IL-23 Is Increased in Dendritic Cells in Multiple Sclerosis and Down-Regulation of IL-23 by Antisense Oligos Increases Dendritic Cell IL-10 Production

Adi Vaknin-Dembinsky, Konstantin Balashov, and Howard L. Weiner[1]

IL-23 is a heterodimeric cytokine comprising a p19 subunit associated with the IL-12/23p40 subunit. Like IL-12, IL-23 is expressed predominantly by activated dendritic cells (DCs) and phagocytic cells, and both cytokines induce IFN-γ secretion by T cells. The induction of experimental autoimmune encephalitis, the animal model of multiple sclerosis (MS), occurs in mice lacking IL-12, but not in mice with targeted disruption of IL-23 or both IL-12 and IL-23. Thus, IL-23 expression in DCs may play an important role in the pathogenesis of human autoimmune diseases such as MS. We quantified the expression of IL-23 in monocyte-derived DCs in MS patients and healthy donors and found that DCs from MS patients secrete elevated amounts of IL-23 and express increased levels of IL-23p19 mRNA. Consistent with this abnormality, we found increased IL-17 production by T cells from MS patients. We then transfected monocyte-derived DCs from healthy donors with antisense oligonucleotides specific for the IL-23p19 and IL-12p35 genes and found potent suppression of gene expression and blockade of bioactive IL-23 and IL-12 production without affecting cellular viability or DCs maturation. Inhibition of IL-23 and IL-12 was associated with increased IL-10 and decreased TNF-α production. Furthermore, transfected DCs were poor allostimulators in the MLR. Our results demonstrate that an abnormal Th1 bias in DCs from MS patients related to IL-23 exists, and that antisense oligonucleotides specific to IL-23 can be used for immune modulation by targeting DC gene expression. *The Journal of Immunology,* **2006, 176: 7768–7774.**

IL-23 is a heterodimeric cytokine with many similarities to IL-12 that was discovered as part of a structure-based bioinformatics search for novel IL-6 family members (1). IL-23 comprises a p19 subunit that associates with the IL-12p40 subunit (1), while IL-12 is a combination of IL-12p35 with the same IL-12p40 subunit (2). Formation of biologically active IL-23 requires the synthesis of both p19 and p40 subunits within the same cell and is expressed predominately by activated dendritic cells (DCs)[2] and phagocytic cells (1). Like IL-12, IL-23 induces IFN-γ secretion from T cells and has been shown to be involved in Th1-type immune responses against mycobacteria (1). In contrast with IL-12, IL-23 preferentially stimulates memory rather than naive T cell populations both in humans and mice (1).

In addition, it has been reported that IL-23, but not IL-12, may be involved in the development of a novel T cell subset characterized by the production of the proinflammatory cytokine IL-17 (3). Of the different types of IL-17, both IL-17A and IL-17F have been shown to be regulated by IL-23. IL-17A is produced primarily by activated memory T cells. Increasing evidence suggests that the IL-17 family plays central role in inflammatory and autoimmune diseases (4).

Multiple sclerosis (MS) is a disabling, inflammatory, demyelinating disease of the CNS postulated to be a Th1 type T cell-mediated autoimmune disease (2, 5, 6). Recently, it has been shown that the induction of experimental autoimmune encephalitis (EAE) which serves as a murine model for MS occurs in mice lacking IL-12, but not in mice with targeted disruption of IL-23 or both IL-12 and IL-23 (7). Thus, it is possible that IL-23 may play an important role in the pathogenesis of MS by affecting immune system in a fashion that polarizes the innate immune response toward a Th1 bias. Previous studies of MS have demonstrated increased amounts of IL-12p40 in the nervous system (8) and increased production of IL-12 by PBMC (9, 10). IL-23 has not yet been studied in MS patients.

DCs are professional APCs and play a key role as part of the innate immune system in the induction of immune responses (11). Although it is known that DCs have functional IL-23Rs, little is known about the autocrine effect IL-23 has on their function. One way to examine the role of IL-23 in DC function is by suppressing IL-23 expression using antisense oligonucleotides (oligos) specific for the different subunits of IL-23 and IL-12. In the present study, we studied the production of IL-23 in monocyte-derived DCs from MS patients and investigated the effect of down-regulating the expression of IL-23 via antisense oligos on DC function.

Materials and Methods

Subjects

Peripheral blood was obtained after informed consent from healthy subjects and MS patients. All patients were seen at the Partners MS Center at Brigham and Women's Hospital (Boston, MA). We isolated monocyte-derived DCs from 30 MS patients: 1) 17 untreated relapsing-remitting (RR) MS patients who were minimally disabled (mean expanded disability status score (EDSS)), 1.5 $\pm$ 1.1 (mean $\pm$ SD), average age 41 $\pm$ 8 years, and who had MS for an average of 12.2 years; 2) 13 patients with secondary progressive (SP) MS (mean EDSS 6.5 $\pm$ 2.3, average age 54 $\pm$ 7.3 years), and who had MS for an average of 9.0 years. Seven of the progressive patients were untreated, and six were being treated with IFN-β1a. In the 30 patients studied, none had received steroids in the 2 mo before blood drawing, and untreated patients were not treated with IFN-β1a in the 10 mo before blood drawing or with immunosuppressive therapy in the 3 years before blood

Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115

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[1] Address correspondence and reprint requests to Dr. Howard L. Weiner, Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115-5817. E-mail address: hweiner@rics.bwh.harvard.edu

[2] Abbreviations used in this paper: DC, dendritic cell; MS, multiple sclerosis; EAE, experimental autoimmune encephalitis; oligo, oligonucleotide; RR, relapsing-remitting; SP, secondary progressive; EDSS, expanded disability status score; h, human.

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drawing. None of the patients were treated with glatiramer acetate before blood drawing. 3) Fourteen healthy donors, average age 35 ± 6.6 years.

For measuring IL-17 and IFN-γ production by $CD4^+$ T cells we studied 25 RR MS patients (mean EDSS, 2.5 ± 1.6) who were receiving IFN therapy and 23 age- and sex-matched healthy donors. None of the patients had received steroid treatment in the 2 mo before blood drawing.

Generation of monocyte-derived DCs

PBMC were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia). $CD14^+$ monocytes were negatively selected from blood mononuclear cells by using magnetic beads (Miltenyi Biotec). A total of 1×10^6 cells were plated in 24-well plates (Falcon) in 1 ml of RPMI 1640 complete medium in the present of recombinant human GM-CSF and rhIL-4 (100 ng/ml and 50 ng/ml; R&D Systems). Cells were fed on days 2 and 4 by changing 500 μl of the medium and keeping the same concentration of IL-4 and GM-CSF. At day 5, DCs were matured by adding 100 ng/ml LPS. Twenty-four hours after stimulation, supernatants were collected for IL-23/IL-12 measurements, and 48 h after stimulation, IL-10/TNF-α levels were measured. In some experiments, rhIL-23 (10 ng/ml) or rhIL-12 (1 ng/ml) was added to the DCs 3 h after the cells were matured with LPS.

Purification of T cells and MLR

Purified $CD4^+$ T cells (>95% $CD4^+$ T cells) from an individual healthy donor were prepared using magnetic beads for negative selection (Miltenyi Biotec). The MLR was set up by culturing the purified CD4 T cells (1×10^5 cells/0.2 ml of RPMI 1640 complete medium per well in triplicate) with different numbers of allogeneic-transfected DCs obtained 24 h after maturation. All the responder T cells were from the same donor at the same time. Five days after initiation of MLR, a fraction of culture medium was harvested to measure the production of IFN-γ by ELISA. One microcurie of [^{3}H]thymidine (NEN) was added to each well during the last 10 h of culture to measure [^{3}H]thymidine incorporation using a liquid scintillation counter. In some experiments, we added rhIL-23 (10 ng/ml) or rhIL-12 (1 ng/ml) to the culture.

IL-17 production by activated T cells

Purified $CD4^+$ T cells were prepared using magnetic beads for negative selection (Miltenyi Biotec). The $CD4^+$ T cells were cultured at a concentration of 2×10^6 cells/ml in 48-well plates coated with anti-CD3 mAb (2.5 μg/ml, 250 μl/well). Cells were cultured for 48 h with and without 10 ng/ml rhIL-23 or 1 ng/ml rhIL-12, the cells were counted, and the supernatants were assayed for IL-17 and IFN-γ. We adjusted the amount of cytokine production by $CD4^+$ T cells to 2×10^6/ml counted at 48 h of culture. To test the biological activity of IL-23, CD4 T cells from healthy donors were processed as described above, and supernatants from monocyte-derived DCs transfected with the different antisense oligos were added to the $CD4^+$ T cells. The rhIL-23 was added to the medium, and soluble IL-23R was added to the supernatants derived from monocyte-derived DCs treated with antisense oligo control at 2 and 5 ng/ml, respectively (R&D Systems). The secreted IL-17 was determined after 48 h of culture by ELISA.

Cytokine ELISA

Secretion of IL-23, IL-23/12p40, and IL-12p70 was determined in 24-h cell culture supernatants, and production of IFN-γ, TNF-α, IL-17, and IL-10 was measured in 48-h supernatants by ELISA. The protocols for IL-10 and IFN-γ ELISA are as described (9). IL-23/12p40, IL-12p70, TNF-α, and IL-17 ELISA were prepared using predesigned kits from R&D Systems, according to the manufacturer's instructions.

Because there is no commercially available ELISA for IL-23, we designed an ELISA to measure IL-23. The IL-23 ELISA was performed by coating plates overnight with 5 ng/ml soluble rhIL-23R/Fc Chimera (NSO-derived) (R&D Systems). For detecting Ab, 2 μg/ml mouse anti-human p40 Ab (clone no. 169516; R&D Systems) was added for 2 h, followed by biotinylated rat anti-mouse mAb (Zymed Laboratories) for an additional 1 h. The sensitivity of the IL-23 ELISA was 78 pg/ml. The IL-23 ELISA standard curve is shown below in Fig. 1. The following recombinant human cytokines were tested at a level of 20 ng without cross-reactivity with IL-23: IL-12p70, p40, IL-17 GM-CSF, IL-4 (all from R&D Systems). The sensitivity of IFN-γ, TNF-α, IL-17, IL-23/12p40, IL-12p70, and IL-10 ELISA was 31.25 pg/ml.

Flow cytometry

Directly conjugated mouse mAbs, Cychrom-conjugated CD86 and CD40, PE-conjugated anti-HLA-DR, anti-CD83, and anti-CD80, were supplied by BD Pharmingen. PE-conjugated anti-CD62L was supplied by R&D Systems. Cellular staining was measured on a FACS Calibur instrument (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences), with results expressed as percentage of cell staining above background staining obtained with isotope control mAb.

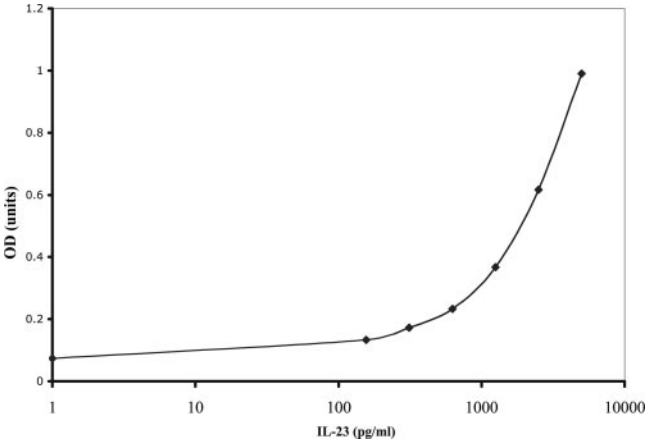


FIGURE 1. IL-23 ELISA calibrating curve. The IL-23 ELISA uses a standard approach. IL-23R/Fc Chimera is used as a capture reagent and anti-human IL-12/IL-23 p40 mAb as a detection Ab (R&D Systems). The sensitivity of the IL-23 ELISA was 78 pg/ml.

Real time (TaqMan) RT-PCR analysis

Total RNA was extracted from 1×10^6 DCs using an RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using an Applied Biosystems kit. The primers and probes for the TaqMan RT-PCR assays for IL-12p40, IL-23p19, and IL-12p35 were generated using primers that were described previously (12). Those primers include: hIL-12p40 (forward), ACGGACAAGACCTCAGCCAC; (reverse) GGGCCC GCACGCTAA; fluorescent probe, TCATCTGCCGCAAAAATGC CAGC. Human IL-12p35 (forward), CCACTCCAGACCCAGGAATG; (reverse) GACGGCCCTCAGCAGGT; fluorescent probe, TCCCATGC CTTCACCACTCCCAA. Human IL-23p19 (forward), GAGCCTTCTCT GCTCCCTGAT; (reverse) AGTTGGCTGAGGCCCAGTAG; fluorescent probe, CCTGTGGGCCAGCTTCATGCCT. RT-PCR were performed according to the manufacturer's directions using an Applied Biosystems PRISM 7700 thermal cycler. The human GAPDH, a housekeeping gene, was used to normalize each sample and each gene.

Antisense oligo transfection

One micromole of morpholino antisense oligos IL-23p19 and IL-12p35 oligos (IL-23p19 antisense oligo, IL-12p35 antisense oligo, respectively), or standard control oligos (control antisense oligo) (Gene Tools) was incubated with transfection medium for 20 min at room temperature. The nucleotide sequences of the p19 and p35 antisense oligo are as follows: IL-23p19, 5′-CATTACAGCTCTGCTCCCCAGCATC-3′; IL-12p35, 5′ATTGTCCCGAGGCGCGGCAGGACTT-3′; and the sequences of the control oligo was 5′-CCTCTTACCTCAGTTACAATTTATA-3′ (scrambled). Transfection was performed for 3 h in serum-free medium, and then the cells were cultured as described above.

Statistical analysis

Results are expressed as mean ± SEM for each group. Statistical significance was calculated using Student's or Welch's *t* tests.

Results

IL-23 expression is elevated in monocyte-derived DCs from MS patients both at the protein and mRNA levels

One of the major cytokines secreted from mature DCs that is believed to play a central role in chronic inflammatory disease is IL-23 (3). IL-23 belongs to the same hetrodimeric cytokine family as IL-12 and both share the IL-23/12p40 subunit (1). Therefore, we compared the secretion of IL-12 and IL-23 from monocyte-derived DCs of MS patients and healthy donors. As

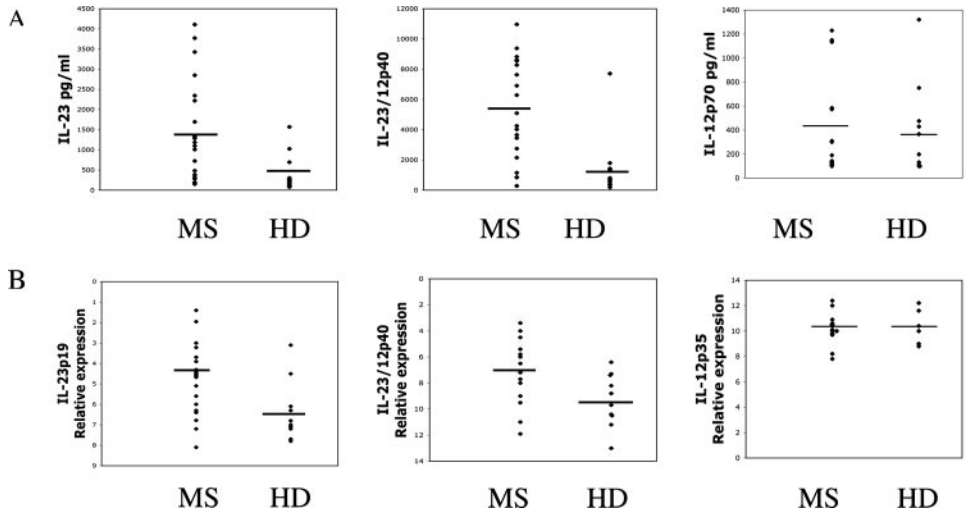


FIGURE 2. IL-23 expression in monocyte-derived DCs from MS patients and control subjects. *A*, IL-23, IL-12, and IL-23/12p40 secretion from mature monocyte-derived DCs in MS patients and health donors. Each dot represents the average amount of the secreted cytokine from three different wells of the same individual. For IL-23, $p = 0.018$ MS vs HC; for IL-23/12p40, $p = 0.003$ MS vs HC. *B*, IL-23p19, IL-12p35, and IL-12p40 mRNA expression was measured by RT-PCR and is presented as ΔCt values. Each dot represents the average ΔCt of a triplicate from the same individual. A lower ΔCt means that there is a higher expression of the gene. For IL-23p19, $p = 0.028$ MS vs HC; for IL-23/12p40, $p = 0.045$ MS vs HC.

shown in Fig. 2*A*, we found increased levels of both IL-23 and IL-23/12p40 in supernatants collected from monocyte-derived DCs of MS patients, compared with the healthy donors. IL-23 (1307.8 pg/ml) in MS vs 407.9 pg/ml in control ($p = 0.018$); IL-23/12p40 (5087.19 pg/ml) in MS vs 1401.31 pg/ml in control ($p = 0.003$). For IL-12p70, there was no significant difference between the two groups, 580 pg/ml in MS vs 536.0 pg/ml in control ($p = 0.3$). The MS groups that we tested included RR ($n = 17$) and SP ($n = 13$) subjects. Levels of IL-23 and IL-23/12p40, but not IL-12p70, were increased in both the RR and SP subjects vs control. For IL-23, RR (1325.34 pg/ml, $p = 0.019$ vs control; SP, 1286 pg/ml, $p = 0.012$ vs control). For IL-23/12p40, RR (5.38 ng/ml, $p = 0.007$ vs control; SP, 4.81 ng/ml, $p = 0.0014$ vs control). No significant difference was found between the treated and untreated patients ($p = 0.47$). Serum levels of IL-23 were undetectable in both MS patients and normal individuals

We than measured the gene expression of IL-23p19, IL-12p35, and IL-23/12p40 by quantative RT-PCR in monocyte-derived DCs from MS patients and healthy donors. The values obtained for each gene were normalized to the housekeeping gene GAPDH. As shown in Fig. 2*B*, we found a significant increase in the expression of the IL-23p19 and p40 subunits in MS subjects vs healthy donors, (IL-23p19, 4.83 ΔCt in MS vs 6.57 in control group, $p = 0.0028$; IL-23/12p40, 7.30 ΔCt in MS vs 9.29 ΔCt in control group, $p = 0.045$ MS vs control; MS to healthy donors in IL-23p19 and IL-23/12p40, respectively). As shown in Fig. 2*C*, we found no significant increase in the expression of the IL-12 p35 in MS subjects vs healthy donors (IL-12p35, 10.33 ΔCt in MS vs 10.20 in control, $p = 0.8$.) Thus, we found increased expression of IL-23 at both the protein and mRNA level in patients with both RR and SP MS.

Activated CD4$^+$ T cells of MS patients produce increased amounts of IL-17

It is known that CD4$^+$ T cells activated with IL-23 produce IL-17 (13). Thus, our finding of increased IL-23 production from monocyte-derived DCs in MS raised the question of whether CD4$^+$ T cells from MS patients produced higher levels of IL-17. To address this question, we purified CD4$^+$ T cells from MS patients and healthy controls and stimulated with plate-bound anti-CD3. We analyzed the supernatants for IL-17 production in the presence or absence of exogenous rhIL-23 or rhIL-12. As shown in Fig. 3*a*, we found increased IL-17 production by CD4$^+$ T cells from MS patients vs healthy donors both with and without the addition of exogenous rhIL-23. In cultures without IL-23, IL-17 was 894.2 pg/ml in MS vs 361.5 pg/ml in control ($p = 0.014$); in cultures

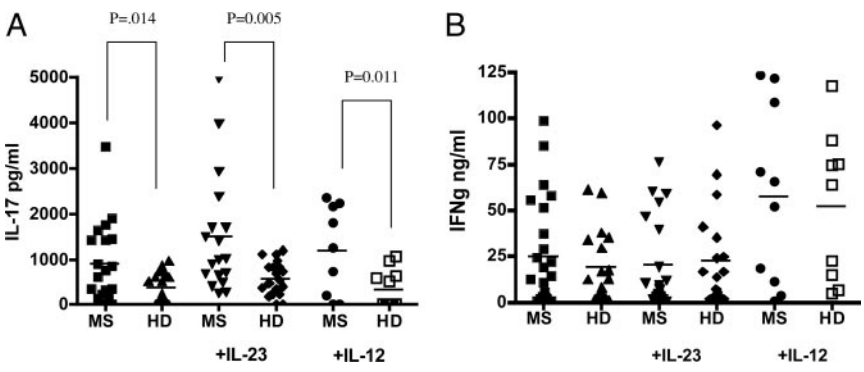
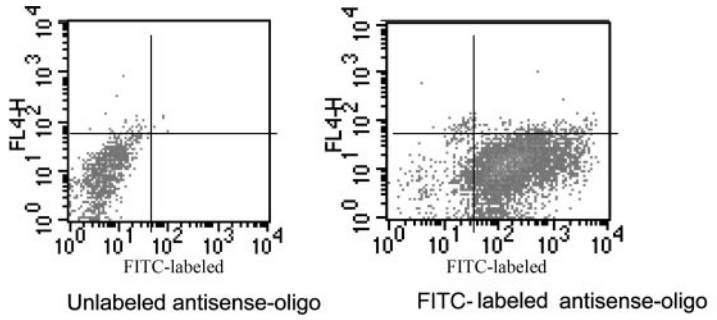


FIGURE 3. IL-17 and IFN-γ secretion from anti-CD3 stimulated CD4$^+$ T cells from MS patients and control subjects with and without the addition of rhIL-23 or rhIL-12. Each dot represents the average amount of the secreted cytokine from three different wells of the same individual. *A*, Without IL-23, IL-17 was 894.2 pg/ml in MS vs 361.5 pg/ml in control ($p = 0.014$); in cultures with IL-23, IL-17 was 1493.2 pg/ml in MS vs 564.3pg/ml in control ($p = 0.005$); in cultures with IL-12, IL-17 was 1190.2 pg/ml in MS vs 595.1 pg/ml in control ($p = 0.011$). *B*, No differences in IFN-γ secretion between healthy donors and MS patients either cultured alone or with the addition of rhIL-23 or rhIL-12.

A



B

	Control	IL-23p19 antisense-oligo	IL-12p35 antisense-oligo
CD80	35 ± 11%	35 ± 7%	32 ± 10%
CD83	58 ± 8%	61 ± 9%	54 ± 7%
CD86	92 ± 10%	92 ± 5%	94 ± 4%
CD40	78 ± 11%	80 ± 12%	83 ± 9%
HLADR	97 ± 1%	96 ± 1%	96 ± 1%

FIGURE 4. Monocyte-derived DCs are efficiently transfected with antisense oligos. *A*, Monocyte-derived DCs were transfected with FITC-labeled or nonlabeled, control antisense oligos. Transfection efficacy was assessed by flow cytometry 48 h after LPS activation. Data are representative of at least three independent experiments. *B*, Effect of p19 and p35 antisense transfection on DC phenotype after maturation. The DC phenotype was assessed by expression analysis of CD83, HLA-DR, CD86, CD40, and CD80 by flow cytometry. Data are representative of three independently performed experiments.

with IL-23, IL-17 was 1493.2 pg/ml in MS vs 564.3pg/ml in control ($p = 0.005$); in cultures with IL-12, IL-17 was 1190.2 pg/ml in MS vs 595.1 pg/ml in control ($p = 0.011$). We also analyzed the supernatants from the cultures reported in Fig. 3*a* for IFN-γ production. As shown in Fig. 3*b*, we found no differences in IFN-γ secretion between healthy donors and MS patients either cultured alone or with the addition of rhIL-23 or rhIL-12.

Thus, we find that in both healthy donors and MS patients addition of IL-23 causes a significant increase of IL-17 ($p < 0.05$), but not IFN-γ, whereas addition of IL-12 causes a significant increase of IFN-γ ($p < 0.03$) but not IL-17. These results are analogous to what has been observed in murine systems (14).

Monocyte-derived DCs are efficiently transfected with antisense oligos with no phenotype alterations following transfection with IL-23p19 or IL-12p35 antisense oligos

Given that there was elevated IL-23 in monocyte-derived DCs from MS subjects, we then embarked on a series of experiment to determine whether we could selectively decrease IL-23 production by DCs and what effect such a decrease had on DC function. We began by evaluating the transfection of human monocyte-derived DCs with control antisense oligos to determine the transfection efficacy using FITC-labeled control antisense oligos. The transfection efficiency was quantified by flow cytometry. As shown in Fig. 4*A*, FITC-labeled antisense successfully transfected >90% of the cells.

To down-regulate the expression of IL-23 or IL-12, we transfected human monocyte-derived DCs with IL-23p19 or IL-12p35 antisense oligos. To assess whether the transfection with IL-23p19 or IL-12p35 antisense oligos changed the maturation status of the monocyte-derived DCs, we stained the transfected cells for surface markers. Monocyte-derived DCs were collected and analyze for their phenotypes by flow cytometry. As shown in Fig. 4*B*, transfection of human monocyte-derived DCs with IL-23p19 or IL-12p35 antisense oligos did not change DC maturation, compared with the control with regard to MHC class II, CD86, CD83, CD40, and CD80 expression.

Transfection of mature DCs with IL-23p19 or IL-12p35 antisense oligos specifically decreases IL-23 and IL-12 production

The specificity of IL-23 p19 and IL-12p35 antisenses in knocking down the expression of the two genes in mature monocyte-derived DCs was investigated. Immature human DCs were prepared as described in *Materials and Methods*, and after maturation, the supernatants of the DCs transfected with the different antisenses were collected and IL-23 and IL-12 production was measured by ELISA. As shown in Fig. 5*A*, IL-23p19 antisense transfection decreased IL-23 protein production significantly ($p < 0.05$) by ~70%, compared with transfection with control antisense. There was no change in the IL-23 production by IL-12p35 antisense oligo transfection. As shown in Fig. 5*B*, when we measured IL-12, there was no significant effect in cells transfected with IL-23p19 antisense, whereas an effect was observed in cells transfected with IL-12p35 antisense ($p = 0.034$). This demonstrates that the IL-23p19 or IL-12p35 antisense oligos specifically down-regulate IL-23 and IL-12, respectively.

Monocyte-derived DCs transfected with p19 antisense produce decreased amounts of biologically active IL-23

In response to IL-23, but not IL-12, stimulation, activated/effectors $CD4^+$ T cells produce IL-17. IL-12 appears to antagonize IL-23-induced IL-17 production (15). To determine whether the down-regulation of IL-23 by antisense oligos also decreased the biological activity of IL-23, we measured the amount of IL-17 secretion by CD4 T cells stimulated by anti-CD3 in the presence of supernatants taken from DCs transfected with different antisense oligos. As shown in Fig. 6, $CD4^+$ T cells stimulated by anti-CD3 secreted increased amounts of IL-17 when cultured in the presence of IL-23 or supernatants from DCs transfected with control antisense. Addition of soluble IL-23R to the cultures reversed this effect. No increase in IL-17 production was observed with supernatants from DCs transfected with antisense oligos to IL-23p19 ($p < 0.05$), whereas an increase was observed with supernatants from DCs transfected with antisense to IL-12p35. Thus, monocyte-derived

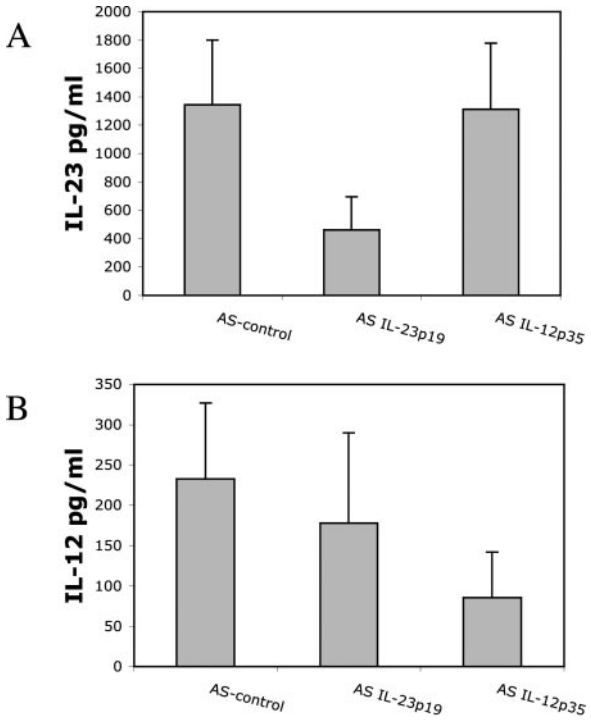


FIGURE 5. IL-23p19 and IL-12p35 antisense oligos efficiently down-regulate the expression of IL-23 and IL-12 in monocyte-derived DCs. *A*, IL-23 was measured by ELISA in supernatants of monocyte-derived DCs transfected with antisense control, antisense oligos to IL-23p19, or antisense oligos to IL-12p35. There was a 65–70% reduction of IL-23 levels in the cells transfected with antisense to IL-23p19 ($p < 0.05$). *B*, IL-12 was measured by ELISA in supernatants of monocyte-derived DCs transfected with AS control, AS IL-23p19, or AS IL-12p35. There was a 52–66% reduction of IL-12 levels in the cells transfected with antisense to IL-12p35 ($p = 0.034$). Data are from three independent experiments.

DCs transfected with IL-23p19 antisense produce decreased amounts of biologically active IL-23.

Down-regulating the expression of IL-23p19 or IL-12p35, increases IL-10 production, and decreases TNF-α production from mature monocyte-derived DCs

Having successfully knocked down the p19 subunit of IL-23 and the p35 subunit of IL-12, we investigated whether this had functional immunologic consequences. Little is known about the autocrine effect IL-23 has on monocyte-derived DC function. IL-23 is believed to induce the production of TNF-α by peritoneal macrophages, and a reciprocal relationship between IL-10 secretion and IL-12 production has been reported previously (16). Therefore, we evaluated the IL-10 and TNF-α production in monocyte-derived DCs treated with either antisense oligo control or antisense oligo to IL-23p19 or IL-12p35. As shown in Fig. 7, down-regulation of IL-23 or IL-12 caused a significant increase of IL-10 (Fig. 7*A*) and decrease of TNF-α secretion (Fig. 7*B*) from monocyte-derived DCs, compared with monocyte-derived DCs treated with control antisense ($p = 0.037$; 0.014 for increase of IL-10 by antisense oligo to IL-23p19 and IL-12p35; $p < 0.05$ TNF-α reduction by antisense oligo to IL-23p19, or IL-12p35). To further investigate this autocrine feedback loop, we also found that treatment of antisense-transfected DCs with IL-23 or IL-12 reverses these effects (Fig. 7). Specifically, the levels of the IL-10 were decreased, and the levels of TNF-α were increased when antisense oligos-treated DCs were then treated with rhIL-23 or rhIL-12.

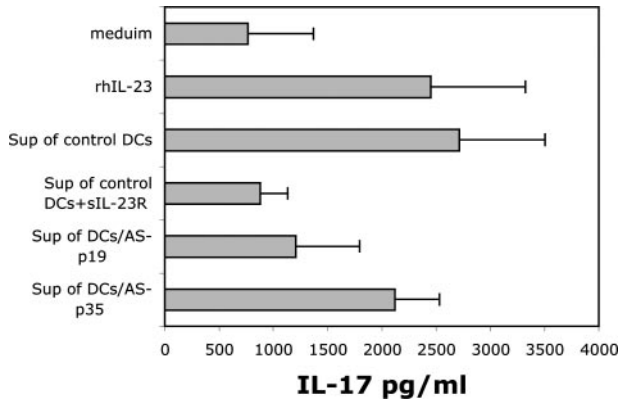


FIGURE 6. Down-regulation of IL-23 production by antisense oligos specifically decreases the secretion of IL-17 by $CD4^+$ T cells. Monocyte-derived DCs were transfected with antisense oligo control and antisense oligos to IL-23p19 and IL-12p35 and then stimulated with LPS. Supernatants were harvested 24 h later and added to negatively isolated $CD4^+$ cells stimulated with plate-bound anti-CD3 mAbs, and IL-17 secretion was measured. Data are representative of three independent experiments.

Down-regulating the expression of IL-23p19 or IL-12p35 in monocyte-derived DCs decreases their Ag presentation activity

DC function can be measured by their ability to stimulate T cells in the MLR. To determine whether down-regulation of IL-23 or IL-12 had an effect on the allostimulatory activity of DCs, an MLR was performed using DCs transfected with either antisense oligos

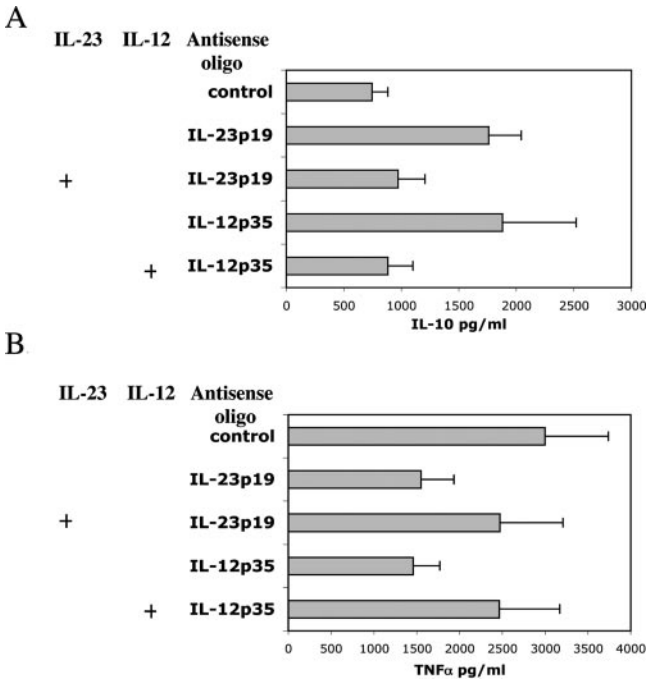


FIGURE 7. Decreasing the expression of IL23p19 and IL-12p35 up-regulates IL-10 production and decreases TNF-α secretion from mature monocyte derived DCs. *A*, Increased IL-10 and decrease of TNF-α secretion (*B*) from control monocyte-derived DCs, compared with monocyte-derived DCs treated with antisense oligos to IL-23p19 and IL-12p35 ($p = 0.037$; 0.014 for increase of IL-10 by antisense oligos to IL-23p19 and IL-12p35; $p < 0.05$ for TNF-α reduction by antisense oligos to IL-23p19, or IL-12p35). By adding rhIL-23 to the monocyte-derived DCs treated with antisense oligos to IL-23p19, or rhIL-12 to the DCs treated with antisense oligos to IL-12p35, we reversed the effect of the antisense oligos.

control or antisense oligos to either IL-23p19 or IL-12p35. Allogeneic negatively isolated $CD4^+$ T cells were cultured for 5 days with mature DCs transfected with different antisense oligos, at which time allostimulation was determined by proliferation and IFN-γ production. As shown in Fig. 8, interference with either IL-23 or IL-12 expression significantly decreased the ability of DCs to induce T cell proliferation or IFN-γ secretion ($p = 0.013$; $p = 0.033$ for proliferation DCs transfected with antisense oligo to IL-23p19 or IL-12p35 and $p = 0.017$; $p = 0.016$ for IFN-γ in DC transfected with antisense oligo to IL-23p19 or IL-12p35 in 1:20 DCs:T cells ratio). We also demonstrate that the addition of rIL-23 or IL-12 can rescue the effects on T cell proliferation observed after both IL-23p19 or IL-12p35 knockdown in DCs. (Fig. 8).

Another way to evaluate the function of the DCs is to determine the activation status of $CD4^+$ cells in the MLR. $CD4^+$ T cells that do not express CD62L are considered activated cells. Because CD62L is not a stable surface marker, T cells rapidly shed this receptor following cellular activation (17). We found an average of 42% decrease in the $CD4^+$CD62L low population when we incubated $CD4^+$ T cells with monocyte-derived DCs treated with antisense oligo to IL-23p19 and IL-12p35, compared with monocyte-derived DCs treated with control antisense oligo.

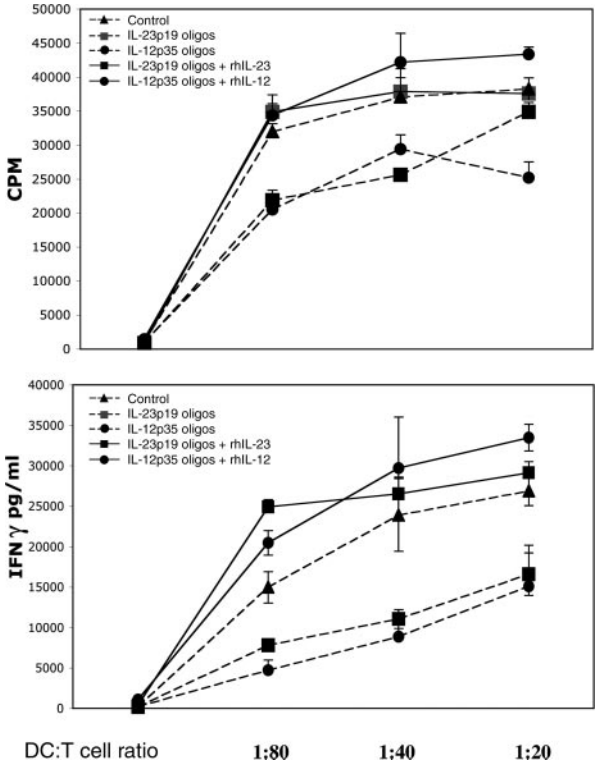


FIGURE 8. Antisense oligos to IL-23p19 and IL-12p35 decrease the allostimulatory ability of monocyte-derived DCs. Allogeneic T cells (1×10^5 cells/well) were incubated for 96 h with increasing numbers of irradiated monocyte-derived DCs transfected with antisense oligo control (dashed line with triangles), antisense oligos to IL-23p19 (dashed line with squares), or IL-12p35 (dashed line with circles). Proliferation was determined using [^{3}H]thymidine incorporation and IFN-γ measured in the supernatants by ELISA. We reversed this effect by adding rhIL-23 to the monocyte-derived DCs treated with antisense oligos to IL-23p19 (solid line with squares) or rhIL-12 to the DCs treated with antisense oligos to IL-12p35 (solid line with circles). Data are representative of five independent experiments. ($p = 0.013$; $p = 0.033$) for proliferation DCs transfected with antisense oligos to IL-23p19 or IL-12p35 ($p = 0.017$; $p = 0.016$) for IFN-γ in DCs transfected with antisense oligos to IL-23p19, or IL-12p35 in 1:20 DC:T cell ratio).

Discussion

In this study, we investigated IL-23 production from monocyte-derived DCs in MS patients and healthy donors. We found an increase in the levels of IL-23 secreted from monocyte-derived DCs as well as increased mRNA production of both IL-23p19 and IL-12/23p40 in MS patients. The increased IL-23 production was observed in patients with both RR and SP MS. We then investigated the biologic role of IL-23 in monocyte-derived DCs by down regulating IL-23 production using antisense oligos. We found that when monocyte-derived DCs produced less IL-23, they had increased production of IL-10 and decreased Ag presentation activity as measured in the MLR.

Accumulating evidence suggests there is a Th1-type bias in MS and that factors associated with Th2-type responses are beneficial in MS (5). In animals, DCs can drive a Th1 type response related to cell-to-cell contact and cytokine production (18). In humans, distinct subsets of DCs have been identified, which preferentially induce or regulate Th1 or Th2 immune responses (19). IL-23 and IL-12 are both proinflammatory cytokines produced mainly by DCs. Through studies that showed a resistant to EAE induction in the IL-23-deficient mice, IL-23 has emerged as the key player in chronic inflammatory autoimmune responses (7).

It has been reported that IL-12 is associated with CNS inflammation in patients with MS (8), and that there is increased production of IL-12 in the peripheral immune compartment (9) that correlates with disease activity (20) (21) and may be related to response to therapy (10). Most of these studies measured the common IL-12p40 subunit shared by IL-12 and IL-23. We have now found that there is increased secretion of IL-23 from monocyte-derived DCs from MS patients vs healthy donors, and that IL-23p19 and p40, but not IL-12p35 mRNA, are overexpressed. Thus, our data suggest that IL-23 may be one of the key cytokine that drives Th1-type responses in MS.

DCs, which can activate naive T cells, are thought to be the most potent APCs. Depending on their state of maturation, DCs may differentially affect the immune system by activating different T cell pathways or inducing anergy. Therefore, the state of differentiation or activation of DCs could play a crucial role in the pathogenesis of autoimmune diseases such as MS (22). In MS, altered phenotypes of circulating blood DC have been described (23, 24). Furthermore, it has been shown that patients with SP MS have increased IL-12 producing DCs after maximal stimulation with LPS and IFN-γ (10, 20). These findings are in agreement with the observation that inflammatory plaques in MS brain lesions have increased IL-12 expression. However, all of the above studies, which reported increased levels of IL-12 in MS, were obtained by quantification of the IL-12 p40 subunit. Because it is now known that IL-12 p40 is shared by both IL-12 and IL-23, based on our findings, it appears that the findings in MS attributable to IL-12 may, in fact, be related to IL-23 or both IL-23 and IL-12.

It is known that $CD4^+$ T cells activated with IL-23 produce IL-17 (13). When we analyzed IL-17 production from $CD4^+$ T cells from MS patients stimulated with anti-CD3 in the presence or absence of exogenous rhIL-23 or rhIL-12, we found increased IL-17 production by $CD4^+$ T cells from MS patients vs healthy donors both with and without the addition of exogenous rhIL-23. We found no differences in IFN-γ secretion between healthy donors and MS patients either cultured alone or with the addition of rhIL-23 or rhIL-12. The presence of increased IL-17 production by $CD4^+$ T cells is consistent with reports that IL-17 is increased in the brains of MS patients (25) and that IL-17 is a central cytokine associated with ecnephalitogenic T cell responses in the murine EAE model (26).

We also have demonstrated that antisense oligos can be successfully used for immune modulation of DCs by targeting expression of the Th1-polarizing cytokines IL-23 and IL-12. Using an antisense oligo specific to the IL-23p19, and IL-12p35 we were able to decrease the production of the endogenous, bioactive IL-23 or IL-12. Suppression was observed at the protein level and resulted in the DCs having higher IL-10 production, less TNF-α production, and less allostimulatory activity. Because the antisense oligos treatment did not change the maturation of the monocyte-derived DCs, our results demonstrate that IL-23 and IL-12 levels influence allogeneic T cell proliferation. IL-12 production by APC was demonstrated to be critical for MLR proliferative response because addition of anti-IL-12 Abs resulted in suppression of proliferation (27). Our study is the first to demonstrate that IL-23 in monocyte-derived DCs is also critical for the proliferation of $CD4^+$ T cells. IL-12 and IL-23 may influence proliferation using the same or complimentary pathways because knocking down either of them resulted in increased DCs IL-10 production. Treatment with antisense oligo to decrease the endogenous expression of both IL-23 and IL-12 results in more IL-10 production which may act directly as an inhibitor of T cell proliferation (24) (28) or by its ability to further decrease IL-12 production by APCs (29, 30). We have shown previously that there is increased production of IL-12 and defective regulation of IFN-γ by endogenous IL-10 in patients with SP MS (31) It also has been shown that low levels of IL-10 production are associated with higher disability and magnetic resonance imaging lesion load in SP MS (32).

In summary, our results demonstrate that an abnormal Th1 bias in DCs from MS patients related to IL-23 exists and that antisense oligos can be used for immune modulation by targeting DC gene expression. The immune effects we obtained by down-regulating IL-23, viz an increase in IL-10 secretion plus less potent allostimulatory properties of DCs, raises the possibility that knocking down IL-23 in monocyte-derived DCs may be a useful therapeutic avenue for treatment of Th1 cell-mediated autoimmune diseases such as MS.

Disclosures

The authors have no financial conflict of interest.

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