29]. IL-1 also functions as a limiting factor in the expansion of IL-17-producing cells in EAE [30]. Even when IL-17 is induced as an effector of inflammatory tissue damage, it is now clear that IL-17-producing cells are not sufficient to induce disease and additional factors are required for EAE susceptibility [31, 32]. Among these, the Th1 transcription factor T-bet plays a critical, non-redundant role. T-bet regulates the development of encephalitogenic cells in EAE, as shown by resistance to disease in mice that lack T-bet [33] or in which its expression is silenced by siRNA [34, 35]. Thus, inhibition of T-bet expression by IL-23 is likely to represent the mechanistic underpinning of the suppressive effect of exogenous IL-23 on EAE. Our data are also supported by the recent independent observation that IL-23 suppresses the expression of T-bet in $CD4^+$ T cells [13]. Most recently, Lovett-Racke's group reported that T-bet is critical for the encephalitogenicity of both Th1 and Th17 cells [36]. Our observation that IL-23 was active on both antigen- and mitogen-induced stimulation *in vitro* (Figs. **3A**, **5**, **S3**) suggests that its effects may not be restricted to our myelin antigen-specific system in particular, but may be relevant to other experimental paradigms as well.

An altered balance between T-bet and Gata-3 may also explain the increased production of IL-6 and IL-4 in IL-23-treated mice (Fig. **5**), thus favoring Th2 immune deviation in these experimental conditions. IL-6 may be directly induced by IL-23 [8, 15] and, in turn, promote IL-4 production, as shown in other experimental paradigms [37]. In agreement with our data, Peng *et al.* demonstrated that IL-23 signaling can regulate allergic airway inflammation by enhancing Th2 polarization [38].

It is noteworthy that, similar to IL-23p19-/- mice, IL-6-deficient mice are resistant to EAE [39], but injection of the cytokine suppressed disease in immunized rats [40].

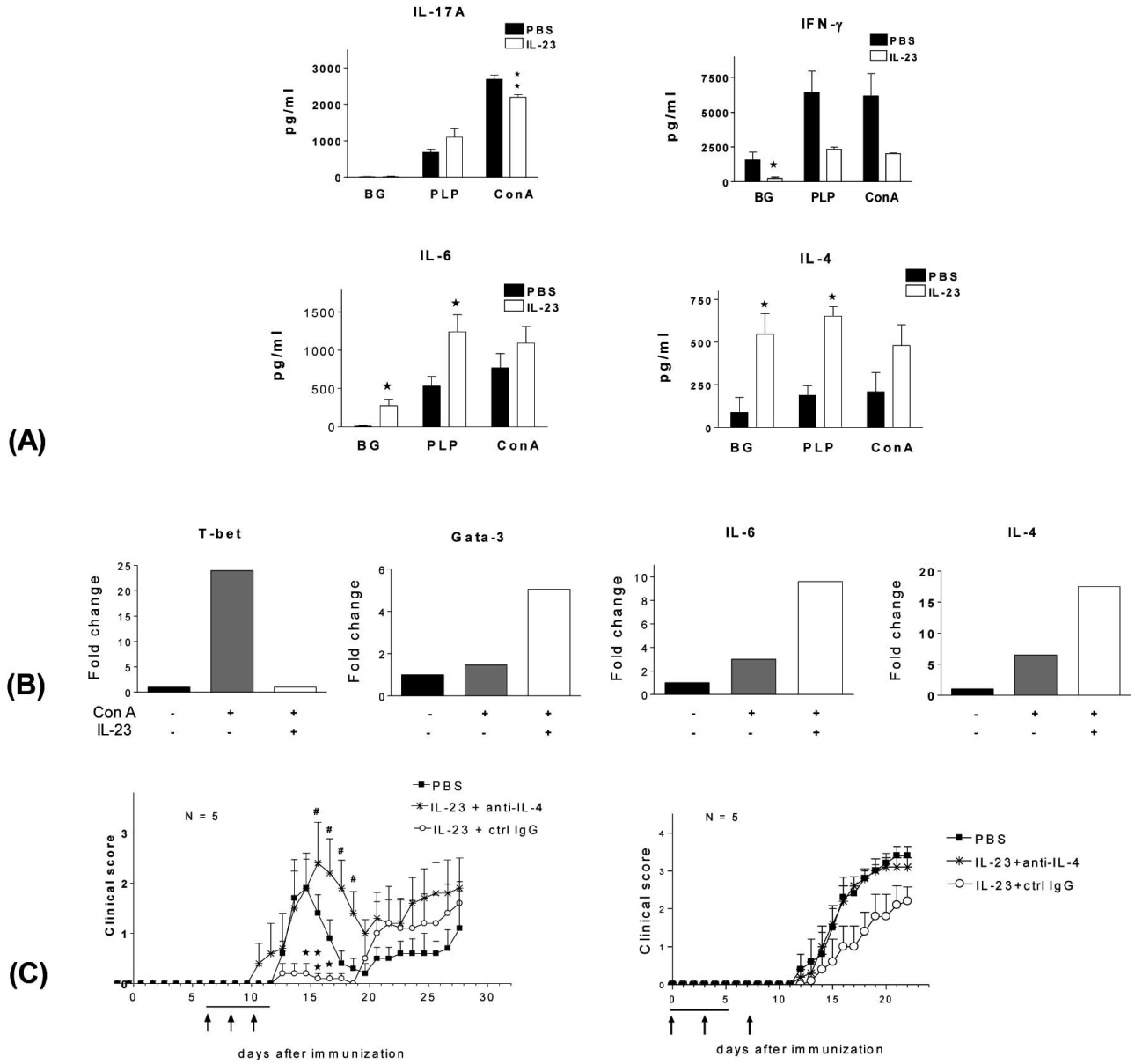


Fig. (5). Effect of IL-23 treatment on the phenotype of the immune response in mice with relapsing EAE. **A**. Splenocytes were harvested from IL-23 and PBS-treated SJL mice (see Fig. **1B**) and cultured in the presence or absence of 60 μg/ml $PLP_{139-151}$, or 2.5 μg/ml Con A. Cytokine concentrations were determined by ELISA in culture supernatants collected at 48 h. Data represent average + SE of triplicate values; ** $p < 0.01$; * $p < 0.05$. **B**. Effects of IL-23 on the expression of Th2 cytokines and transcription factors *in vitro*. Splenocytes from healthy SJL mice were cultured in the presence or the absence of Con A and 100 ng/ml IL-23. Relative expression of mRNA for IL-6, IL-4, and GATA-3, were determined by real-time PCR. Data represent relative gene expression in splenocytes after normalization for 18S ribosomal RNA. **C.** Effect of IL-4 neutralization on IL-23-induced suppression of EAE. Mice were immunized with $PLP_{139-151}$ (SJL mice, left panel) or MOG_{35-55} (C57BL/6 mice, right panel) and treated at the indicated days with PBS-0.1% BSA (PBS), 200 ng IL-23 + a neutralizing anti-IL-4 IgG (dissolved in PBS-0.1% BSA; arrows), or 200 ng IL-23 + a control IgG (horizontal bars). Mice were scored daily and data represent average scores ± SE. ★ refers to differences between PBS- and IL-23 + IgG groups; # refers to differences between IL-23 + IgG and IL-23 + anti-IL-4 groups. ★ or #, $p \leq 0.05$. ★★, $p < 0.01$.

Systemically administered IL-23 does not reverse EAE-resistance in IL-23-deficient mice [8]. However, expression of IL-23 in the target organ is essential for the maintenance of the encephalitogenic Th phenotype [17, 41]. Thus, the impact of systemic IL-23 application contrasts the role of IL-23 generated at the inflammatory site. An additional mechanism by which IL-23 may be exerting immune regulation is an alteration of the spleen architecture and composition (Fig. **4**). Indeed, both in rodent and marmoset EAE, immunization has been reported to disrupt spleen immune-architecture through T cell depletion and apoptosis [24] [25]. Interestingly, an immunoregulatory role for IL-23 has also been demonstrated in immune reactivity to cancer [11].

We found that IL-23:Fc was more potent than recombinant IL-23 in suppressing chronic EAE (Fig. **1F-G**, **5C**, and **S1**), suggesting that Fc-binding and potential uptake of IL-23:Fc by APC did not play a significant role in bioactivity. Most likely, it is the higher doses of IL-23:Fc that led to more potent suppression of chronic EAE.

In summary, while recent data have shown that CNS-produced IL-23 has clearly disease-promoting effects [8, 41] that correlate with the expansion of Th17 cells [8, 15, 42], we conclude that systemically administered IL-23 induces a distinct immunoregulatory pathway that limits the expansion of autoreactive T cells, leads to immune deviation, and limits the extent of organ-specific autoimmune disease.

ABBREVIATIONS

EAE = experimental autoimmune encephalomyelitis

MS = multiple sclerosis

PLP = proteolipid protein

MOG = myelin oligodendrocyte glycoprotein

PT = pertussis toxin

IL-23:Fc = IL-23-IgG3:Fc fusion protein

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health (to A.M.R.), the National Multiple Sclerosis Society (to B.G. and A.M.R.), the M.E. Groff Surgical Medical Research and Education Charitable Trust (to B.G.) the Swiss National Center for competence in research NCCR-plasticity and repair (to B.B.), the Swiss national MS-society (to B.B.), and an unrestricted grant by Serono Pharma (to B.B.). B.B. is a Harry Weaver neuroscience scholar of the National Multiple Sclerosis Society (USA).

CONFLICT OF INTEREST

The authors have no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

REFERENCES

[1] Steinman L. Assessment of animal models for MS and demyelinating disease in the design of rational therapy. Neuron 1999; 24: 511-4.

[2] Pettinelli CB, McFarlin DE. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after *in vitro* activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. J Immunol 1981; 127: 1420-3.

[3] Gran B, Zhang GX, Rostami AM. Role of the IL-12/IL-23 system in the regulation of T-cell responses in central nervous system inflammatory demyelination. Crit Rev Immunol 2004; 24: 111-28.

[4] Segal BM, Dwyer BK, Shevach EM. An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. J Exp Med 1998; 187: 537-46.

[5] Oppmann B, Lesley R, Blom B, *et al.* Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000; 13: 715-25.

[6] Becher B, Durell BG, Noelle RJ. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. J Clin Invest 2002; 110: 493-7.

[7] Gran B, Zhang GX, Yu S, *et al*. IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. J Immunol 2002; 169: 7104-10.

[8] Cua DJ, Sherlock J, Chen Y, *et al*. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 2003; 421: 744-8.

[9] Zhang GX, Gran B, Yu S, *et al*. Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. J Immunol 2003; 170: 2153-60.

[10] Zhang GX, Yu S, Gran B, *et al*. Role of IL-12 receptor beta 1 in regulation of T cell response by APC in experimental autoimmune encephalomyelitis. J Immunol 2003; 171: 4485-92.

[11] Langowski JL, Zhang X, Wu L, *et al*. IL-23 promotes tumour incidence and growth. Nature 2006; 442: 461-5.

[12] Aggarwal S, Ghilardi N, Xie MH, *et al*. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 2003; 278: 1910-4.

[13] Harrington LE, Hatton RD, Mangan PR, *et al.* Interleukin 17-producing CD4+ effector T cells develop *via* a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005; 6: 1123-32.

[14] Park H, Li Z, Yang XO, *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005; 6: 1133-41.

[15] Langrish CL, Chen Y, Blumenschein WM, *et al*. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005; 201: 233-40.

[16] Haak S, Croxford AL, Kreymborg K, *et al.* IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. J Clin Invest 2009; 119: 61-9.

[17] Gyulveszi G, Haak S, Becher B. Eur J Immunol 2009; 39: 1864-9.

[18] McGeachy MJ, Chen Y, Tato CM, *et al*. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells *in vivo*. Nat Immunol 2009; 10: 314-24.

[19] Vanderlugt CL, Neville KL, Nikcevich KM, *et al*. Pathologic role and temporal appearance of newly emerging autoepitopes in relapsing experimental autoimmune encephalomyelitis. J Immunol 2000; 164: 670-8.

[20] Belladonna ML, Renauld JC, Bianchi R, *et al.* IL-23 and IL-12 have overlapping, but distinct, effects on murine dendritic cells. J Immunol 2002; 168: 5448-54.

[21] Deloire MS, Touil T, Brochet B, *et al*. Macrophage brain infiltration in experimental autoimmune encephalomyelitis is not completely compromised by suppressed T-cell invasion: *in vivo* magnetic resonance imaging illustration in effective anti-VLA-4 antibody treatment. Mult Scler 2004; 10: 540-8.

[22] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-8.

[23] Sedgwick JD, Schwender S, Imrich H, *et al*. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proc Natl Acad Sci USA 1991; 88: 7438-42.

[24] De Vos AF, van Riel DA, van Meurs M, *et al*. Severe T-cell depletion from the PALS leads to altered spleen composition in common marmosets with experimental autoimmune encephalomyelitis (EAE). J Neuroimmunol 2005; 161: 29-39.

[25] Tsunoda I, Libbey JE, Kuang LQ, Terry EJ, Fujinami RS. Massive apoptosis in lymphoid organs in animal models for primary and secondary progressive multiple sclerosis. Am J Pathol 2005; 167: 1631-46.

[26] Zhang GX, Yu S, Li Y, *et al.* A paradoxical role of APCs in the induction of intravenous tolerance in experimental autoimmune encephalomyelitis. J Neuroimmunol 2005; 161: 101-12.

[27] Gran B, Chu N, Zhang GX, *et al.* Early administration of IL-12 suppresses EAE through induction of interferon-gamma. J Neuroimmunol 2004; 156: 123-31.

[28] Bettelli E, Carrier Y, Gao W, *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006; 441: 235-8.

[29] Veldhoen M, Hocking RJ, Atkins CJ, *et al*. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006; 24: 179-89.

[30] Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T

cells that mediate autoimmune encephalomyelitis. J Exp Med 2006; 203: 1685-91.
[31] Chen Y, Langrish CL, McKenzie B, *et al*. Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. J Clin Invest 2006; 116: 1317-26.
[32] Iwakura Y, Ishigame H. The IL-23/IL-17 axis in inflammation. J Clin Invest 2006; 116: 1218-22.
[33] Bettelli E, Sullivan B, Szabo SJ, *et al*. Silencing T-bet defines a critical role in the differentiation of autoreactive T lymphocytes. Exp Med 2004; 200: 79-87.
[34] Lovett-Racke AE, Rocchini AE, Choy J, *et al*. Silencing T-bet defines a critical role in the differentiation of autoreactive T lymphocytes. Immunity 2004; 21: 719-31.
[35] Gocke AR, Cravens PD, Ben LH, *et al*. T-bet regulates the fate of Th1 and Th17 lymphocytes in autoimmunity. J Immunol 2007; 178: 1341-8.
[36] Yang Y, Weiner J, Liu Y, *et al*. T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. J Exp Med 2009; 206: 1549-64.
[37] Diehl S, Chow CW, Weiss L, *et al*. Induction of NFATc2 expression by interleukin 6 promotes T helper type 2 differentiation. J Exp Med 2002; 196: 39-49.
[38] Peng J, Yang XO, Chang SH, Yang J, Dong C. IL-23 signaling enhances Th2 polarization and regulates allergic airway inflammation. Cell Res 2010; 20(1): 62-71.
[39] Samoilova EB, Horton JL, Hilliard B, Liu TS, Chen Y. IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. J Immunol 1998; 161: 6480-6.
[40] Di Marco R, Khademi M, Wallstrom E, *et al*. Curative effects of recombinant human Interleukin-6 in DA rats with protracted relapsing experimental allergic encephalomyelitis. J Neuroimmunol 2001; 116: 168-77.
[41] Becher B, Durell BG, Noelle RJ. IL-23 produced by CNS-resident cells controls T cell encephalitogenicity during the effector phase of experimental autoimmune encephalomyelitis. J Clin Invest 2003; 112: 1186-91.
[42] Murphy CA, Langrish CL, Chen Y, *et al*. Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 2003; 198: 1951-7.

Received: November 30, 2009 Revised: February 03, 2010 Accepted: February 16, 2010