Vasoactive intestinal peptide induces regulatory dendritic cells with therapeutic effects on autoimmune disorders

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The induction of antigen-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune tolerance. In addition to their classical role as sentinels of the immune-response-inducing T cell reactivity, dendritic cells (DCs) play an important role in maintaining peripheral tolerance through the induction/activation of regulatory T cells (Tr). The possibility to generate tolerogenic DCs opens new therapeutic perspectives in autoimmune/inflammatory diseases. Therefore, the characterization of the endogenous factors that contribute to the development of tolerogenic DCs is highly relevant. In this study, we report on the use of the known immunosuppressive neuropeptide, the vasoactive intestinal peptide, as a new approach to induce tolerogenic DCs with capacity to generate Tr cells, to restore tolerance in vivo, and to reduce the progression of rheumatoid arthritis and experimental autoimmune encephalomyelitis.

autoimmunity | regulatory T cell | tolerance

The immune system is faced with the daunting job of protecting the host from an array of pathogens, while maintaining tolerance to self-antigens (Ags). The induction of Ag-specific tolerance is essential to maintain immune homeostasis, to control autoreactive T cells, preventing the onset of autoimmune diseases, and to achieve tolerance toward transplants. Both thymic and peripheral mechanisms account for the ability of the immune system to induce tolerance. Attention has been focused recently on induction of active suppression by regulatory T cells (Tr) (1), and dendritic cells (DCs) have been shown to contribute to T cell tolerance (2, 3). The maturation/activation state of DCs might be the control point for the induction of peripheral tolerance, by promoting Tr differentiation. Thus, whereas mature DCs (mDCs) are potent Ag-presenting cells enhancing T cell immunity, immature DCs (iDCs) are involved in the induction of peripheral T cell tolerance under steady-state conditions (2–6). However, the clinical use of iDCs may not be suitable for the treatment of autoimmune diseases, because iDCs are likely to mature in inflammatory conditions (6), emphasizing the need to develop tolerogenic DCs with a strong potential to induce Tr. Immunosuppressive therapy, traditionally focused on lymphocytes, has been revolutionized by targeting the development and key functions of DC, and the generation of tolerogenic DCs in the laboratory has become the focus of new therapies (7).

Vasoactive intestinal peptide (VIP) is a neuropeptide released by both innervation and immune cells, particularly T helper (Th)2 cells, in response to Ag stimulation and under inflammatory/autoimmune conditions (8). VIP elicits a broad spectrum of biological functions, including immunomodulation, predominantly acting as a potent antiinflammatory factor and a suppressive agent for Th1 responses (9). Therefore, VIP has emerged as a promising therapeutic factor for the treatment of autoimmune/inflammatory diseases, including rheumatoid arthritis (RA), ulcerative colitis, uveoretinitis, and experimental autoimmune encephalomyelitis (EAE) (10–12). In this study, we investigated whether the presence of VIP during the early phases of DC differentiation induces the generation of regulatory DCs with the capacity to induce Tr and to prevent autoimmunity.

Materials and Methods

Cell Isolation and Cultures. Bone marrow (BM)-derived DCs (BM-DCs) were generated as described in ref. 13. Briefly, BM cells (2 × 10⁶) obtained from BALB/c (H-2b), C57BL/6 (H-2b), or DBA/1 (H-2b) mice were incubated in complete medium (RPMI medium 1640 supplemented with 100 units/ml penicillin/streptomycin, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, and 10% heat-inactivated FCS) containing 20 ng/ml granulocyte macrophage colony-stimulating factor in the presence or absence of VIP (10⁻⁸ M). At day 6, nonadherent cells were collected (routinely containing 80–90% CD11c⁺ cells) and stimulated for 48 h with LPS (1 μg/ml) to induce activation/maturaion. In some experiments, DCs were pulsed with ovalumalin (OVA), collagen II (CII), or myelin oligodendrocyte glycoprotein (MOG) (20 μg/ml) for 12 h. Allogeneic naïve CD4 T cells were purified from C57BL/6 mice by positive immunomagnetic selection (MACS, Miltenyi Biotec, Auburn, CA).

Flow Cytometry. Cells were incubated with various peridinin–chlorophyll–protein complex (PerCP)-, FITC- and phycoerythrin (PE)-labeled mAbs (BD Pharmingen), diluted at optimal concentration for immunostaining, fixed in 1% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). We used isotype-matched Abs as controls and IgG block (Sigma) to avoid the nonspecific binding to Fc-receptors.

Cytokine Assays. Cytokine contents in the culture supernatants were determined by specific sandwich ELISAs by using capture/biotinylated detection Abs from BD Pharmingen, diluted at optimal concentration for immunostaining, fixed in 1% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). We used isotype-matched Abs as controls and IgG block (Sigma) to avoid the nonspecific binding to Fc-receptors.

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intracellular cytokine content was determined as described above. In some experiments, DCs (10^5) were cultured with purified allogeneic CD4 T cells (5 x 10^5). One week later, CD4 T cells were recovered by immunodepletion of CD11c^+ DCs and cultured in different numbers with syngeneic CD4 T cells (5 x 10^5) in the presence of allogeneic mDC (10^5), and the proliferative response was determined. Some cultures were performed in the presence of blocking anti-IL-10 (10 μg/ml) and/or anti-TGFβ1 (40 μg/ml) mAbs. To determine the cell-contact-dependence of the regulatory response, we placed responder CD4 T cells (5 x 10^5) with LPS-matured DC (10^5) in the bottom well of a Transwell system (Millipore) and syngeneic TrVIP (2 x 10^5) with allogeneic mDC (10^5) in the upper Transwell chamber. After 72 h, we measured the proliferative response of the bystander reactive CD4 T cells in the bottom well. To generate CII- and MOG-specific Tr cells, DBA/1/J and C57BL/6 DCs (10^5) pulsed with CII or MOG, respectively, were cultured with syngeneic CD4 T cells (5 x 10^5) for 1 week in the presence of CII or MOG (20 μg/ml).

**Immunization Model.** BALB/c mice were injected s.c. with different numbers (from 50 to 5 x 10^5) of cells of methylated BSA (mBSA)-pulsed DCcontrol or DCVIP, followed a week later by s.c. immunization with the Ags mBSA or OVA (60 μg) in complete Freund’s adjuvant. Five days after Ag immunization, serum Ag-specific Ab, draining lymph nodes (DLN) T cell proliferative responses, and delayed type hypersensitivity (DTH) responses were measured. For the DTH responses, mice were injected i.d. with Ag (5 μg) or saline into the ears, and ear swelling was measured 24 h later by using a caliper. Ag-specific T cell proliferative responses were measured after ex vivo stimulation of DLN cells (4 x 10^5) with 10 μM Ag. Levels of mBSA-specific IgG in serum were determined by ELISA, as described in ref. 16.

**Model for RA and EAE.** RA was induced in DBA/1/J mice by s.c. injection of CII, as described in ref. 10. Chronic EAE was induced in C57BL/6 mice by s.c. immunization with MOG35–55, as described in ref. 17. Mice with established arthritis (with a clinical score of 2) were injected i.v. with different numbers of syngeneic CII-pulsed DCcontrol or DCVIP or with CII-specific Tcontrol or TrVIP. Mice with established EAE (with a clinical score of 1) were injected i.v. with different numbers of syngeneic MOG35–55-pulsed DCcontrol or DCVIP or with MOG35–55-specific Tcontrol or TrVIP. The clinical score was determined daily, based on joint inflammation for RA and tail/leg paralysis for EAE, as described in ref. 17. DLN cells were isolated at the peak of the diseases, stimulated with CII or MOG35–55 (20 μg/ml), and assayed for proliferation and cytokine production, as described above. The content of serum anti-CII or anti-MOG35–55 IgG antibodies was determined by ELISA, as described in refs. 10 and 18. To assess Ag-specificity, arthritic mice were injected with unpulsed, OVA-pulsed, or CII- or MOG35–55-pulsed DCcontrol or DCVIP and immunized s.c. with OVA, CII, or MOG35–55 (150 μg of Ag in complete Freund’s adjuvant) one week later. After 5 d, mice received 5 μg of Ag i.d. in the ear pinna, and the DTH response was determined, as described above. In some experiments, collagen-induced arthritis (CIA) and EAE mice received i.v. injections of neutralizing anti-IL-10 polyclonal Ab, neutralizing anti-TGFβ mAb, or preimmune rat IgG used as control Ig (500 μg of Ab per mouse) on alternate days up to 8 d after onset of disease.

**Results and Discussion.**

The induction of Ag-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune tolerance. In addition to their classical role as sentinels of the immune response inducing T cell reactivity, increasing evidence now indicates that DCs can induce specific T cell tolerance. Although underlying mechanisms are not fully elucidated, the capacity to induce Tr cells is an important property of tolerogenic/regulatory DCs. The generation of “designer” DCs with tolerogenic properties in the laboratory by using specific cytokines or immunologic and pharmacologic reagents is a desirable goal and represents the subject of intensive investigations. Because of its immunosuppressive action, VIP is a candidate for the induction of regulatory DCs with capacity to generate Tr. In a previous study, we showed that VIP treatment of activated DCs reduces their capacity to activate allogeneic and syngeneic T cells, an effect associated with the prevention of CD80/CD86 up-regulation (19). VIP treatment of iDC in the absence of activation resulted in DCs with increased capacity to induce Th2 responses (19). However, other immunomodulatory factors with capacity to induce tolerogenic DCs have been found to be effective when administered during the differentiation of DCs (6, 7). Therefore, we determined whether exposure to VIP during DC differentiation results in DC phenotypic and functional changes.

**BM-DC Differentiated with VIP Induce Regulatory Tr1-Like Cells and Tolerance in Vivo.** We first compared murine BM-derived DCs generated in the presence or absence of VIP in terms of surface markers and cytokine production. As previously described, BM cells cultured with granulocyte macrophage colony-stimulating factor for 6 d differentiate into iDCs (data not shown). Upon LPS stimulation, iDCs mature to DCs expressing high levels of DC markers (CD11c), MHC molecules (class I and class II), and costimulatory molecules (CD40, CD80, and CD86) (Fig. 1a, DCcontrol). However, DCs generated in the presence of VIP (DCVIP) were resistant to the LPS-induced up-regulation of the costimulatory molecules (CD40, CD80, and CD86) (Fig. 1a). Upon
tolll-like receptor activation, iDCs mature into cells capable of producing high levels of inflammatory cytokines. In contrast to DCcontrol, which produce TNF and IL-12, and low levels of IL-10, DCVIP produce very low levels of proinflammatory cytokines (TNF and IL-12) but secrete significant levels of the antiinflammatory cytokine IL-10 (Fig. 1b). Taken together, these results indicate that the DCs generated in the presence of VIP are resistant to LPS-induced up-regulation of costimulatory molecules and produce IL-10. These characteristics are quite similar to those reported for tolerogenic DCs generated with other immunomodulatory factors, such as IL-10 or the activated form of vitamin D 1,25(OH)2D3 (2–4, 20–24).

Tolerogenic DCs are poor stimulators of T cell proliferation and cytokine production (20, 25–28). To examine the capacity of the DCVIP to stimulate T cells, we cocultured DCcontrol or DCVIP with alloreceptive CD4 T cells. Priming with DCcontrol results in a strong proliferation of allogeneic CD4 T cells, whereas DCVIP induce only weak proliferation (Fig. 2a). In contrast, CD4 T cells primed with DCVIP are not proliferative (data not shown), indicating that DCVIP induces anergic T cells and/or Tr. Although Tr generated by exposure to regulatory/tolerogenic DCs do not proliferate in response to the Ag, they can release antiinflammatory cytokines, such as IL-10 and TGFβ. Therefore, we assessed the cytokine profile of T cells cocultured with DCVIP. In contrast to T cells exposed to DCcontrol, which show a predominant Th1 cytokine profile, with high levels of IFNγ and IL-12, CD4 T cells primed with allogeneic DCVIP exhibit a Th1-like phenotype, characterized by IL-10 and TGFβ but not IL-2 and IFNγ production (Fig. 2b).

After TCR stimulation, Tr cells suppress the proliferation and IL-2 production of Ag-specific effector T cells. To determine whether T cells exposed to DCVIP become functional Tr, we restimulated CD4 T cells with allogeneic mDCs in the presence of syngeneic responder CD4 T cells previously exposed to allogeneic DCVIP (TrVIP). The resulting regulatory CD4 T cells (Tr) were restimulated for 1 week with allogeneic DCcontrol or DCVIP. The proliferative response of CD4 T cells (Tr) was incubated with syngeneic responder CD4 T cells (rCD4) in the presence of allogeneic mDCs, and the proliferative response was determined (n = 4). (d) CD4 T cells were cocultured with syngeneic TrVIP and allogeneic mDCs in the absence or presence of blocking anti-IL10 and/or anti-TGFβ. Additionally, CD4+mDCs were separated from TrVIP+mDC in a Transwell system. The proliferative response of responder CD4 T cells was determined (n = 4). (e) Sorted CD4 T cells generated with DCcontrol or DCVIP were analyzed for neuropilin 1 and Foxp3 mRNA expression by real-time RT-PCR and for surface CD103 and glucocorticoid-induced TNF receptor (GITR) expression by flow cytometry. Open histograms and dashed lines represent isotype controls. One representative experiment of two is shown. (f) Mice were injected s.c. with increasing numbers (from 50 to 5 × 105 cells) of Ag-pulsed DCcontrol or DCVIP 1 week before priming with Ag. Five days later, mice were tested for DLN Ag-specific T cell proliferation, serum antibody levels, and DTH responses. Mice injected with Ag alone (None) were used as controls. Results are the mean ± SD for each group (n = 4) tested separately and are representative of three experiments.
The effect of TrVIP on responder CD4 T cell proliferation might be mediated through soluble factors produced. When TrVIP and responder CD4 T cells were separated in transwell experiments by a semipermeable membrane that allows the free exchange of soluble factors but excludes direct cell contact of responder CD4 T cells and TrVIP, the proliferation of effector CD4 cells was still inhibited, indicating that soluble factors mediate the inhibitory effect (Fig. 2d). In regular cocultures, the addition of anti-TGFβ or anti-IL-10 Abs reversed inhibition modestly. However, the addition of both anti-IL-10 and anti-TGFβ Abs reverses the inhibitory effect almost completely (Fig. 2d).

Several populations of CD4 Tr have been described and characterized, including the naturally occurring thymic-born CD4+CD25+ Tr and the induced peripheral Tr, consisting of IL-10-producing Tr1 and TGFβ-secreting Th3/Tr2 (29). Regulatory DCs do not participate in the generation of naturally occurring CD4+CD25+ Tr; however, they play an important role in the differentiation of peripherally induced Tr1 and Th3/Tr2 Tr (30–32). Although the CD4+CD25+ population is slightly increased in TrVIP, the fact that TrVIP did not express significant levels of the CD4+CD25+ Tr markers Foxp3, neuropilin-1, glucocorticoid-induced TNF-receptor-family-related gene, and CD103 (Fig. 2e), argues against the possibility that DCVIP induce the generation of CD4+CD25+ Tr cells. There are no reports on the expression of neuropilin-1 in IL-10-induced Tr1 cells. However, in contrast to CD4+CD25+ Tr, and in agreement with our results, Tr1 cells generated by repetitive stimulation with IL-10-secreting regulatory DCs have been shown to express low levels of CD25 and Foxp3 (33).

Although the precise mechanisms remain unknown, several possibilities may account for the generation of Tr cells by DCVIP. The activation of naïve CD4 T lymphocytes requires several signals delivered by mDCs and mediated through Ag/MHCII–TCR, CD80/CD86–CD28, and CD40–CD40L interactions. Costimulatory molecules, especially CD40, appear to be key determinants of the decision between tolerance and immunity (34). The characteristic phenotype of DCVIP, i.e., high levels of MHC plus poor expression of costimulatory molecules, which will deliver stimulatory but not costimulatory signals, is in agreement with DCVIP’s tolerogenic-

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**Fig. 3.** Therapeutic effect of DC differentiated with VIP in RA and EAE. (a) DBA1/J mice (H-2k) with established CIA or C57BL/6 mice (H-2b) with established EAE were treated (arrows) with syngeneic CII-pulsed DCs or MOG-pulsed DCs, respectively, generated in the absence (DCcontrol, ○) or presence (DCVIP, ▽) of VIP. Untreated CIA and EAE mice (none, ●) were used as controls. Clinical score was monitored (n = 12). (b) CII- and MOG-pulsed DCVIP were injected at different doses. (c) CII-induced proliferation and IFNγ production by spleen T cells, and the levels of anti-CII IgG in sera were determined in CIA mice injected with DCcontrol or DCVIP (n = 5). (d) The effect of DCVIP is Ag-specific. Arthritic mice were treated with unpulsed, CII-pulsed, or OVA-pulsed DCcontrol or DCVIP after disease onset. One week later, mice were immunized s.c. with OVA or CII and challenged i.d. in the ear pinna with the respective Ag 5 d later. Clinical score and DTH responses were determined 24 h later (n = 5). (e) Untreated CIA or EAE mice or animals injected with DCVIP and treated with control Ig, anti-IL10, anti-TGFβ, or a combination of both mAbs (10 mice per group).
followed by in vivo autoimmune diseases. Recently proposed the possibility of using regulatory in vivo lack of CD40 expression or signaling has been demonstrated both in vivo and in vitro (28). Expression of CD40 depends on NF-κBp65 nuclear translocation and NF-κB phosphorylation are inhibited in DCVIP (M.D., E.G.-R., and D.G., unpublished data). The connection among NF-κB transactivating activity, CD40 expression, and DC function (including TNF-α and IL-12 production) has been established in a number of recent studies. The association between tolerance, particularly tolerogenic DCs, and lack of CD40 expression or signaling has been demonstrated both in vivo and in vitro (9). One feature with RA. For MS, we used the EAE model induced by immunization with CII, an experimental disease model induced by immunization with CII, and we have recently found that syn genesic CII and MOG-specific Trcontrol (10) or with different doses of CII- or MOG-specific DCVIP (106 cells, n = 5 × 10^5 cells; n = 5 × 10^6 cells, n = 5). Untreated mice (n) were used as CIA and EAE controls. Clinical score was determined (10). (b) Untreated CIA/EAE mice or CIA/EAE mice injected with TrVIP and treated with control Ig, anti-IL10, anti-TGFβ, or anti-IL10 plus anti-TFβ Abs. Clinical score was measured at the peak of the disease (n = 10).

Several reports have recently proposed the possibility of using regulatory/tolerogenic DCs generated in vivo as a therapeutic tool to prevent organ-specific autoimmune diseases (3, 7, 21). Interestingly, DCVIP retained their T cell regulatory capacity in vitro and in vivo under inflammatory conditions. This observation is particularly relevant for conditions in which ongoing Ag presentation is associated with chronic inflammation, including autoimmune diseases. Therefore, we tested the therapeutic effect of DCVIP in two murine models of RA and multiple sclerosis (MS). For RA, we used the CIA, an experimental disease model induced by immunization with CII, which shares a number of clinical, histologic, and immunological features with RA. For MS, we used the EAE model induced by MOG35–55 in C57BL/6 mice that mirror different clinical characteristics of MS. Inoculation of DCcontrol does not ameliorate arthritis (i.e., joint inflammation, cartilage destruction, and bone erosion) or EAE (i.e., tail and leg paralysis) (Fig. 3a). In contrast, administration of syn genesic DCVIP after the onset of disease abrogates arthritis and EAE progression in a dose-dependent manner (Fig. 3a and b).

The therapeutic effect of DCVIP was associated with the down-regulation of the autoimmune component of both diseases, because
of anti-IL10 and/or anti-TGFβ Abs abrogated the protective effect (Fig. 4b). In both models, the protective effect of TrVIP was Ag-specific, because OVA-specific TrVIP did not efficiently ameliorate arthritis or paralysis (data not shown). These results indicate that Ag-specific Tr1-like cells generated in vitro with DCVIP can efficiently modulate pathogenic immune responses in vivo.

VIP has been previously found to ameliorate CIA and EAE, mainly by down-regulating the two components of both diseases, inflammation and Th1-mediated autoimmunity (ref. 10 and E.G.-R., A.F.-M., D.G., and M.D., unpublished results). The involvement of Tr cells in the therapeutic effect of VIP was demonstrated by the fact that CD4+ T cells isolated by VIP-treated CIA or EAE mice showed an increased regulatory activity against self-reactive Th1 cells. Phenotypic analysis of these Tr cells indicated that they consist of a mix of Foxp3+CD4+CD25+ and IL-10+ Tr1-like cells (E.G.-R., A.F.-M., D.G., and M.D., unpublished results). In addition, by using a transgenic TCR murine model, we found that VIP induces the in vivo generation of Ag-specific tolerogenic IL-10-producing DCs with capacity to generate/activate Tr1-like cells (M.D., E.G.-R., D.G., and M.D., unpublished results). These findings validate the data obtained in this study, demonstrating that the pharmacological use of VIP in the treatment of autoimmunity is exerted partially through the induction of tolerogenic DCs and Tr1-like cells.

It has been proposed that tolerance induction by DCs requires maturation signals different from microbial or inflammatory stimuli. In steady-state conditions, VIP could represent one of the endogenous maturation signals driving the differentiation of tolerogenic DCs with a regulatory phenotype. VIP is secreted in the lymphoid microenvironment, mainly by Th2 cells, after Ag stimulation, and VIP levels are increased in immunopathologic conditions, such as autoimmunity and inflammation (8, 9). Therefore, DCVIP may represent a population of DCs that have matured to display a stable tolerogenic phenotype. Under steady-state conditions, DCVIP could be loaded with self- and commonly encountered Ags, and, after migration to the lymphoid organs, they could induce Tr1 differentiation and tolerance. Interestingly, in subjects with various autoimmune disorders, reduced serum VIP levels and increased VIP-specific autoantibodies have been reported (41).

Numerous strategies based on immunosuppressive agents, such as vitamin-D3, IL-10, TGFβ, glucocorticoids, and N-acetyl-l-cysteine, alone or in combinations, have been used to induce tolerogenic DCs (7). However, in the case of regulatory DCs induced with vitamin D analogs, it looks as if these regulatory DCs induce CD4+CD25+ Tr cells rather than Tr1-like cells (7). Our data demonstrate that VIP is very efficient at the induction of regulatory DCs, in comparison with current strategies, and we propose that the addition of VIP to cocktails of immunomodulatory agents will increase their effectiveness.

In conclusion, the possibility of generating tolerogenic DCVIP opens therapeutic perspectives for the treatment of autoimmune/inflammatory diseases and in allogeneic transplantation. In vivo pulsing of tolerogenic DCVIP with self-Ags, followed by in vivo injection, leads to the differentiation of Ag-specific Tr cells. Therefore, the inclusion of tolerogenic DCVIP in future therapeutic regimens may minimize the dependence on nonspecific immunosuppressive drugs used currently for autoimmune disorders.

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