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 (2×10^6) obtained from BALB/c (H-2^d), C57BL/6 (H-2^b), or DBA/1 (H-2^q) mice were incubated in complete medium (RPMI medium 1640 supplemented with 100 units/ml penicillin/strectomycin, 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/maturation. In some experiments, DCs were pulsed with ovoalbumin (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. For intracellular analysis of cytokines in restimulated CD4 T cells, 10^6 cells per ml were collected and stimulated with phorbol 12-myristate 13-acetate (1 ng/ml) plus ionomycin (20 ng/ml) for 8 h in the presence of monensin. Cells were stained with PerCP-anti-CD4 mAbs for 30 min at 4°C, washed, fixed/saponin-permeabilized with Cytofix/Cytoperm, stained with 0.5 μg per sample FITC- and PE-conjugated anticytokine-specific mAbs, and analyzed by flow cytometry. To distinguish between DC and T cell sources,

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Abbreviations: Ag, antigen; BM, bone marrow; DC, dedritic cell; BM-DC, BM-derived DC; CIA, collagen-induced arthritis; CII, collagen II; DLN, draining lymph nodes; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; iDC, immature DC; mDC, mature DC; MOG, myelin oligodendrocyte glycoprotein; OVA, ovalbumin; RA, rheumatoid arthritis; Tr, regulatory T cells; Th, T helper; VIP, vasoactive intestinal peptide

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intracellular cytokine analysis was done exclusively in the PerCP-labeled CD4 T cell population.

mRNA Analysis. Total RNA was isolated from CD4 T cells, and real-time PCR was used to determine Foxp3 and neuropilin mRNA expression, as described in refs. 14 and 15.

Mixed Leukocyte Reaction and Analysis of Tr Cell Function. Naïve CD4 T cells (2×10^5) were cultured with allogeneic $DC_{control}$ or DC_{VIP} at various T:DC ratios in the presence of IL-2 (100 units/ml) for 3 d. Cell proliferation was evaluated by using a cell-proliferation assay (BrdUrd) from Roche Diagnostics (Mannheim, Germany), and intracellular cytokine content was determined as described above. In some experiments, DCs (10^5) were cultured with purified allogeneic CD4 T cells (5×10^5). One week later, CD4 T cells were recovered by immuodepletion of $CD11c^+$ DCs and cultured in different numbers with syngeneic CD4 T cells (5×10^5) 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 (5×10^5) with LPS-matured DC (10^5) in the bottom well of a Transwell system (Millipore) and syngeneic Tr_{VIP} (2×10^5) 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, DBA1/J and C57BL/6 DCs (10^5) pulsed with CII or MOG, respectively, were cultured with syngeneic CD4 T cells (5×10^5) for 1week 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 5×10^5) of cells of methylated BSA (mBSA)-pulsed $DC_{control}$ or DC_{VIP}, 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 (4×10^5) 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 DBA1/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 MOG_{35-55}, 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 $DC_{control}$ or DC_{VIP} or with CII-specific $Tr_{control}$ or Tr_{VIP}. Mice with established EAE (with a clinical score of 1) were injected i.v. with different numbers of syngeneic MOG_{35-55}-pulsed $DC_{control}$ or DC_{VIP} or with MOG_{35-55}-specific $Tr_{control}$ or Tr_{VIP}. 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 MOG_{35-55} (20 μg/ml), and assayed for proliferation and cytokine production, as described above. The content of serum anti-CII or anti-MOG_{35-55} 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 MOG_{35-55}-pulsed $DC_{control}$ or DC_{VIP} and immunized s.c. with OVA, CII, or MOG_{35-55} (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. 1*a*, $DC_{control}$). However, DCs generated in the presence of VIP (DC_{VIP}) were resistant to the LPS-induced up-regulation of the costimulatory molecules (CD40, CD80, and CD86) (Fig. 1*a*). Upon

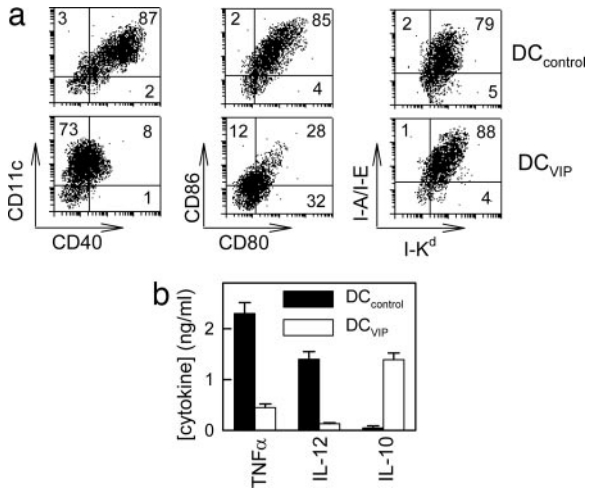

Fig. 1. VIP induces a stable ''semimature'' phenotype in BM-DCs. DCs were generated from mouse BM cells in the absence ($DC_{control}$) or presence (DC_{VIP}) of VIP and activated with LPS to induce DC maturation. (*a*) $DC_{control}$ and DC_{VIP} were double-labeled for different markers and analyzed by flow cytometry. Numbers represent the percentage of positive cells ($n = 4$). (*b*) Cytokine content in the DC supernatants was determined by ELISA ($n = 4$).

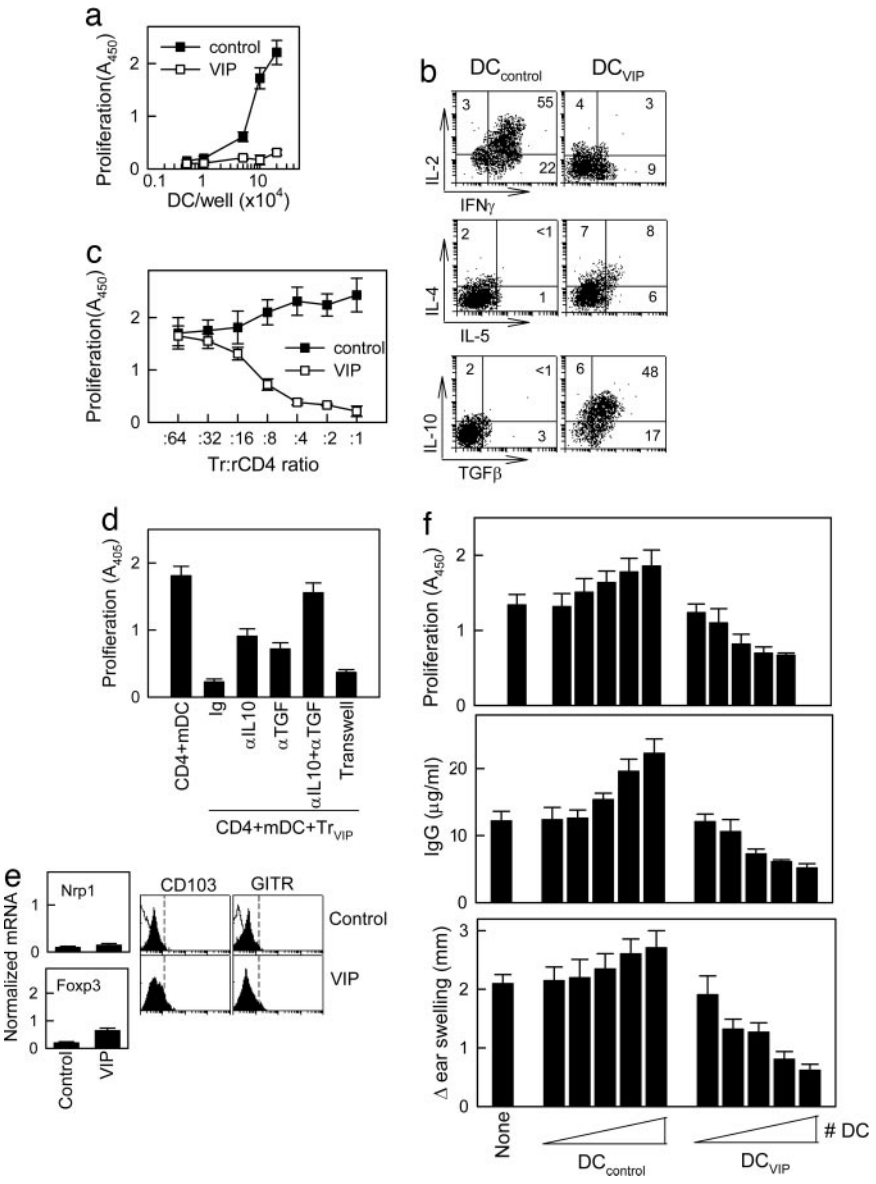

Fig. 2. Murine DCs differentiated in the presence of VIP induce regulatory Tr1 cells and tolerance *in vivo*. DCs were generated from mouse BM cells in the absence ($DC_{control}$) or presence (DC_{VIP}) of VIP and activated with LPS to induce DC maturation. (*a*) $DC_{control}$ or DC_{VIP} was added to allogeneic CD4 T cells (5×10^5), and the proliferative response was determined. $DC_{control}$ or DC_{VIP} without T cells did not proliferate. Each result is the mean ± SD of three experiments performed in duplicate. (*b*) Purified CD4 T cells were exposed to allogeneic $DC_{control}$ or DC_{VIP} and activated with phorbol 12-myristate 13-acetate plus ionomycin. Intracellular cytokines were determined in CD4-gated cells by flow cytometry. Numbers represent percentage of positive cells ($n = 5$). (*c*) Purified CD4 T cells were stimulated for 1 week with allogeneic $DC_{control}$ or DC_{VIP}. The resulting regulatory CD4 T cells (Tr) were incubated with syngeneic responder CD4 T cells (rCD4) in the presence of allogeneic mDCs, and the proliferative response was determined ($n = 4$). (*d*) Isolated CD4 T cells were cocultured with syngeneic Tr_{VIP} and allogeneic mDCs in the presence or absence of blocking anti-IL10 and/or anti-TGFβ. Additionally, CD4+mDCs were separated from Tr_{VIP}+mDC in a Transwell system. The proliferative response of responder CD4 T cells was determined ($n = 4$). (*e*) Sorted CD4 T cells generated with $DC_{control}$ or DC_{VIP} 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 5×10^5 cells) of Ag-pulsed $DC_{control}$ or DC_{VIP} 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.

toll-like receptor activation, iDCs mature into cells capable of producing high levels of inflammatory cytokines. In contrast to $DC_{control}$, which produce TNF and IL-12, and low levels of IL-10, DC_{VIP} produce very low levels of proinflammatory cytokines (TNF and IL-12) but secrete significant levels of the antiinflammatory cytokine IL-10 (Fig. 1*b*). 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 $1,25(OH)_2D_3$ (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 DC_{VIP} to stimulate T cells, we cocultured $DC_{control}$ or DC_{VIP} with alloreactive CD4 T cells. Priming with $DC_{control}$ results in a strong proliferation of allogeneic CD4 T cells, whereas DC_{VIP} induce only weak proliferation (Fig. 2*a*). In addition, CD4 T cells primed with DC_{VIP} reexposed to fresh LPS-stimulated allogeneic DCs (mDC) did not proliferate (data not shown), indicating that DC_{VIP} 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 DC_{VIP}. In contrast to T cells exposed to $DC_{control}$, which show a predominant Th1 cytokine profile, with high levels of IFNγ and IL-2, CD4 T cells primed with allogeneic DC_{VIP} exhibit a Tr1-like phenotype, characterized by IL-10 and TGFβ but not IL-2 and IFNγ production (Fig. 2*b*).

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 DC_{VIP} become functional Tr, we restimulated CD4 T cells with allogeneic mDCs in the presence of syngeneic CD4 T cells previously exposed to allogeneic $DC_{control}$ ($Tr_{control}$) or DC_{VIP} (Tr_{VIP}). Tr_{VIP} inhibit the proliferation of syngeneic responder CD4 cells in response to allogeneic mDCs in a dose-dependent manner, whereas $Tr_{control}$ are not suppressive (Fig. 2*c*). Similar results were obtained in respect to IL-2 production (data not shown). Therefore, the phenotype of Tr_{VIP} correlates with their regulatory T cell activity.

The observation that Tr_{VIP} produce high levels of the immunosuppressive cytokines IL-10 and TGFβ suggests that the inhibitory

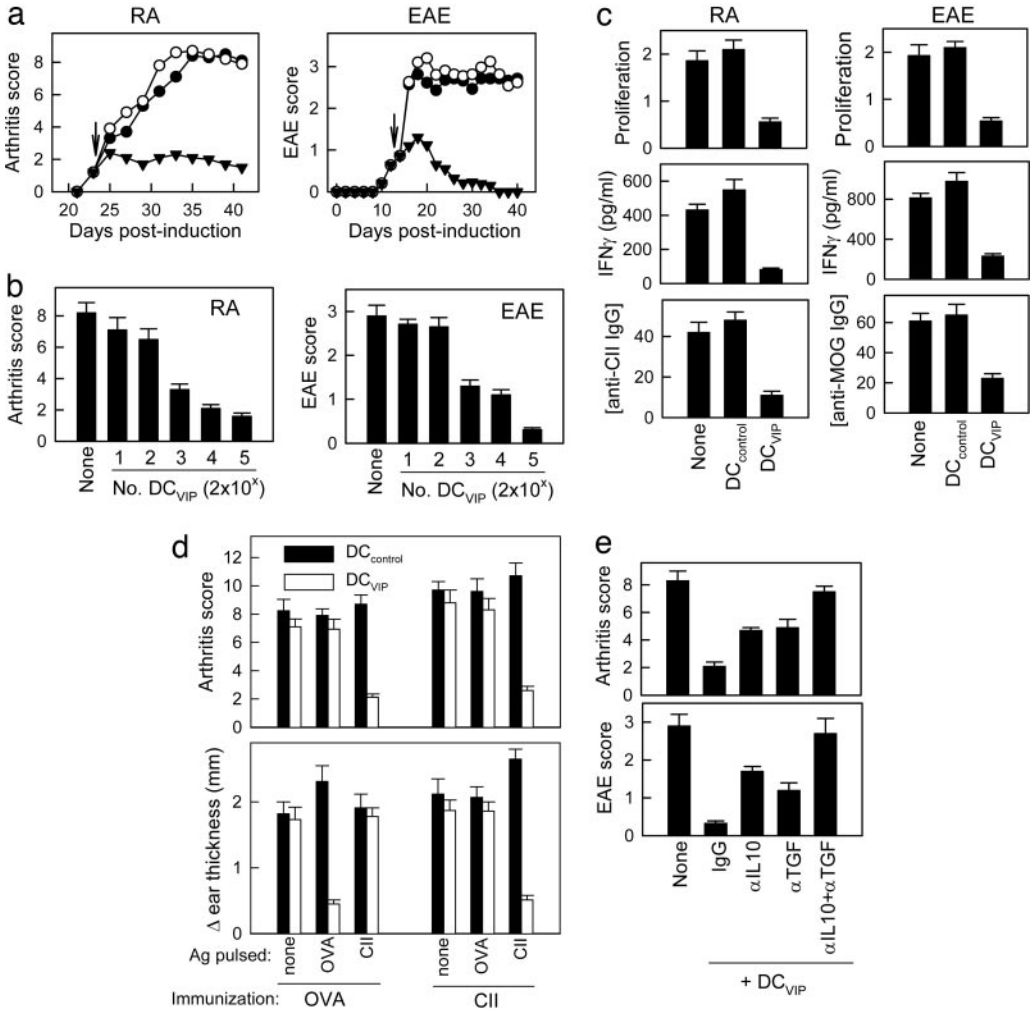

Fig. 3. Therapeutic effect of DC differentiated with VIP in RA and EAE. (*a*) DBA1/J mice (H-2^q) with established CIA or C57BL/6 mice (H-2^b) with established EAE were treated (arrows) with syngeneic CII-pulsed DCs or MOG-pulsed DCs, respectively, generated in the absence ($DC_{control}$, ○) or presence (DC_{VIP}, ▼ of VIP. Untreated CIA and EAE mice (none, ●) were used as controls. Clinical score was monitored ($n = 12$). (*b*) CII- and MOG-pulsed DC_{VIP} 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 $DC_{control}$ or DC_{VIP} ($n = 5$). (*d*) The effect of DC_{VIP} is Ag-specific. Arthritic mice were treated with unpulsed, CII-pulsed, or OVA-pulsed $DC_{control}$ or DC_{VIP} 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 DC_{VIP} and treated with control Ig, anti-IL10, anti-TGFβ, or a combination of both mAbs (10 mice per group).

effect of Tr_{VIP} on responder CD4 T cell proliferation might be mediated through soluble factors produced. When Tr_{VIP} 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 Tr_{VIP}, the proliferation of effector CD4 cells was still inhibited, indicating that soluble factors mediate the inhibitory effect (Fig. 2*d*). 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. 2*d*).

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 Tr_{VIP}, the fact that Tr_{VIP} did not express significant levels of the $CD4^+CD25^+$ Tr markers Foxp3, neuropilin-1, glucocorticoid-induced TNF-receptor-family-related gene, and CD103 (Fig. 2*e*), argues against the possibility that DC_{VIP} 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 DC_{VIP}. 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 DC_{VIP}, i.e., high levels of MHC plus poor expression of costimulatory molecules, which will deliver stimulatory but not costimulatory signals, is in agreement with DC_{VIP}'s tolerance-inducing ability. In addition, the observation that DC_{VIP} secrete IL-10 may be linked to the stability of DC_{VIP}'s tolerogenic-

like phenotype (20, 35–37). Previously, VIP has been reported to inhibit NF-κBp65 nuclear translocation, DNA-binding, and transactivating activity in macrophages (9), and we have recently found that both NF-κBp65 nuclear translocation and IkB phosphorylation are inhibited in DC_{VIP} (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* (28). Expression of CD40 depends on NF-κBp65 (38), and the inhibition of NF-κB in DCs leads to failure of CD40, CD80, and CD86 expression upon LPS-stimulation and to the generation of tolerogenic DCs (39). In addition, a recent study suggests that VIP treatment induced a decrease of toll-like receptors (TLR-2/4) expressions in DCs in a murine model of Crohn's disease by a mechanism that would involve a decrease of NF-κB activation (40). Therefore, we would like to propose that the mechanism by which VIP induces tolerogenic DCs involves the cAMP/PKA-mediated inhibition of IκB phosphorylation and NF-κBp65 nuclear translocation, leading to lack of CD40 expression, TLR-2/4 signaling, and inflammatory cytokine production.

Because DC_{VIP} appear to have a predominantly negative effect on Th1 cells, we determined the effect of DC_{VIP} in an *in vivo* model of DTH. Ag (methylated-BSA)-pulsed DC_{VIP} and $DC_{control}$ were administered i.v., followed a week later by s.c. antigenic immunization. We determined T cell proliferation in response to *ex vivo* restimulation, Ab production, and DTH after a secondary s.c. Ag administration (Fig. 2*f*). Mice that received $DC_{control}$ developed DTH reactions higher than controls (no DCs), whereas those receiving DC_{VIP} exhibited reduced DTH. In addition, DLN T cells from mice inoculated with $DC_{control}$ proliferated at higher levels than controls (no DCs), and, again, inoculation of DC_{VIP} resulted in a substantial reduction in T cell proliferation after *ex vivo* restimulation with the Ag. Similarly, mice inoculated with $DC_{control}$ produced high levels of anti-mBSA Abs, whereas those inoculated with DC_{VIP} had anti-mBSA Ab levels below control (no DCs) (Fig. 2*f*). These results indicate that DC_{VIP} induce tolerance *in vivo*. The induction of tolerance is restricted to the Ag presented by the inoculated DC, because we did not observe reduction in DTH in mice injected with mBSA-pulsed DC_{VIP} when we used an unrelated Ag (OVA) for immunization and *ex vivo* T cell restimulation (data not shown). These experiments suggest the possibility of using a VIP *in vitro* system to generate Ag-specific tolerogenic DCs, followed by *in vivo* administration of these cells to patients with autoimmune diseases.

Therapeutic Effect of DC_{VIP} in Autoimmunity. Several reports have recently proposed the possibility of using regulatory/tolerogenic DCs generated *ex vivo* as a therapeutic tool to prevent organ-specific autoimmune diseases (3, 7, 21). Interestingly, DC_{VIP} 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 DC_{VIP} 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 $MOG_{35–55}$ in C57BL/6 mice that mirror different clinical characteristics of MS. Inoculation of $DC_{control}$ does not ameliorate arthritis (i.e., joint inflammation, cartilage destruction, and bone erosion) or EAE (i.e., tail and leg paralysis) (Fig. 3*a*). In contrast, administration of syngeneic DC_{VIP} after the onset of disease abrogates arthritis and EAE progression in a dose-dependent manner (Fig. 3 *a* and *b*). The therapeutic effect of DC_{VIP} was associated with the down-regulation of the autoimmune component of both diseases, because

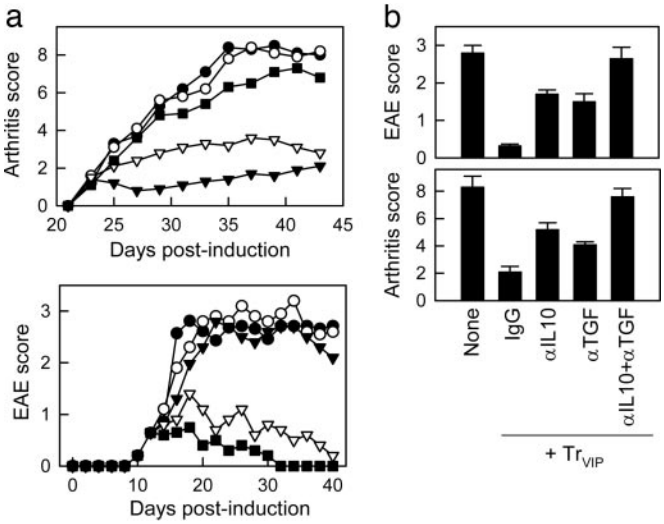

Fig. 4. DC_{VIP}-induced Tr prevent autoimmunity. (*a*) Therapeutic effect on arthritis. CIA (H-2^q) or EAE mice (H-2^b) with established disease were treated with syngeneic CII- and MOG-specific $Tr_{control}$ (○) or with different doses of CII- or MOG-specific Tr_{VIP} (10^6 cells, ▼; 5×10^5 cells, ▽; 5×10^4 cells, ■). Untreated mice (●) were used as CIA and EAE controls. Clinical score was determined (n = 10). (*b*) Untreated CIA/EAE mice or CIA/EAE mice injected with Tr_{VIP} and treated with control Ig, anti-IL10, anti-TGFβ, or anti-IL10 plus anti-TGFβ Abs. Clinical score was measured at the peak of the disease (n = 10).

DLN T cells from DC_{VIP}-treated mice showed weak proliferation and IFNγ production in response to the autoantigen (Fig. 3*c*). Furthermore, this inhibition of the Th1-type autoreactive response correlates with decreased levels of CII- and MOG-specific autoantibodies (Fig. 3*c*). The effect of DC_{VIP} was Ag-specific. Unpulsed or OVA-pulsed DC_{VIP} showed a weak therapeutic effect on arthritis, while reducing OVA-specific, but not CII-specific, DTH responses. In contrast, CII-pulsed DC_{VIP} inhibit arthritis and DTH response toward CII but not toward OVA (Fig. 3*d*). Similar Ag-dependence was observed in the EAE model (data not shown). These results indicate that DC_{VIP} generated *ex vivo* could prevent organ-specific autoimmune disorders in matched subjects, probably by inducing Ag-specific Tr cells, which suppress the ongoing autoreactive/inflammatory response. The participation of Tr cells in the therapeutic effect of DC_{VIP} correlated with the fact that DC_{VIP} induce *in vitro* the generation of IL-10/TGFβ-producing regulatory CD4 T cells (Fig. 2). Therefore, we further examined the role of Tr in the therapeutic effect of DC_{VIP} on both CIA and EAE. *In vivo* blockade experiments showed that treatment with anti-IL-10 or anti-TGFβ Abs significantly decrease disease amelioration, and treatment with both Abs abrogates the beneficial effects exerted by DC_{VIP} (Fig. 3*e*), suggesting the partial involvement of newly generated Tr cells in such action.

DC_{VIP}-Induced Tr Ameliorate Autoimmunity. In certain circumstances, successful suppression of an autoimmune response might require high numbers of Tr, and the *in vivo* administration of DC_{VIP} might not be sufficient for a complete and rapid suppression. Therefore, we decided to generate *in vitro* Ag-specific DC_{VIP}-induced Tr cells and to subsequently determine their suppressive capacity *in vivo* in both the CIA and EAE models. We generated CII- or MOG-specific Tr_{VIP} through stimulations of CD4 T cells with syngeneic CII- or MOG-pulsed DC_{VIP}. $Tr_{control}$ were generated in the same manner with $DC_{control}$. Treatment with Tr_{VIP}, but not $Tr_{control}$, of mice with established CIA or EAE prevented disease progression in a dose-dependent manner (Fig. 4*a*). This effect was mainly mediated through TGFβ and IL-10, because *in vivo* administration

of anti-IL10 and/or anti-TGFβ Abs abrogated the protective effect (Fig. 4*b*). In both models, the protective effect of Tr_{VIP} was Ag-specific, because OVA-specific Tr_{VIP} did not efficiently ameliorate arthritis or paralysis (data not shown). These results indicate that Ag-specific Tr1-like cells generated *in vitro* with DC_{VIP} 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., A.C., D.P., 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/suppressive 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.C., 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., and D.G., 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, DC_{VIP} may represent a population of DCs that have matured to display a stable tolerogenic phenotype. Under steady-state conditions, DC_{VIP} 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 DC_{VIP} opens therapeutic perspectives for the treatment of autoimmune/inflammatory diseases and in allogeneic transplantation. *In vitro* pulsing of tolerogenic DC_{VIP} with self-Ags, followed by *in vivo* injection, leads to the differentiation of Ag-specific Tr cells. Therefore, the inclusion of tolerogenic DC_{VIP} in future therapeutic regimens may minimize the dependence on nonspecific immunosuppressive drugs used currently for autoimmune disorders.

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