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Prednisolone Treatment Induces Tolerogenic Dendritic Cells and a Regulatory Milieu in Myasthenia Gravis Patients

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FOXP3-expressing naturally occurring CD4+CD25high T regulatory cells (Treg) are relevant in the control of autoimmunity, and a defect in this cell population has been observed in several human autoimmune diseases. We hypothesized that altered functions of peripheral Treg cells might play a role in the immunopathogenesis of myasthenia gravis, a T cell-dependent autoimmune disease characterized by the presence of pathogenic autoantibodies specific for the nicotinic acetylcholine receptor. We report in this study a significant decrease in the in vitro suppressive function of peripheral Treg cells isolated from myasthenia patients in comparison to those from healthy donors. Interestingly, Treg cells from prednisolone-treated myasthenia gravis patients showed an improved suppressive function compared with untreated patients, suggesting that prednisolone may play a role in the control of the peripheral regulatory network. Indeed, prednisolone treatment prevents LPS-induced maturation of monocyte-derived dendritic cells by hampering the up-regulation of costimulatory molecules and by limiting secretion of IL-12 and IL-23, and enhancing suppressive function compared with untreated patients, suggesting that prednisolone may play a role in the control of the peripheral regulatory network. Indeed, prednisolone treatment prevents LPS-induced maturation of monocyte-derived dendritic cells by hampering the up-regulation of costimulatory molecules and by limiting secretion of IL-12 and IL-23, and enhancing suppressive function compared with untreated patients, suggesting that prednisolone may play a role in the control of the peripheral regulatory network. Indeed, prednisolone treatment prevents LPS-induced maturation of monocyte-derived dendritic cells by hampering the up-regulation of costimulatory molecules and by limiting secretion of IL-12 and IL-23, and enhancing suppressive function compared with untreated patients, suggesting that prednisolone may play a role in the control of the peripheral regulatory network. Indeed, prednisolone treatment prevents LPS-induced maturation of monocyte-derived dendritic cells by hampering the up-regulation of costimulatory molecules and by limiting secretion of IL-12 and IL-23, and enhancing suppressive function compared with untreated patients, suggesting that prednisolone may play a role in the control of the peripheral regulatory network. Indeed, prednisolone treatment prevents LPS-induced maturation of monocyte-derived dendritic cells by hampering the up-regulation of costimulatory molecules and by limiting secretion of IL-12 and IL-23, and enhancing suppressive function compared with untreated patients, suggesting that prednisolone may play a role in the control of the peripheral regulatory network.

Central thymic tolerance has evolved to prevent autoreactive cells from escaping into the periphery. However, self-specific T cells are frequently found in the peripheral blood even of healthy individuals. Thus, autoreactivity is an intrinsic feature of T cells and active suppression is required to maintain tolerance to self.

Among CD4+ T cells, different types of regulatory T cells (Treg) can be distinguished, such as naturally occurring and induced Treg (iTreg). Naturally occurring CD4+CD25high Treg (nTreg) cells originate from the thymus as a functionally distinct population. They express constitutively high levels of CD25 and FOXP3, a transcription factor that regulates the expression of genes involved in immune suppression. iTregs are different from nTreg cells as they develop in the thymus from the precursor cells (Treg)6 and can be induced by various stimuli such as TGF-β and IL-10. iTregs are characterized by a low expression level of costimulatory molecules and an immunosuppressive cytokine milieu that in turn promotes the induction of iTregs.

remission in a large cohort of patients. The effects of corticosteroids, and pred in particular, are manifold, including inhibition of IL-2 production and dendritic cell (DC) function (23, 24).

We have previously shown that the peripheral frequency of Treg cells in MG patients is unaltered when compared with age-matched healthy donors (25). Moreover, Balandiana and colleagues (26) showed reduced suppression by thymic Treg from MG patients. Peripheral Treg were not assessed in this study. In this study, we show that the suppressive capability of peripheral blood Treg in untreated MG patients is profoundly impaired. Even more striking, MG patients treated with pred showed a considerable improvement in their Treg cell function. Our data support the hypothesis that pred-induced tolerogenic DCs are involved in the generation of peripheral Tregs that in turn ameliorate the clinical picture of MG.

Materials and Methods

Patients

Peripheral blood was obtained upon informed consent from 19 patients diagnosed with MG according to standard clinical criteria and all positive for AChR Abs (age range 35 to 85), and 27 age-matched healthy donors (age range 27 to 71). MG patients were divided into two groups according to their treatment: 1) MG patients treated with (t-MG) with azathioprine (AzA) and pred or pred alone (supplemental Table Ia) and 2) MG patients that were not treated (ut-MG) with any immunomodulatory drug for at least 1 year before blood collection (supplemental data, Table Ib).

Isolation of Tregs

CD4+CD25+ and CD4+CD25high cells were isolated from freshly prepared PBMCs using Ab-tagged magnetic microbeads (CD4+CD25+ Regulatory T Cell Isolation Kit, Miltenyi Biotec). CD4+ T cells were first isolated by negative selection, and subsequently separated into CD4+CD25− and CD4+CD25+ fractions using anti-CD25 magnetic beads. The positively selected CD25high T cells were run over a second column to ensure higher purity. Alternatively, cells were sorted on the basis of CD4+ and CD25high after staining with CD4-Pacific Blue (DakoCytomation) and CD25-PE (Miltenyi Biotec) in the FACS Aria (BD Biosciences). In all cases, the purity of the Treg population was between 90 and 98% as assessed by flow cytometry.

Proliferation and suppression assays

CD4+CD25+ responder T cells (Tresp, 1 × 106 per well) were cultured with different ratios of CD4+CD25high Treg cells (Treg:Tresp=1:1, 1:1.5, 1:2.5, and 1:0.125) in the presence of 1 μg/ml anti-CD3 (clone OKT3) and 10 U/ml IL-2 (purchased from PAA Laboratories) and antibiotics (PAA Laboratories). Cells were cultured in triplicates or quadruplicates in BD Falcon flat-bottom 96-well plates (BD Biosciences) in a total volume of 200 μl T cell medium. After 3 days in culture, T cells were collected, washed, and restimulated with CD4-Pacific Blue (DakoCytomation) and CD25-PE (Miltenyi Biotec) in the FACS Aria (BD Biosciences). In all cases, the purity of the Treg population was between 90 and 98% as assessed by flow cytometry.

Flow cytometry analysis

FOXP3 staining was performed using the eBioscience human FOXP3 staining kit (clone PCH101, NatuTec) following the manufacturer’s instructions. In brief, cells were fixed and permeabilized for 1 h at 4°C in the dark. After washing twice with permeabilization buffer, cells were blocked with normal rat serum and stained with anti-FOXP3-PE and anti-CD4-PB (DakoCytomation). Anti-CD25-FITC was added when necessary. Simultaneously, cells from the same donor were also stained with CD4+ PB, CD8-allophycocyanin, and CD25-PE (Miltenyi Biotec) on the cell surface to assess the percentage of CD4+CD25high Treg cells. FACS analysis was performed using the Cyan and the Summit software (DakoCytomation).

The percentage of Treg cells on the basis of CD25 expression was calculated with the CD4+ and CD25high population or, if not possible, a threshold was set at 0.1% on the non-CD4 cells (used as a control) in the CD4/CD25 dot plot (25). FOXP3 expression in CD4+ and CD25+ T cells was assessed after intracellular staining for CD4, CD8, and FOXP3. FOXP3 expression was calculated after gating on CD4+ and CD8+ T cells by setting the threshold with the negative population, which is clearly distinguishable from the FOXP3+ cells.

Generation of DCs

Monocytes were isolated from PBMCs using the human CD14+ microbeads from Miltenyi Biotec. Monocytes were cultured in RPMI 1640 containing 10% FCS supplemented with 100 ng/ml GM-CSF (Leukomed; and rhIL-4 (40 ng/ml, ImmunoTools) for 5 days. DC maturation was induced by the addition of the 5 μg/ml LPS (Streptococcus typhi, Sigma-Aldrich) for 24 h. Pred (1 μM, Solu-Decorin H, Merck) was added for 48 h before LPS stimulation. DCs were collected, irradiated at 60 Gy and either used directly or cryopreserved until needed for in vitro stimulation.

Phenotype and maturation state of differentially treated DCs was assessed by staining the expression level of CD1c, CD80, CD86, CD83, and HLA-DR (all Abs were purchased from BD Biosciences) by FACS.

Real-time PCR

Total RNA was extracted with the RNeasy Micro Kit from Qiagen and subsequently reverse transcribed for mRNA analysis. The template cDNA was amplified using specific primers, the MGB probe system and the TaqMan Universal Master Mix in the ABI Prism 7000 Detection System (Applied Biosystems). PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 × 95°C for 15 s, and 60°C for 1 min. Samples were run in duplicates. The FoxP3 specific primer and MGB-probe system was purchased from Applied Biosystems (Assay-on-Demand, Applied Biosystems).

Additional oligonucleotides used in that study are as follows: IL-1β forward 5′-TGA TGG CTT ATT ACA GTG GCA ATG-3′; IL-1β reverse 5′-GTA GTG GTG GTG GCA GAT TCG-3′; IL-6 forward 5′-TCT CCA CAA GAC GCT TCG-3′; IL-6 reverse 5′-CTC AGG GCT GAT GAT CCG-3′; IL-10 forward 5′-GTT TTA CCT GGA GGA GGT GAT-3′; IL-10 reverse 5′-GCC CTT GCT GCT TGT TTC ACC-3′; TGF-β forward 5′-GCC GTC GAC ACC AAC TAT TG-3′; TGF-β reverse 5′-CTG GTC CAG GCC GCA AAC TAT-3′; and the SybrGreen PCR Master Mix (Applied Biosystems).

ELISA

Supernatants were collected from DCimm, DCmat, and DCpred,LPS after 7 days of culture to assess the amount of IL-12 and IL-23. Cytokine production was determined using the respective capture ELISA following the manufacturer’s instructions (IL-12: R&D Systems; IL-23: ebioscience). The detection limits were 62.5 pg/ml for IL-12 and 15 pg/ml for IL-23. The analysis was performed in triplicates and the cytokine levels were calculated from the standard curve.

T cell differentiation assay

In brief, 0.4 × 106 DCimm, DCmat, or DCpred,LPS were cultured with magnetic bead-purified CD4+CD25− T cells at a ratio of 10:1 in T cell medium in a 24-well plate. After 7 days, 40 U/ml human rIL-2 (Chiron) was added, and cells were expanded for another 7 days. Fourteen days after initiation of the culture, T cells were collected, washed, and restimulated with DCimm, DCmat, and DCpred,LPS from the same allogeneic donor used in the primary culture. After 3 days, 40 U/ml rIL-2 were added. One week after the second stimulation, CD4+ T cells were collected and their phenotype as well as their proliferative and suppressive capacity were analyzed. In some experiments, CD4+ T cells were restimulated for a third time. In some experiments, 20 μg/ml blocking IL-10R Ab (BD Biosciences) were added every 2–3 days for the entire culture period.

Suppression assay

To test the suppressive activity of the CD4+CD25highCells generated in the T cell differentiation assay, freshly isolated CD4+CD25− T cells were labeled with 10 μM CFSE (carboxy-fluorescein diacetate-succinimidyl ester) (Molecular Probes, Eugene, OR) for 15 min at room temperature. The

7 The online version of this article contains supplemental material.
FIGURE 1. Impaired suppressive function of nTreg derived from MG patients. A, CD4+CD25\textsuperscript{high} Treg cells from 27 healthy controls and 19 MG patients were analyzed for their suppressive potential. Tresp and Treg cells were cultured for 4 days at different ratios in the presence of allogeneic stimulation and 1 μg/ml soluble anti-CD3. Proliferation was measured by [\textsuperscript{3}H]thymidine incorporation. A, The diagram represents the average suppressive potential at a 1:1 ratio of Tresp:Treg ± SEM in healthy donors (■) and MG patients (□). B and C, Five representative individual proliferation assays from HD and MG patients, respectively.

Statistical analysis
The mean [\textsuperscript{3}H]thymidine uptake was calculated from triplicates or quadruplicates. Unpaired two-tailed Student’s t test was used to compare the suppression and percentages of CD25\textsuperscript{high} and FOXP3-expressing cells between MG patients and healthy donors, or between treated and untreated patients. The chosen global significance level was p < 0.05. Error bars represent the SEM.

Results
nTreg-mediated suppression is impaired in patients with MG
Previous studies have shown that Treg cells are present with the same frequency in the periphery of both MG patients and healthy donors (HD) (25). To analyze the functional capabilities of these cells, we cultured highly pure CD4+CD25\textsuperscript{high} Treg cells from MG patients and healthy donors together with autologous responder CD4+CD25\textsuperscript{−} T cells (Tresp) (Fig. 1A). CD4+CD25\textsuperscript{−} Tregs from healthy donors consistently suppressed the proliferation of Tresp cells (86.47% average at 1:1 ratio, range 50.03–100%). As expected, decreasing the numbers of CD4+CD25\textsuperscript{high} in the coculture system resulted in decreased suppression, but even at a 1:0.25 ratio suppression was detectable in all cases (range 10.38–70.73%; Fig. 1B). In striking contrast, inhibition of Tresp proliferation by Treg isolated from MG patients was very poor in most patients, with an average suppression of 35.09% at a 1:1 ratio (range 0–87.19%, p < 0.0001; Fig. 1A), and with no clear trend at different Tresp:Treg cell ratios (Fig. 1C).

These data suggest an impaired suppression of CD4+ T cell proliferation by Treg cells isolated from MG patients, as it has been shown for other autoimmune diseases.

Steroid treatment partially restores nTreg-mediated suppression
Pred is the treatment of choice for patients that do not respond to oral anti-cholinesterase treatment. To investigate whether treatment with pred has any effect on Treg-mediated suppression, we compared the suppressive potential of CD4+CD25\textsuperscript{high} cells isolated from ut-MG and from pred-treated MG patients. Although the CD4+CD25\textsuperscript{high} population purified from ut-MG patients showed only marginal suppression at a 1:1 ratio of Tresp and Treg cells (average suppression 34.44%; Fig. 2A), a much stronger suppression was observed when using CD4+CD25\textsuperscript{high} cells from t-MG patients (Tresp:Treg 1:1; 51.06%, Fig. 2A), although it did not reach the levels of suppression achieved by the healthy donor.

FIGURE 2. Impaired Treg-mediated suppression is partially restored in pred-treated MG patients. A, CD4+CD25\textsuperscript{high} cells from nine ut-MG patients, 10 t-MG patients, and 11 HD were cultured at different ratios with Tresp cells in the presence of allogeneic stimulation and 1 μg/ml soluble anti-CD3. After 4 days in culture, thymidine incorporation was measured for the last 18 h. The graph shows the average percentage of suppression ± SEM at 1:1, 1:0.5, and 1:0.25 Tresp:Treg ratios. The statistical difference is * p = 0.0027 and ** p < 0.0001. B, B cells from three HD, three ut- and three t-MG patients were cocultured with or without autologous CD4+CD25\textsuperscript{−} Treg cells and IgG production measured after 5 days in culture by ELISA. The graph shows the average percentage of IgG production ± SEM at 1:1 Treg:B cell ratio. The asterisk denotes statistical significance. * p = 0.028.
cohort. However, suppression mediated by CD4^+CD25^{high} cells from t-MG patients was enhanced at all tested ratios of Tresp and Treg cells when compared with ut-MG patients.

Although MG is a CD4 T cell-dependent disease, the pathophysiology is mediated by autoantibodies directed against the AChR. To assess whether the failure of Treg in MG patients to suppress T cell responses also extends to the B cell compartment, and whether this defect in suppression can be reversed by in vivo pred treatment, we measured direct Treg-mediated suppression of Ab production (from HD and MG patients). We found that Treg cells from ut-MG are compromised in suppressing IgG production by B cells (average suppression of IgG production is 2.8%; Fig. 2B) in comparison to HD (20.9%, p < 0.05). In contrast, two of the three patients in the treated group showed suppression values close to healthy donors. Our data show that CD4^+CD25^{high}-mediated suppression of both T and B cell responses is especially compromised in ut-MG patients and it is partially restored in MG patients treated with pred.

The levels of FOXP3 expression are similar in healthy donors and MG patients

FOXP3 is considered a master regulator for the control of Treg development and function (6). We hypothesized that the defect in the suppressive capacity of CD4^+CD25^{high} cells from MG patients could be related to lower than normal levels of FOXP3 that would result in an impaired Treg function in patients. To check this, peripheral blood from 14 MG patients (range from 35–80 years of age) and 17 healthy controls (range from 21–48 years of age) was analyzed for the expression of CD25 and of FOXP3 in CD4^+ T cells at the protein level by flow cytometry. A triple surface staining for CD8, CD4, and CD25 was used to identify CD25^{high} expressing CD4^+ T cells (Fig. 3A, left), while intracellular staining for CD4, CD8, and FOXP3 was used for the detection of FOXP3-expressing cells (Fig. 3A, right). The frequency of CD4^+CD25^{high} cells in healthy donors averaged 3.48% (range 0.70–6.98%) and in MG patients 2.45% (range 0.61–6.85%; Fig. 3B), differences that did not reach statistical significance (p = 0.1895). Similarly, the average frequency of circulating FOXP3^+ in CD4^+ T cells from healthy controls was 6.82% (range 3.26–9.88%) and in MG patients 6.71% (range 2.03–16.84%), which was again not statistically significant (p = 0.38; Fig. 4C). Of note, the frequency of FOXP3^+ cells was in all cases higher than the frequency of CD25^{high} cells.

To analyze the expression level of FOXP3 on a per cell basis, we calculated the ratio of the median fluorescence intensity (MFI) between CD4^+FOXP3^− and CD4^+FOXP3^+ cells. The average MFI for healthy controls was 5.35 (range 2.05–11.54) and from MG patients 6.65 (range 2.13–10.34; Fig. 3D) representing a non-statistically significant difference. To correlate the levels of FOXP3 expression with suppressive activity, we categorized the expression levels of FOXP3 in CD4^+ T cells in three different groups according to the MFI (low: MFI < 4, medium: MFI ≥ 4 and ≤ 7 and high: MFI > 7) and calculated whether the quality of CD4^+CD25^{high}-mediated suppression is reflected by the expression level of FOXP3. We found that the high variation among FOXP3 expression did not mirror Treg-mediated suppression (Fig. 3E). For instance, CD4^+CD25^{high} cells isolated from a donor with medium FOXP3 expression (D6; MFI 5.82) showed the highest suppression (suppression of 97.12%) while CD4^+CD25^{high} cells from donors with a high FOXP3 MFI (D2; MFI of FOXP3 11.54, suppression of 63.24%) and low FOXP3 MFI (D1; MFI of FOXP3 3.92, suppression of 64.09%) showed almost the same suppressive capability. We also compared average FOXP3 MFI from t-MG patients (average 7.61 ± 1.16) with that from ut-MG patients (average 5.51 ± 0.49) and found a slight increase (1.38-fold) that did not reach significance (data not shown). These data demonstrate that the expression level of FOXP3 in CD4^+CD25^{high} cells does not necessarily correlate with the level of their suppressive activity in terms of proliferation inhibition of Tresp in vitro, and that differences in FOXP3 levels cannot account for impaired suppression in MG patients.

In vitro treatment with pred prevents full maturation of DCs and inhibits the production of proinflammatory cytokines

After finding an improved suppressive capacity of CD4^+CD25^{high} cells isolated from pred-treated MG patients, we wondered whether pred has a direct impact on the CD4^+CD25^{high} cells. However, pretreatment of CD4^+CD25^{high} cells with pred before a suppression assay did not result in enhanced suppressive capability, nor could we detect changes in FOXP3 expression (data not shown). To investigate whether pred can influence T cells by regulating Ag-presenting cell maturation and function, we isolated monocytes from peripheral blood of healthy donors and generated monocyte-derived DCs (mo-DCs). After 5 days in culture, DCs were pretreated with 1 µM pred for 24 h and subsequently stimulated with 5 µg/ml LPS for another 24 h (DC_{pred,LPS}). As controls, DCs were left unstimulated (immature DC, DC_{imm}) or...
treated with either 1 μM pred (DCpred) or 5 μg/ml LPS (mature DCs, DCmat). Phenotypical analysis revealed that immature DCs expressed low levels of CD80, CD83, and CD86 (Fig. 4A). Treatment of DCs with pred alone did not alter the expression level of these costimulatory molecules when compared with DCimm. As expected, LPS-stimulated DCs up-regulated surface expression of CD80, CD83, and CD86. Interestingly, pred-pretreated DCs showed only a moderate up-regulation of CD86 after induction of maturation with LPS while the expression level of CD80 was comparable to that of DCmat (Fig. 4A). We could not detect any differences in the MFI of HLA-DR between DCimm, DCmat, DCpred, and DCpred,LPS (data not shown). Next, we studied the cytokine profile of differentially treated DCs and found that transcripts for the proinflammatory cytokines IL-6 and IL-1β were most abundant in DCmat, and practically absent in DCimm. Preincubation of DCs with pred before LPS stimulation totally abrogated IL-6 expression in DCs, and it reduced the transcription of IL-1β by 50% (Fig. 4B). Similarly, when supernatants from the DC cultures were assessed for IL-12 and IL-23, cytokines that promote differentiation of CD4+ T cells into Th1 (IFN-γ-producing cells) and Th17 (IL-17-producing cells), respectively, we found that LPS-matured DCs showed highest secretion levels of IL-12 (2051 pg/ml) and IL-23 (1661.4 pg/ml) while preincubation with pred resulted in a marked decrease in IL-12 (300.25 pg/ml) and IL-23 (662.4 pg/ml) (Fig. 4C). In contrast, RNA transcripts for the immunomodulatory cytokines IL-10 and TGF-β were higher in the cells that had been preincubated with pred, independently of the maturation stimulus provided by LPS.

Taken together, these data demonstrate that DCs pretreated with pred maintain an immature state even in the presence of the LPS, and produce preferentially the immunomodulatory cytokines IL-10 and TGF-β rather than their proinflammatory counterparts IL-6, IL-12, or IL-23, suggesting that DCpred,LPS may induce CD4+ T cell tolerance rather than a potent immune reaction.

Modulation of DC function in patients treated with pred

In vitro treatment of mo-DCs with pred before LPS challenge prevents these cells from full maturation. We wondered whether the same effect is observed in cells derived from MG patients, and, more importantly, if DCs generated from patients treated with pred show any signs of impaired maturation to LPS stimulus. To test this, mo-DCs generated from ut-MG and t-MG blood samples were treated with pred in the presence or absence of LPS as described above and compared with mo-DC from HD treated in the same way. Phenotypic analysis revealed that the expression of CD80 was markedly reduced in all HD and in five of the six ut-MG patients upon pred preincubation before LPS stimulation (Fig. 5A). Not surprisingly, there was less decrease in the expression of CD86 upon LPS stimulation in the t-MG group (p = 0.03; Fig. 5A). The expression level of CD80 on mo-DCs from ut-MG patients
after LPS stimulation was comparable to that of HD. In contrast, DCs generated from t-MG patients up-regulated CD80 only marginally upon LPS stimulation. Similarly, the production of IL-12 by mo-DCs from ut-MG was comparable to that of HD upon LPS challenge. However, in vivo pred treatment of patients severely compromised the ability of mo-DCs to produce IL-12 upon stimulation with LPS (Fig. 5B). As expected, in vitro addition of pred to these cells had no further effect in IL-12 production. Altogether, these data show that pred, both in vitro and in vivo, modulates the maturation of DCs, preventing the up-regulation of costimulatory molecules, and reducing the production of proinflammatory cytokines.

**Pred-treated DCs render CD4+ T cells hyporesponsive**

CD4+ T cells primed with allogeneic immature DCs are hyporesponsive to stimulation whereas CD4+ T cells cultured in the presence of LPS-stimulated DCs do proliferate (28). We hypothesized that DCpred,LPS, given the reduced costimulatory potential and the immunomodulatory cytokines produced, would behave tolerogenic. Thus, we assessed the capacity of DCpred,LPS to stimulate the proliferation of allogeneic CD4+ T cells. CD25-depleted CD4+ T cells were cultured in the presence of DCimm, DCmat, or DCpred,LPS at a 10:1 ratio. One week after the second stimulation, the CD4+ T cell lines were harvested and tested for their proliferative capacity in response to 1 μg/ml anti-CD3 and allogeneic irradiated PBMCs. In agreement with published data, CD4+ T cell lines stimulated by mature DCs proliferate upon polyclonal stimulation while CD4+ T cells stimulated with DCimm are hyporesponsive. In contrast, CD4+ T cells primed with DCpred,LPS were hyporesponsive to polyclonal stimulation (Fig. 6).

**CD4+ T cells differentiated in the presence of pred-treated DC are suppressive**

Low proliferation to stimulation is a characteristic of Tregs. We therefore tested the suppressive capacity of CD4+ T cells generated after repetitive stimulation with DCpred,LPS (CD4+-DCpred,LPS). To this end, freshly isolated CD4+CD25− T cells were cultured with irradiated allogeneic APCs and soluble anti-CD3 for 4 days in the presence or absence of purified CD4+-DCpred,LPS generated as above, and proliferation was measured by CFSE dilution. As expected, CD4+CD25− had no suppressive effect on CD4 Tresp proliferation, and CD4+CD25− showed minimal suppression capability (<10%). In contrast, DCpred,LPS elicited a relevant 24.7% suppression of proliferation of autologous CD4+CD25− responder cells (Fig. 7), which was abrogated when the regulatory CD4+ cells were generated in the presence of blocking the IL-10R Ab during stimulation with DCpred,LPS (data not shown). These data show that CD4+ T cells generated by repeated stimulation with pred-treated LPS-stimulated DCs acquire the potential to suppress Tresp proliferation, and that the induction of the regulatory phenotype is dependent on IL-10.

**Discussion**

Immune tolerance to self and innocuous Ags is a basic mechanism to prevent self-destruction. Tregs play a critical role in the maintenance of peripheral tolerance and a distorted function of these cells can contribute to the development and course of autoimmune diseases. Several published studies have reported abnormalities in the Treg population, either in the frequency or in their in vitro function, or both, that could be related to different types of autoimmune diseases (15, 18). In MG, a T cell-dependent autoimmune disease, nTreg cells were shown to be present with the same frequency in the periphery when compared with healthy donors but their functional attributes were not yet assessed (25). Balandina et al. (26) highlighted a defect in the regulatory activity of CD4+CD25+ thymocytes and a decrease in the FOXP3 expression within this population when isolated directly from the thymus of MG patients. FOXP3 is the only known lineage-specific marker for nTreg cells (5, 29). The importance of FOXP3 in Treg cell function is underlined in patients suffering from an immunodeficiency caused by mutations in the foxp3 gene (30, 31). Although we could demonstrate that the defective regulatory capacity is also present in peripheral Treg in MG patients, our data show that this is not due to a decreased expression of FOXP3: 1) there is no significant difference in the percentage of FOXP3+ cells between patients and healthy donors, 2) the levels of expression (MFI) are also not different, and 3) the level of FOXP3 expression did not correlate with suppressive function, as described also by others (32). There is conflicting data about the role of FOXP3 in suppression, with recent work showing that transient FOXP3 expression in recently activated CD4+CD25+ T cells could (33) or could not (34) exert suppressive functions. FOXP3 in humans exists in two isoforms, the full length and a splice variant lacking exon 2 (FOXP3Δ2). Retroviral transfer of either or both FOXP3 isoforms into naïve CD4+ T cells led to the development of a regulatory phenotype. However, these converted Treg cells did not reach the suppressive potential of ex vivo isolated CD4+CD25high T cells (35). Thus, it is conceivable that FOXP3 does not represent the only regulator of Treg function, but rather contributes to regulate a network of molecules that control the development and function of nTreg cells.

MG is known to be a heterogeneous disease and the patient cohort we analyzed was fairly different according to MG classification. However, no significant difference in Treg-mediated suppression was observed when MG samples were subdivided according to disease onset.
or the presence of a MG-associated thymoma. We found, however, that Tregs isolated from MG patients treated with pred showed an improved regulatory function compared with the Tregs from untreated patients. This effect was not only observed with respect to T cell proliferation but also to the B cell response, i.e., IgG production.

Corticosteroids are well known for their anti-inflammatory and immunomodulatory properties (36–38). Pred is the treatment of choice for patients that do not respond to acetycholinesterase inhibitors, and it is often given together with AzA, to avoid some of the secondary effects that accompany steroid treatment. AzA is an inhibitor of DNA and RNA synthesis, thus affecting the proliferation of immune cells (39), but with no immunomodulatory effect. Because of the lack of immunomodulatory properties of AzA, and that the presence or absence of this complementary drug did not change the improved suppression in the treated patients, we did not separate patients treated with one or with the two drugs any further. The improved suppression potential of Tregs from pred-treated patients led us to hypothesize that 1) pred has a direct positive impact on Treg cells by the up-regulation of FOXP3 or other yet unknown molecules that direct their function and/or 2) Treg cells are generated de novo by pred-induced tolerogenic APCs. Because we could not demonstrate any direct effects of pred on Treg function, we sought to assess whether other cells could contribute to the improvement of regulatory function observed in pred-treated patients.

DCs represent the most potent APCs and are essential in immune system regulation (40). DCs can have dual functions; while mature DCs are potent T cell stimulators (reviewed in Ref. 41), immature DCs can induce T cell tolerance (28, 42, 43). Anti-inflammatory glucocorticoids are promising agents for developing tolerogenic DCs and thus the effect of pred on mo-DCs was evaluated (44, 45). In agreement with previous data (24), we found that pred-treated DCs maintain an immature state in the presence of maturation stimuli such as LPS. The up-regulation of costimulatory molecules was diminished suggesting that DCpred,LPS may induce T cell tolerance rather than a potent immune response. This supposition was also based on increased RNA levels of IL-10 and TGF-β in DCpred,LPS, known immunomodulatory cytokines, and reduced secretion of the proinflammatory cytokines IL-12 and IL-23 (46). Although IL-12 is known for activating DCs and promoting the differentiation of CD4⁺ T cells into Th1 cells, IL-23 is involved in the generation of Th17 cells and the induction of chronic (autoimmune) inflammation (47). We further studied the consequence of in vitro pred treatment on mo-DCs generated from patients that were untreated or pred treated. Mo-DCs generated from untreated MG responded to the treatment applied in vitro in the same way as mo-DCs from HD did. Interestingly, we observed in this study for the first time an impaired up-regulation of CD80 and IL-12 production by mo-DCs from pred-treated MG patients, suggesting that in vivo pred treatment can effectively modulate DC maturation.

The tolerogenic properties of DCpred,LPS were documented with the hyporesponsiveness of CD4⁺ T cell lines raised using treated DCs as APCs. The extend of induced anergy was similar to that of CD4⁺ T cells that have been precultured with DCimm in agreement with published results (48, 49). Induced Tregs develop from conventional CD4⁺ T cells in the periphery under tolerizing conditions such a suboptimal antigenic stimulation (50), in the presence of immunomodulatory cytokines like IL-10 and TGF-β (51) or after administration of immunosuppressive drugs (10, 52). Based on that observation, it was of great interest to test the potential of DCpred,LPS to generate Tregs, especially after having disclosed their potential to induce anergy. After repetitive exposure of CD25-depleted CD4⁺ T cells to allogeneic DCpred,LPS, we found that CD4⁺·DCpred,LPS were able to down-modulate CD4⁺ T cell proliferation in the presence of polyclonal stimulation to a greater extend than their counterparts that were cultured with DCimm. Blocking IL-10R during the generation of the Tregs resulted in the partial abrogation of their suppressive capacity, confirming IL-10 as a necessary factor for the induction of a regulatory phenotype (53). In these experiments, great emphasis was given to the depletion of CD25-expressing cells from the starting CD4⁺ T cell population and the purity of the remainder CD4⁺CD25⁻ cell population was monitored before coculturing with DCs. Although unlikely, we cannot completely exclude the possibility that limited numbers of residual CD4⁺CD25high cells after depletion from the CD4⁺ pool survive and expand in cultures with DCimm and DCpred,LPS and contribute to the down-modulation of Tresp proliferation. Given the fact that CD4⁺ T cells cultured with DCimm do not suppress proliferation of freshly isolated Tresp cells while CD4⁺ T cells cultured with DCpred,LPS do, we favor a scenario in which Tregs are newly generated from the starting CD4⁺CD25⁻ T cell population. Supporting our finding, the in vivo administration of a pred derivate prevented the development of colitis in mice due to potent IL-10 production and probably Treg induction (54).

Adaptive Tregs can be of different nature (11, 50, 55). Phenotypically, they are diverse in terms of FOXP3 expression, and in the cytokines that they produce, probably depending on the conditions that induce their differentiation. In addition, it is now known that FOXP3 can be expressed transiently in non-Treg cells upon activation, suggesting that this molecule may harbor a more diverse set of functions as assumed originally (56, 57). In our experiments, the expression of FOXP3 was comparable in all three CD4⁺ T cell lines after stimulation with differentially generated DCs (data not shown). This fact could just simply reflect recently activated and dividing cells present at each time point while the actual Tregs generated in the presence of DCpred,LPS do evolve and exert their suppressor function independent of FOXP3 (33). This assumption is supported by the finding that in vitro generated Tr1 cells do not constitutively express FOXP3 but they still suppress T cell proliferation efficiently (53, 58).

We demonstrate in this study for the first time an impaired suppressive potential of peripheral Treg cells from MG patients. In addition, we show that patients treated with pred displayed better suppression than untreated patients, although not to the levels of healthy donors. We had the chance to measure suppression before and after steroid treatment in two MG patients, and in both cases, the suppressive ability of Treg cells improved after pred treatment (data not shown). In addition, we have shown a modulatory effect of pred on mo-derived DC and the induction of regulatory cells after exposure to pred-treated DCs, and that this mechanism is IL-10 dependent, as it has been shown using different models (43, 59). Multiple factors might contribute to the improved suppression of T cell proliferation in treated patients, but we favor de novo generation of induced Tregs.

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Disclosures
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