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Induction of Alloantigen-Specific Human T Regulatory Cells by Vasoactive Intestinal Peptide

David Pozo,*, Per Anderson,† and Elena Gonzalez-Rey‡‡

T regulatory cells (Tregs) are instrumental in the maintenance of immunological tolerance. Although Treg-based immunotherapy proved successful in preclinical autoimmunity and transplantation, factors involved in the generation of human Ag-specific Tregs are poorly known. In this study, we show that treatment of human CD4+CD25− T cells with the cytokine-like vasoactive intestinal peptide (VIP) during in vitro stimulation induces an anergic FoxP3+CD4+CD25high T cell subset displaying potent regulatory activities against allospecific effector T cells, irrespective of the presence of naturally occurring Tregs. VIP-tolerant T cells are characterized by incapability to progress to S phase of cell cycle during stimulation with HLA-disparate APCs by negatively affecting the synthesis of cyclins D3 and E, the activation of cyclin-dependent kinases (cdk)2 and cdk4, and the down-regulation of the cdk inhibitor p27kip1. VIP interaction with the type 1 VIP receptor and subsequent activation of cAMP/protein kinase A pathway play a major role in all these effects. Moreover, VIP-tolerant T cells protect against acute graft-vs-host disease in a mouse model of allogeneic bone marrow transplantation. The infusion of VIP-tolerant T cells together with the graft significantly reduces the clinical signs and mortality rate typical of the graft-vs-host disease. These effects are mediated by impairing allogeneic haplotype-specific responses of donor CD4+ cells in the transplanted animals. Our results suggest that including alloantigen-specific VIP-generated Tregs may be a valuable tool in therapeutic interventions to promote immunotolerance toward allogeneic grafts and to reduce the need of general immunosuppressive drugs. The Journal of Immunology, 2009, 183: 4346–4359.

afi induction of Ag-specific long-term tolerance is essential to maintain homeostasis, to control autoreactive T cells in autoimmune diseases, and to achieve transplantation tolerance (1, 2). Tolerance is mainly maintained through the intrathymic deletion of self-reactive T cells and through the induction of T cell anergy and differentiation of T regulatory cells (Tregs).3

Two major populations of Tregs, with complementary and overlapping functions in the control of immune response in vivo, exist, as follows: natural (or constitutive) and inducible (or adaptive) Tregs. Numerous studies have demonstrated the therapeutic use of Ag-specific Tregs in various experimental models of autoimmune diseases and allogeneic transplantation, providing long-term tolerance by active and specific regulation of self-Ag and alloantigen-specific T cells (3–5). These findings have opened up exciting opportunities for new therapies in several human diseases that are associated with Treg dysfunction. However, the translation of important biological findings about Tregs to the clinic has been mainly limited by the inability to define their antigenic specificities and by the scarcity of circulating Tregs. The solution to this problem might lie in expanding the Treg population in vitro, and making the Tregs Ag specific. However, although Tregs replicate efficiently in vivo, they are anergic and refractory to stimulation in vitro (3, 5). Thus, to efficiently expand Treg populations in vitro while maintaining their immunoregulatory properties in vivo, new protocols must be developed that reproduce the conditions that enable replication in vivo, including TCR occupancy, crucial co-stimulatory signals, and the presence of selective growth factors. However, although the ontogeny and mechanisms involved in the suppressive action of Tregs on self- and alloreactive lymphocytes are widely described in the literature (2, 3, 5), the endogenous factors and mechanisms controlling their peripheral generation or expansion are mostly unknown.

Allogeneic bone marrow transplantation (BMT) is the treatment of choice in many hematopoietic malignancies. Following high-dose chemotherapy or irradiation, the host is reconstituted with bone marrow cells, and the donor T cells are responsible for the graft-vs-tumor effects that eliminate the remaining malignant cells in the host. However, the same donor T cells, which recognize MHC disparities in the recipient, expand and initiate a multiorgan system distraction known as graft-vs-host disease (GvHD). In fact, acute GvHD is a major cause of morbidity and mortality in patients undergoing allogeneic BMT (6). Most therapeutic approaches designed to reduce acute GvHD have focused on the development of nonspecific immunosuppressive drugs and the ex vivo removal of donor T cells from the transplant (7). However, removal of these T cells before grafting was shown to lead to transplant failure and leukemia relapse, and although successful in controlling T cell alloreactivity during transplantation, prolonged administration of many of the immunosuppressive drugs results in adverse side effects derived from sustained immunosuppression (8). An alternative approach to improve the allogeneic transplantation outcome is

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3 Abbreviations used in this paper: Treg, T regulatory cell; BMT, bone marrow transplantation; cdk, cyclin-dependent kinase; GvHD, graft-vs-host disease; int, intermediate; PCCF, pigeon cytochrome c fragment; PKA, protein kinase A; Rb, retinoblastoma gene product; pRb, phosphorylated Rb; TCD-BM, T cell-depleted bone marrow cell; VIP, vasoactive intestinal peptide; VPAC1, type 1 VIP receptor.

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the induction of graft tolerance by selectively inactivating alloreactive donor T cells in the absence of chronic immunosuppression. Recently, the use of alloantigen-specific Tregs as a therapeutic strategy to limit the pathologic effects of donor-alloreactive T cells has been proposed (1, 3, 9). Given the potential clinical importance of the induction of tolerance after the transplant, it is crucial to identify immunosuppressive agents that do not interfere with the development of Tregs, and ideally improve the function or generation of the Treg compartment.

Vasoactive intestinal peptide (VIP), a neuropeptide released from the VIP-ergic innervation of immunocompetent tissues, such as thymus, spleen, and lymph nodes as well as from Th2 cells in response to Ag stimulation and under inflammatory conditions, is a potent cytokine-like agent that affects both innate and adaptive immunity (10–12). Indeed, VIP has been used for the treatment of various experimental models of inflammatory and autoimmune diseases (12, 13). The therapeutic effect of VIP was initially attributed to the down-regulation of a wide panel of inflammatory mediators and to the inhibition of autoreactive Th1 cells. However, recent evidence has demonstrated the involvement of Tregs in the beneficial effect of VIP in immune disorders (14). VIP has been shown to induce the emergence of Ag-specific Tregs in vivo with suppressive activity on effector T cells (15–17). However, the mechanisms involved in the generation or expansion of this Treg population are not fully understood. Moreover, the tolerance/energy-promoting potential of VIP in human T cells is still unknown. In this study, we investigate whether VIP is able to induce human Tregs with suppressive action on alloantigen-specific effector T cells and the molecular mechanisms involved in such an effect. We also describe the therapeutic applicability of the VIP-tolerated T cells in a murine model of histoincompatible BMT.

Materials and Methods

Abs and reagents

VIP, Sp-8-Br-cAMP, H-89, and histone H1 were purchased from Calbiochem. The type 1 VIP receptor (VPAC1) antagonist (Ac-His2-D-Phe4-Lys6)-VIP(3–7)-GRF(8–27) was previously described (18). FITC-, PerCP-, and PE-conjugated Abs against CTLA4, TGF-β1, IFN-γ, IL-10, IL-17, IL-2, IL-4, Vβ3 (clone KJ25), CD154, CD25, and CD4, and Abs against p80, CD3, and IL-10 were obtained from BD Pharmingen. Human rHL-2 and rHL-15 were obtained from Roche Biomedical and PeproTech. Protein A/G agarose, and Abs against cyclin E, cyclin A, cyclin-dependent kinase (cdk)2, cyclin D2, cyclin D3, cdk4, cdk6, and p27kip1 were purchased from Santa Cruz Biotechnology, FITC- and Alexa-conjugated anti-mouse FoxP3 Ab (clone FJK-16s) and anti-human FoxP3 Abs (clones PCH101 and 236A/E7) were obtained from eBioscience. Pegeon cytome cytochrome c fragment (PCCF) was synthesized and purified in our facilities.

Cell isolation

PBMCs were isolated from buffy coat preparations derived from the whole blood of healthy volunteers by density sedimentation on Ficoll-Hypaque gradient (g). Cells recovered from the gradient interface were washed twice in RPMI 1640 medium and immediately used for culture or further purification. To isolate T cells (purity >96%), PBMCs were incubated with anti-CD8, -CD14, -CD19, -CD20, and -CD56 mAbs (Coulter Immunotech) for 1 h at 4°C, followed by 1-h incubation at 4°C with anti-mouse IgG-coated magnetic beads, and bead-bound cells removed with a magnetic device. To minimize stimulation of cells, all the purification steps were conducted in the absence of serum. CD4+ T cells (purity of 94–98%) were isolated by negative selection from the PBMCs using the CD4 isolation kit (Miltenyi Biotech). CD4+CD25− and CD4+CD25+ T cell fractions were isolated (purity >96%) using the CD4+CD25+ T Regulatory Cell Isolation kit from Miltenyi Biotech. In some experiments, the different T cell populations (CD4+, CD4+CD25+, CD4+CD25−) were isolated by sorting using a FACSCalibur flow cytometer (BD Biosciences) after labeling with PE anti-CD25 and PerCP anti-CD4 Abs, as described below. All samples of CD4+CD25− T cells isolated with either beads or sorting used in the study were negative for FoxP3 expression, as assessed by flow cytometry (FoxP3+ cells were <1%) and RT-PCR analysis. ACPs were obtained from PBMCs after cell adhesion on plastic dishes for 2 h, followed by T cell depletion with anti-CD3-coated magnetic beads.

Murine CD4+CD25− and CD4+CD25+ cells were isolated from C57BL/6 mice (The Jackson Laboratory), as described previously (15). Spleens were gently minced in complete DMEM containing 10% FBS (BioWhittaker), and CD4+ T cells were purified using a mouse CD4+ T cell column system (R&D Systems). T cell-depleted spleen cells (irradiated, 3000 rad) of BALB/c or C57BL/6 mice were used as ACPs.

Cell cultures

All cultures were conducted in complete medium consisting of RPMI 1640 medium supplemented with heat-inactivated human pooled serum (8%), 1-glutamine (20 mM), sodium pyruvate (1%), nonessential amino acids (1%), and penicillin/streptomycin (1%) (all from Invitrogen) in a 5% CO2 humidified atmosphere at 37°C.

Allogeneic stimulation

Primary MLCs were performed in 96-well, round-bottom plates by stimulating responder PBMCs (105) with allogeneic HLA-mismatched 30 Gy γ-irradiated stimulator PBMCs (105) in 200 μl medium in the presence or absence of VIP (10−7 M). Cells were pulsed with 0.5 μCi (0.0185 MBq)/well [3H]thyidine for the last 8 h of the culture and harvested onto membranes, and proliferation was determined by measuring [3H]thyridine uptake. Some primary MLCs were established with CD4+CD25− T cells (5 × 104) and were stimulated with allogeneic HLA-mismatched γ-irradiated PBMCs (5 × 104) without or with VIP. Secondary MLCs were performed to determine the memory response of allogeneic primed T cells. Cells were harvested after 6 days of primary stimulation, washed three times, and rested for 2 days. The viable cells (5 × 104) were recovered by density gradient centrifugation with Lymphoprep (Nycomed Pharma) and restimulated in a secondary culture with HLA-mismatched γ-irradiated PBMCs (2 × 105) in the absence or presence of IL-2 (20 U/ml). [3H]Thyridine incorporation was determined at different time points of secondary MLC. When indicated, recovered viable cells (105/ml) from primary MLC were stimulated in a secondary culture with anti-CD3/anti-CD28 mAb-coated magnetic beads (Invitrogen; 1 bead/cell). Cells were collected at different time points after anti-CD3/CD28 restimulation and lysed for protein and kinase activity determinations, as described below, and the proliferative response was determined as above.

T cell suppression assays

The suppressive capacity of VIP-treated cells was analyzed in a coculture assay. After primary stimulation of responder cells (105 PBMCs or CD4+CD25− T cells, from donor A) in MLCs with HLA-mismatched 30 Gy γ-irradiated stimulator PBMCs (105, from donor B) in the presence or absence of VIP for 6 days, the cells were harvested and allowed to reculture for 2 days. The recovered T cells were added at different ratios to a newly set-up primary MLC (consisting of 105 original responder PBMCs from donor A and 105 stimulator PBMCs from donor B), and proliferation was measured by [3H]thyridine uptake. Some cultures were performed in the presence of blocking anti-IL-10 (10 μg/ml), anti-TGF-β (10 μg/ml), and/or anti-CTLA4 (10 μg/ml) mAbs, or human rHL-2 (100 U/ml). To determine the cell-contact dependence of the suppressive response, we placed responder PBMCs (5 × 105) with allogeneic stimulator PBMCs (5 × 105) in the bottom well of a Transwell system (Millipore; 0.4 μm pore), and the recovered T cells (2 × 104) with or without allogeneic stimulator PBMCs (5 × 105) in the upper Transwell insert. At day 4, the proliferative response of the responder PBMCs in the lower compartment was determined.

In similar experiments, responder PBMCs and T cells were labeled with 2.5 μM CFSE (Molecular Probes) before setting up cocultures, and proliferating cells were determined by CFSE dilution by flow cytometry.

Assessment of apoptosis and cell viability

Quantitative determination of viability was performed using an annexin V-based apoptosis detection kit (R&D Systems) and subsequently analyzed by flow cytometry. Moreover, cell numbers were determined by counting cells excluding trypan blue after 72 h of culture.

Cell cycle analysis

Cells (105) were fixed in an ice-cold solution of 70% ethanol for at least 1 h and incubated with 0.1% RNase at 37°C for 45 min. Cells were then incubated with 50 μg/ml propidium iodide at 37°C for 30 min, before analysis for DNA content by flow cytometry using CellQuest software.
Flow cytometry

Cells were incubated with various PerCP-, FITC-, and PE-labeled mAbs diluted at optimal concentration for immunostaining, fixed in 1% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer. We used isotype-matched Abs as controls, and IgG block (Sigma-Aldrich) to avoid the nonspecific binding to Fc receptors. For analysis of intracellular CTLA4 and FoxP3, cells were stained first for surface CD4 and CD25 with PerCP anti-CD4 and PE anti-CD25, fixed with Cytofix/Cytoperm solution (BD Pharmingen), incubated with FITC anti-CTLA4 or FITC anti-FoxP3 mAb diluted in 0.5% saponin, and analyzed by flow cytometry. Because Tran et al. (19) recently reported that anti-FoxP3 mAb clone PCH101 could result in nonspecific staining of activated T cells, two different anti-FoxP3 mAbs were used in this study (clones eBio5A6 and 236/6E7) to confirm our results. In addition, mRNA expression of FoxP3 was determined, as described below.

For intracellular cytokine analysis, viable T cells were recovered after stimulation by gradient centrifugation and stimulated at 107 cells/ml with PMA (10 ng/ml) plus ionomycin (50 ng/ml) for 6 h. Monensin (1.33 μM) was added for the last 4 h of culture. Cells were stained with PerCP anti-CTLA4 and PE anti-CD25, fixed with Cytofix/Cytoperm solution (BD Pharmingen), incubated with FITC anti-CTLA4 or FITC anti-FoxP3 mAb diluted in 0.5% saponin, and analyzed by flow cytometry. Because foxp3 primers and probe were as follows: forward primer, 5′-GAGCGACAGGCGAGCACAAGG-3′ and 5′-CTCAATGGATGAAATTTAATAAGCGTATTCGTG-3′, PCR conditions were as follows: 94°C for 5 min, 30 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 7 min. The amplified fragments were separated on 1.5% agarose gel and visualized by ethidium bromide. RNA integrity and cDNA synthesis were verified by amplifying β2-microglobulin cDNA. The intensity of the re-vealed bands was quantified by ImageQuant software (Amersham Biosciences), normalized to those of β2-microglobulin, and expressed as arbitrary units. Human FoxP3 gene expression was quantitated by real-time PCR in triplicate according to the TaqMan Universal 2× master mix and run on the ABI/PRISM 7900 Sequence Detector System (Applied Biosystems) (20). The amount of FoxP3 mRNA expression was normalized with the β-actin and calculated according to the comparative cycle threshold method. FoxP3 primers and probe are as follows: forward primer, 5′-GGCCATCGTGCAACCTGATTCCCGC-3′, reverse primer, 5′-GGCCATCGTGCAACCTGATTCCCGC-3′, PCR conditions were as follows: 94°C for 5 min, 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 7 min. The amplified fragments were separated on 1.5% agarose gel and visualized by ethidium bromide. RNA integrity and cDNA synthesis were verified by amplifying β2-microglobulin cDNA. The intensity of the re-vealed bands was quantified by ImageQuant software (Amersham Biosciences), normalized to those of β2-microglobulin, and expressed as arbitrary units. Human FoxP3 gene expression was quantitated by real-time PCR in triplicate according to the TaqMan Universal 2× master mix and run on the ABI/PRISM 7900 Sequence Detector System (Applied Biosystems) (20).

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Cytokine determination

Cytokine contents in the culture supernatants or sera were determined by specific sandwich ELISAs using capture/biotinylated detection Abs from BD Pharmingen. Cytokine activities, whole protein extracts (500 μg/sample), and expressed as arbitrary units. To determine the Cdk2- , Cdk4-, cyclin E-, and cyclin D2-associated kinase activities, whole protein extracts (500 μg) were immunoprecipitated with 1 μg of the following Abs: anti-cdk2, anti-cyclin E, anti-cdk4, and anti-cyclin D2, for 2 h at 4°C under constant agitation. The immune complexes were collected by incubation with 10 μg of protein A/G-Sepharose beads for 45 min at 4°C under constant agitation. The beads were extensively washed with the lysis buffer, and kinase activity was assayed by incubating the immunoprecipitates 30 min at 30°C with 50 mM HEPES (pH 7.5), 10 mM MgCl2, 2.5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 0.1 mM Na3VO4, 1 mM NaF, 50 μM cold ATP, 10 μCi of [γ-32P]ATP (6000 Ci/mmol), and 5 μg of histone H1 for cdk2 or 2 μg of retinoblastoma gene product (Rb)-GST for cdk4. The phosphorylated proteins were separated by SDS-PAGE, transferred to Immobilon P membranes (Millipore), and subsequently used for Western blot analysis.

GVHD model

All experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research and approved by Consejo Superior de Investigaciones Científicas. CD4+CD25+ T cells isolated from C57BL/6 mice (H-2b) were cultured with anti-CD3 (0.5 μg/ml) in the presence of syngeneic APCs in complete medium in the absence (Tcontrol) or presence of 10−7 M VIP (TVIP) for 4 days, then extensively washed and rested in medium containing IL-2 (10 U/ml) for 3 additional days. Viable Tcontrol or TVIP (106) recovered by gradient centrifugation were injected i.v. into BALB/c mice (H-2d) that were lethally irradiated (8 Gy total body irradiation with a 200 Kv x-ray source) and reconstituted i.v. with allogeneic T-cell-depleted bone marrow cells (TCDF-BM; 5 × 106) from C57BL/6 mice. The survival and appearance of the BALB/c hosts were monitored daily, and body weight was measured weekly. In other experiments, allogeneic transplantation was performed by a single i.v. injection of TCD-BM supplemented with 1.5 × 106 spleen mononuclear cells (1.5 × 107/mouse) isolated from C57BL/6 into recipient BALB/c mice lethally irradiated (10 Gy total body irradiation from a 200 Kv x-ray source). Two hours after transplantation, recipients received a single i.v. injection of CD4+ T cells (106) from C57BL/6 mice that were stimulated in a primary MLC with allogeneic spleen cells from BALB/c mice in the absence or presence of 10−7 M VIP for 6 days, harvested, and rested for an additional 2 days. Recipients were monitored from the day of transplantation until they succumbed naturally to GVHD. Serum and spleen cells were harvested on 5 days following transplantation, and splenic donor H-2KbCD4+ and H-2KbCD8+ T cells were isolated by immunomagnetic selection, as described (22). Donor H-2KbCD4+ cells (5 × 104) were stimulated with allogeneic splenic APCs (H-2b, 5 × 105), and the proliferative response was determined by [3H]thymidine incorporation and intracellular cytokine expression by flow cytometry, as described above. Cytokine contents in serum were determined by ELISA, as above. The percentages of CD4+, CD25+, and CTLA4+ T cells in the spleen of recipient mice were determined by flow cytometry, as described above.

Transgenic mice

TCR-Cyt-ChCC7-IRag1 transgenic (I-Eβ) mice (PCCF TCR transgenic) were obtained from Taconic Farms. PCCF TCR transgenic mice were injected i.p. on days 0 and +2 with Ag (PCCF, 50 μg) and with or without VIP (5 nmol). At different times after initial Ag stimulation, spleen and lymph nodes (inguinal, mesenteric, and popliteal) were isolated and analyzed from C57/BL6, CD25, or C57/BL6-CD25− mice by flow cytometry. In some experiments, CD25 cells were depleted (in vivo depletion >98%) from PCCF TCR transgenic mice by treating mice i.v. with anti-CD25 Ab (clone 29Cl1, 1 mg) 3 days before Ag stimulation.

Data analysis

All values are expressed as mean ± SD. Differences in survival of treatment groups were analyzed using the log-rank test. Differences in proliferation and cytokine production by cultures, serum cytokine levels, and percentage of cells were analyzed using two-tailed Student’s t test. Values of p < 0.05 were considered significant.

Results

VIP inhibits proliferation and induces cell cycle arrest in allogeneic activated human T cells

Anergy, the in vitro counterpart of tolerance in vivo, is defined as the inability of T cells to expand after stimulation with fully competent APCs delivering TCR and costimulatory molecules (23). To investigate the capacity of VIP to induce anergic human T cells, freshly isolated PBMCs were stimulated with γ-irradiated HLA-mismatched stimulator PBMCs in the absence or presence of VIP,
and the incorporation of $[^3H]$thymidine to DNA was measured to estimate entry into S phase and cell proliferation. VIP inhibited the proliferative response of PBMCs activated in primary MLCs in a dose-dependent manner (Fig. 1A). The suppressive action of VIP on clonal expansion was maintained for a prolonged period of time (Fig. 1B), and was not due to an effect on survival or apoptosis (Fig. 1C), indicating that VIP did not promote deletion of the alloreactive effector T cell pool. Cell cycle analysis showed that VIP strongly reduced the number of T cells in S phase induced by alloantigenic stimulation and prevented the decrease of cells in $G_0/G_1$ observed after stimulation (Fig. 1D), suggesting that the antiproliferative action of VIP is due to a sustained blocking in the transition from $G_0/G_1$ to $S$ phase.

We next investigated whether VIP also affected the potential of alloreactive T cells to produce effector cytokines by determining the intracellular cytokine staining in CD4$^+$ T cells. Whereas alloantigen-primed control T cells showed increased production of the effector cytokines IL-2, IL-4, IFN-$\gamma$, and TNF-$\alpha$, treatment with VIP resulted in a substantial reduction in the number of CD4$^+$ T cells producing all these cytokines (Fig. 1E). In contrast, VIP increased the number of IL-10- and TGF-$\beta$1-producing CD4$^+$ T cells (Fig. 1E).

VIP generates anergic T cells with suppressive functions on alloantigen-stimulated cells

Once we confirmed the immunosuppressive activity of VIP on alloantigen-specific T cell responses, we investigated its effect on the induction of functional memory T cells typical of allogenic activation. To address this issue, alloantigen-primed PBMCs in the presence of VIP were restimulated with alloantigen in a secondary MLC in the absence of the neuropeptide. Restimulation of primed T cells with alloantigen resulted in the induction of functional memory T cells, characterized by a rapid proliferative response after the rechallenge (Fig. 2A). However, the cells that were primed in the presence of VIP did not proliferate in response to an alloantigen in a secondary MLC (Fig. 2A). These results indicate that VIP induces anergic T cells. In agreement with this finding, T cells primed in the presence of VIP expressed significantly lower levels of the effector cytokines IL-2, IL-4, IFN-$\gamma$, and TNF-$\alpha$ upon restimulation (data not shown).

Previous studies have shown that T cell anergy is closely related to a Treg phenotype (21, 24–33). Therefore, we examined whether the anergic T cells induced upon VIP treatment were able to exert suppressor activity on effector T cells. PBMCs allogenically primed in the absence ($T_{control}$) or presence of VIP ($T_{VIP}$) were added at different ratios to a new set of primary MLCs consisting of responder PBMCs and allogeneic stimulator PBMCs. Whereas $T_{control}$ significantly contributed to the proliferative response of responder T cells, $T_{VIP}$ dose dependently suppressed their clonal expansion (Fig. 2B). This suggests that VIP treatment during primary allostimulation of T cells induced anergic CD4$^+$ T cells that
possessed regulatory capacities. Moreover, because the VIP-indu
ced suppressive activity on allostimulated T cells seems to be
very efficient (observed at a 1:8 suppressive:effector ratio), VIP
could generate enough suppressive cells to significantly contribute
to the anergic state observed on these cells, especially upon re-
stimulation (Fig. 2A).

VIP induces the emergence of human CD4⁺CD25high Tregs
from the CD4⁺CD25⁻ T cell compartment

Because PBMCs comprise both CD4⁺CD25⁻ alloreactive T cells
and naturally occurring CD4⁺CD25⁺ Tregs, VIP could induce the
emergence of suppressive T cell effects by inducing regulatory
functions within the CD4⁺CD25⁻ T cell population, or by simply
reducing the alloreactive effector T cell pool vs Tregs, thereby
favoring the activity of the latter. To address this question, we used
CD4⁺CD25⁻ T cells during alloreactive priming. We observed
identical results when CD4⁺CD25⁻ cells isolated by either im-
munomagnetic separation or flow cytometry cell sorting were used
in the assays. CD4⁺CD25⁻ T cells primed in the presence of VIP
showed hyporesponsiveness upon secondary restimulation (data
not shown) and inhibited, in a dose-dependent manner, the proli-
erative response and the potential to secrete IFN-γ of alloreactive
effector T cells (Fig. 3A). These results were confirmed in cocul-
tures using CFSE-labeled responder T cells (data not shown).
These findings indicate that the induction of regulatory activity by
VIP occurs within the CD4⁺CD25⁻ T cell fraction, independently
of the presence and expansion of naturally occurring CD4⁺CD25⁺
Tregs. Separation of TVIP from responder T cells by a semiper-
membrable membrane in Transwells significantly reversed the sup-
pressive effect of TVIP on alloantigen reactions (Fig. 3B), suggest-
ing a cell-to-cell contact-dependent mechanism. Previous studies
have shown that the membrane-bound molecule CTLA4 plays a
major role in the cell contact-dependent suppressive activity of
Tregs (2, 3, 5). In agreement with the cell-to-cell contact-depend-
ent effect observed, neutralizing Abs for CTLA4, but not for
IL-10 and TGF-β1, fully reversed the regulatory action of TVIP
on allostimulated responder T cells (Fig. 3C). As expected, the addi-
tion of IL-2 to cocultures bypassed their suppressor activity (Fig.
3C). Interestingly, TVIP suppressed the proliferation of third party
stimulated PBMCs and T cells, but only in the presence of the
original allogeneic PBMCs or APCs used in the priming of TVIP
(Fig. 3D), suggesting that activation of TVIP with the correspond-
ing alloantigen is a requisite for their suppressive activity.

Flow cytometry analysis of the CD4⁺ T cells generated from
allogeneic primed CD4⁺CD25⁻ T cells in the presence of VIP
revealed three different cell subsets based on the level of CD25
expression (CD25negative, CD25int, and CD25high) (Fig. 4A). We
subsequently sorted the three populations and examined the ex-
pression of FoxP3 and CTLA4 as markers of Tregs and their func-
tional activities. An elevated percentage of the CD25high popu-
lation generated with VIP expressed FoxP3 and CTLA4 high,i n
comparison with the CD25high cells purified from the untreated
controls (Fig. 4A). This was due to an increase in the absolute
number of both CD4⁺FoxP3⁺ and CD4⁺CTLA4⁺ T cell subsets,
but not to an enrichment of these cells due to a decrease in the
number of effector cells. Again, this indicates the new generation
of Tregs by VIP rather than a mere enrichment of existing cells. In
addition, VIP-induced CD4⁺CD25high T cells showed an effector-
memory phenotype characterized by expression of CD45RO, but
not CD62L and CD27 (data not shown), making these cells suit-
able to act at a site of inflammation.

The CD25negative population mainly compromised ignorant al-
loantigen-unspecific CD4⁺ T cells because they did not respond to
alloantigenic restimulation in the presence of IL-2, whereas the
VIP-induced CD25<sup>int</sup> population slightly proliferated in response to allogeneic stimulation. A, Human CD4<sup>+</sup>CD25<sup>-</sup> T cells isolated from donor A were stimulated in a primary MLC with allogeneic PBMCs from donor B in the absence (T<sub>control</sub>) or presence of VIP (T<sub>VIP</sub>) for 6 days. After the 2-day resting period, increased numbers of the recovered T<sub>control</sub> or T<sub>VIP</sub> were added to a coculture of responder T cells (10<sup>5</sup> from donor B) and allogeneic stimulator PBMCs (10<sup>5</sup> from donor C). Proliferation and IFN-γ production by responder T cells were determined after 4 days of coculture. PBMCs (10<sup>5</sup> from donor B) cultured with syngeneic PBMCs (10<sup>5</sup> from donor B) were used as controls of the background stimulation. *p < 0.001 vs medium or T<sub>control</sub>, #p < 0.001. B, T<sub>VIP</sub> (2 x 10<sup>4</sup> from donor A) were added to a coculture of responder T cells (5 x 10<sup>4</sup> from donor B) and allogeneic stimulator PBMCs (5 x 10<sup>4</sup> from donor C). When indicated, T<sub>VIP</sub> were separated from responder-stimulator PBMC cocultures by a semipermeable membrane in Transwells. Proliferation and IFN-γ production were determined in the lower compartment of the Transwells. *p < 0.001. C, Recovered T<sub>control</sub> or T<sub>VIP</sub> (2 x 10<sup>4</sup>) were added to a coculture of responder T cells (5 x 10<sup>4</sup> from donor B) and allogeneic stimulator PBMCs (5 x 10<sup>4</sup> from donor C) in the presence of IL-2, anti-TGF-β1, anti-CTLA4, or anti-IL-10-blocking Abs, or an isotype control Ab. Proliferation and IFN-γ production by responder T cells were determined after 4 days. PBMCs (10<sup>5</sup> from donor B) cultured with syngeneic PBMCs (10<sup>5</sup> from donor B) were used as controls of the background stimulation (dotted lines). *p < 0.001 vs medium. #p < 0.01 vs T<sub>VIP</sub>. D, T<sub>VIP</sub> (5 x 10<sup>4</sup> from donor A generated in MLC with PBMCs from donor B) were added to a coculture of responder PBMCs (10<sup>5</sup> from donor C) and allogeneic stimulator PBMCs (10<sup>5</sup> from donor B or donor D). Alternatively, T cell-depleted APCs (5 x 10<sup>4</sup> from donor B or donor C) were used as allogeneic stimulators for isolated T cells (10<sup>5</sup> from donor B or donor C). When indicated, PBMCs and T cells from donors B and C were CFSE labeled before their addition to coculture. After 96-h culture, total number of cycling cells (percentage of CFSE-positive cells that had divided the total number) was determined. n = 3–4 experiments performed in duplicate. *p < 0.05; **p < 0.001.

VIP-induced CD25<sup>int</sup> population slightly proliferated in response to allogeneic PBMCs and the CD25<sup>high</sup> subset remained anergic (data not shown). The administration of the VIP-induced CD25<sup>high</sup> cells to allogeneic responder-stimulator PBMC cocultures efficiently inhibited the proliferation of the responder T cells (Fig. 4A). However, the CD25<sup>int</sup> population induced by VIP showed weak suppressive activity, whereas it showed increased expression of CTLA4 and moderate FoxP3 (Fig. 4A). The increase of CTLA4 expression by VIP was rapid, sustained on time, and correlated with the suppressive activity of T<sub>VIP</sub> (Fig. 4B). CTLA4 was previously involved in the induction of FoxP3 expression and in the generation and function of Tregs (34). By using anti-CTLA4 Abs, we demonstrated that CTLA4 is needed for VIP to induce FoxP3 in allogeneic-activated CD4<sup>+</sup>CD25<sup>-</sup> T cells (Fig. 4C) and to generate T cells with suppressive activity (Fig. 4D). However, neutralization of TGF-β1, a well-known factor involved in the induction of Tregs (21), did not significantly affect VIP-induced FoxP3 expression (Fig. 4C), CD4<sup>+</sup>CD25<sup>+</sup> Treg generation (data not shown), or T cell suppression (Fig. 4D).

To further investigate whether VIP induced de novo Tregs, CD4<sup>+</sup>CD25<sup>-</sup> cells were CFSE labeled and stimulated with allogeneic PBMCs in the absence or presence of VIP, and the expression of FoxP3 and CD25 was analyzed in the CFSE<sup>high</sup> (nondividing cells) and CFSE<sup>mid</sup> (cycling cells) cells. VIP treatment resulted in low numbers of cycling cells and confined FoxP3 and CD25<sup>high</sup> expression to the CFSE<sup>high</sup> nondividing CD4 cells.
VIP-PROMOTED IMMUNE TOLERANCE TOWARD HUMAN ALLOANTIGENS

CD4+ CD25high Tregs that express CTLA4 and FoxP3 from the CD4+ CD25– T cell repertory. A. Human CD4+ CD25– T cells were stimulated in a primary MLC with allogeneic PBMCs in the absence (control) or presence of VIP, and after 5 days, CD25 expression was analyzed by flow cytometry in the CD4+ T cell fraction. The subsets formed (CD25low, CD25high, and CD25+Ag) were sorted and analyzed for FoxP3 (results with clone PCH101 are shown, and were confirmed with clone 236A) and CTLA4 expression. Expression of FoxP3 and CD25 in the starting population (t = 0) is shown. Numbers in dot plots represent the percentage of positive cells for each marker in each subset. Sorted cells were restimulated at different cell ratios with allogeneic PBMCs (5 × 10^5 from donor B) and allogeneic stimulator PBMCs (2 × 10^5 from donor C). After 96-h culture, total number of cycling cells (percentage of CFSE-positive cells that had divided the total number) was determined. At different times after primary MLC, CTLA4 protein expression was determined by flow cytometry in gated CD4+ T cells and expressed as the mean fluorescence intensity (MFI), and mRNA expression of CTLA4 was determined by quantitative PCR and expressed as arbitrary units per 10^6 cells. Unstimulated CD4+ CD25– and CD4+ CD25+ T cells were used as negative and positive controls, respectively. n = 3 experiments performed in duplicate. *, p < 0.001 vs control. C. Purified CD4+ CD25– T cells were stimulated in a primary MLC with allogeneic PBMCs in the absence (none) or presence of VIP, with or without anti-CTLA4 (10 μg/ml), anti-TGF–β1, or control IgG (10 μg/ml). After 4 days, FoxP3 expression was quantitated by real-time PCR and normalized to the β-actin gene. Unstimulated CD4+ CD25– and CD4+ CD25+ T cells were used as negative and positive controls, respectively. n = 3 experiments performed in duplicate. *, p < 0.001.

B. Human CD4+ CD25– T cells isolated from donor A were stimulated in a primary MLC with allogeneic PBMCs from donor B in the absence (Tcontrol) or presence of VIP (Tvip), with or without anti-CTLA4 (10 μg/ml), anti-TGF–β1, or control IgG (10 μg/ml) for 6 days. After the 2-day resting period, the recovered Tcontrol or Tvip (4 × 10^5) were added to a coculture of responder CFSE-labeled PBMCs (2 × 10^5 from donor B) and allogeneic stimulator PBMCs (2 × 10^5 from donor C). After 96-h culture, total number of cycling cells (percentage of CFSE-positive cells that had divided the total number) was determined. CFSE-labeled PBMCs (2 × 10^5 from donor B) cultured with syngeneic PBMCs (2 × 10^5 from donor B) were used as controls of the background stimulation (first bar). n = 3 experiments performed in duplicate. *, p < 0.001 vs medium or Tcontrol. #, p < 0.001 vs Tvip.

VIP converts mouse CD4+ CD25+ T cells on CD4+ CD25– FoxP3+ T cells with regulatory functions

To confirm these results in a mouse model, we used PCCF TCR transgenic Rag–/– mice that were injected with the corresponding Ag (PCCF) in the absence or presence of VIP. The administration of VIP induced the emergence of CD4+ CD25+ FoxP3+ cells in the spleen (Fig. 6A) and lymph nodes (data not shown), even when the mice were previously depleted of CD25+ T cells (Fig. 6A). This suggests that VIP could convert CD4+ CD25+ cells on CD4+ CD25– FoxP3+ T cells in vivo. This was confirmed in vitro starting with CD4+ CD25+ T cells isolated from the Rag–/– mice, which is a bona fide FoxP3+ population (Fig. 6B). As expected, stimulation with anti-CD3 and APCs led to transient increase in the percentages of CD4+ CD25+ and CD4+ CTLA4+ cells in the first 48-h culture, which declined later (Fig. 6B). Treatment with VIP maintained the elevated percentages of CD4+ CD25+ and CD4+ CTLA4+ cells long time and significantly increased the
percentage of CD4⁺FoxP³ cells (Fig. 6B). We observed similar results by using CD4⁺CD25⁻ cells isolated from nontransgenic C57BL/6 mice (Fig. 6C). In addition, VIP increased the mRNA expression of FoxP³ in the CD4⁺CD25⁺ cell population, but it failed to significantly increase FoxP³ in isolated CD4⁺CD25⁻ T cells stimulated with IL-2 plus IL-15 were used as positive controls of cell expansion. After 4 days, FoxP³ expression was quantitated by real-time PCR and normalized to the β-actin gene, and the number of FoxP³⁺ cells was determined by flow cytometry. The percentage of CD25⁺ cells in the CFSEmild/low cycling cells was determined by flow cytometry. 

**Protective effect of VIP-tolerant CD4⁺ suppressor cells in acute GvHD**

Based on these results, we investigated the potential therapeutic effects of VIP-treated T cells generated in an alloantigen-driven system in a model of acute GvHD following allogeneic BMT. The injection of activated CD4⁺CD25⁺ T cells (T_control) from C57BL/6 mice (H²b) to irradiated BALB/c mice (H²d) transplanted with allogeneic TCD-BM caused severe signs of GvHD in all of the animals, including weight loss, reduced mobility, hunched posture, diarrhea, ruffled fur, and death within 30 days, in comparison with mice receiving only TCD-BM (Fig. 7A). However, only 20% of the animals injected with allogeneic C57BL/6 CD4⁺CD25⁻ T cells stimulated in the presence of VIP (T_vip) died as a consequence of acute GvHD (Fig. 7A), reflecting the hyporesponsiveness of the T_vip in vivo. More importantly, H2b CD4⁺CD25⁻ T cells primed with allogeneic H2d spleen cells in the presence of VIP (T_vip) significantly prevented the mortality that occurred as a consequence of the GvHD in animals receiving allogeneic BMT (Fig. 7B). This suggests that T_vip could impair allogeneic Ag-specific responses of donor CD4 T cells in mice that have received transplants. This suppressive activity belongs to the CD4⁺CD25⁺ population, because when the T_vip were fractionated as CD25⁻ and CD25⁺ populations before transfer, only the CD4⁺CD25⁺ fraction mimicked the protective effect of T_vip on GvHD (Fig. 7C).

A hallmark of acute GvHD is the expansion of alloreactive T cells. Disease progression is characterized by the differentiation of alloreactive CD4⁺ and CD8⁺ T cells into effector cells, leading to tissue damage, recruitment of additional inflammatory cells, and further cytokine unbalance (7, 8, 35). We therefore investigated...
whether T\textsubscript{VIP} regulated the differentiation of GvHD-causing alloreactive T-effector cells in the grafted mice. We examined the subpopulations of transplanted H2-K\textsuperscript{b} T cells and their ability to produce cytokines in T\textsubscript{control}-or T\textsubscript{VIP}-treated recipients (H-2\textsuperscript{b}). Inoculation of T\textsubscript{VIP} decreased the number of H2-K\textsuperscript{b}CD4\textsuperscript{+} and H2-K\textsuperscript{b}CD8\textsuperscript{+} donor-derived T cells (Fig. 7D) and reduced the percentage of activated IFN-\gamma, IL-17, and IL-2-producing CD154\textsuperscript{+} (CD40L) T cells in the H2-K\textsuperscript{b}CD4\textsuperscript{+} T cell population (Fig. 7E). At the same time, T\textsubscript{VIP} increased the percentage of CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} and CD4\textsuperscript{+}CTLA4\textsuperscript{+} T cells in spleen of the recipients (Fig. 7F). H2-K\textsuperscript{b}CD4\textsuperscript{+} T cells obtained from untreated or T\textsubscript{control}-treated mice that had received transplants responded vigorously to allogeneic PBMCs (H-2\textsuperscript{b}), whereas H2-K\textsuperscript{b}CD4\textsuperscript{+} T cells from T\textsubscript{VIP}-treated recipients were hyporesponsive (Fig. 7G). T\textsubscript{VIP} treatment also reduced the levels of the proinflammatory cytokine TNF-\alpha in the serum of grafted mice (Fig. 7H). These data indicate that the treatment of mice given transplants with VIP-tolerant T\textsubscript{VIP} reduced the number/activation of transplanted Th1, Th17, and CD8; the inflammatory response against the recipient tissue; and the subsequent GvHD lethality, while increasing the number of CD25\textsuperscript{+}FoxP3\textsuperscript{+} Tregs in the host.

VIP-tolerant T cells are arrested in G\textsubscript{1} phase and fail to progress through S phase of the cell cycle

Because these findings have potential clinical significance and applicability in therapeutic approaches, we sought to determine the molecular basis of VIP-induced hyporesponsiveness. This information should give relevant insights not only into the anergy-evoked mechanisms, but into the improvement of patient-oriented research. It has been previously shown that alloantigen-specific human T cells rendered anergic by lack of costimulation through CD28 are arrested at the G1 phase of the cell cycle (24). Moreover, alloreactive mouse T cell clones that have been rendered tolerant by CD40 blockade or TGF-\beta1/IL-10 treatment and do not induce GvHD in vivo fail to progress into the late G\textsubscript{1} phase and to enter the S phase of the cell cycle (21, 24, 25, 36). Our findings indicate that VIP-tolerant T cells are cell cycle arrested (Fig. 1), and do not proliferate upon restimulation with anti-CD3/CD28-coated beads (Fig. 2). These results indicate that VIP must regulate the signals controlling cell cycle progression. TCR/CD3 plus CD28 costimulation regulates the entry of T cells into the cell cycle and progression from G\textsubscript{1} to S phase by up-regulating D-type cyclins and cyclin E (23, 37). Complexes formed between cyclin D2 and cdk4 or cdk6, and between cyclin E and cdk2 are involved in hyperphosphorylation of Rb, which ultimately leads to activation of E2F transcription factor, expression of S phase genes, and cell cycle progression (23). Analysis of the cell cycle regulatory molecules showed that VIP-tolerated T cells were capable of expressing cyclin D2 and the cyclin D-associated cdk4 and cdk6 upon CD3/CD28 restimulation, indicating that they could enter in the G\textsubscript{1} phase (Fig. 8A). However, progression through the G\textsubscript{1} restriction point to the late G\textsubscript{1} and S phases was impaired in the VIP-tolerant T cells. VIP-tolerant T cells showed sustained decrease in the expression of cyclin D3, which is synthesized in the late G\textsubscript{1} phase, and cyclin E, which is expressed in the G\textsubscript{1} restriction point (Fig. 8A). Consequently, the levels of the S phase cyclin A and hyperphosphorylated Rb were dramatically reduced in VIP-tolerant T cells in comparison with primed T cells (Fig. 8A). These results show that VIP-tolerant T cells enter in G\textsubscript{1}, but fail to progress into the late G\textsubscript{1} and enter the S phase of the cycle.

**FIGURE 6.** VIP converts mouse CD4\textsuperscript{+}CD25\textsuperscript{+} T cells on CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} T cells with regulatory functions. A, PCCF transgenic Rag\textsuperscript{−/−} mice (6 mice per group) were injected i.p. on days 0 and 2 with Ag (PCCF, 50 μg) and with or without VIP (5 nmol). When indicated, mice were depleted on CD25 cells before immunization with the Ag. Spleen were collected at the indicated days after initial Ag stimulation and analyzed for CD25, FoxP3, and the transgenic TCR using the clonotypic mAb KJ25. Percentage and total numbers of splenic CD25\textsuperscript{+}FoxP3\textsuperscript{+} transgenic T cells were determined by flow cytometry. B, CD4\textsuperscript{+}CD25\textsuperscript{−} T cells isolated from PCCF transgenic Rag\textsuperscript{−/−} mice were cultured with anti-CD3 and APCs in the absence or presence of 10\textsuperscript{−7} M VIP for 4–5 days and analyzed for the percentages of CD25\textsuperscript{+}, FoxP3\textsuperscript{+}, and CTLA4\textsuperscript{+} T cells. n = 3 experiments performed in duplicate. C, CD4\textsuperscript{+}CD25\textsuperscript{−} and CD4\textsuperscript{+}CD25\textsuperscript{+} T cells isolated from C57BL/6 mice were cultured with anti-CD3 and APCs in the absence or presence of 10\textsuperscript{−7} M VIP for 4–5 days and analyzed for the percentages of FoxP3\textsuperscript{+} and CD4\textsuperscript{+} CTLA4\textsuperscript{+} T cells. The expression of FoxP3 was determined by real-time RT-PCR and normalized using hypoxanthine phosphoribosyltransferase expression. Unstimulated cells were used as basal controls. n = 3–4 experiments. D, CD4\textsuperscript{+}CD25\textsuperscript{−} T cells isolated from C57BL/6 mice were cultured with anti-CD3 and APCs in the absence or presence of 10\textsuperscript{−7} M VIP (T\textsubscript{control}) for 4–5 days. Viable T cells were stained with FITC anti-CD25 mAb, and the resultant CD25\textsuperscript{−} T cells before they were added to responder T cells, and showed the following percentage of FoxP3\textsuperscript{+} cells: <0.4% in CD25\textsuperscript{−} T\textsubscript{control}, 2.3% in CD25\textsuperscript{+} T\textsubscript{control}, 1.1% in CD25\textsuperscript{−} T\textsubscript{VIP}, and 24% in CD25\textsuperscript{+} T\textsubscript{VIP}. n = 3 experiments performed in duplicate.

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VIP treatment prevents down-regulation of p27kip1 and results in defective activation of cdks

We next investigated whether the effect observed for VIP in the cell cycle progression was accompanied by impaired activation of cdk2 and cdk4. VIP-tolerant T cells showed decreased levels of both cyclin D2-associated cdk4 and cyclin E-associated cdk2 kinase activities, in comparison with activated primed T cells (Fig. 8B). These results confirm and extend the observation that VIP affects the function of the molecular players involved in the G1 phase, thereby preventing the progression to the S phase.

Besides cyclins and cdks, several cdk inhibitors also play a prominent role in the regulation of the G1 phase. Because defective cdk4 and cdk2 kinase activities were observed in VIP-treated T cells, we investigated the possible regulation of cdk inhibitors by VIP. In T cells, the major inhibitory proteins of cdk4 and cdk2 activities are the members of the cip/kip (p21cip1, p27kip1, and p57kip2) family (37–42). As previously described (23), activation of control primed T cells down-regulated p27kip1 (Fig. 8C). In contrast, VIP treatment not only prevented the degradation of p27kip1, but increased its levels over the background expression found in unstimulated cells (Fig. 8C).

VIP induces tolerant T cells by elevating cAMP levels

The immunological actions of VIP are exerted through a family of receptors, consisting of VPAC1 and type 2 VPAC (10, 13, 43), coupled to adenylate cyclase and the elevation of intracellular cAMP levels and subsequent protein kinase A (PKA) activation. To determine whether the cAMP/PKA pathway mediates the suppressive actions for VIP on human T cells described in this work,
we assayed the effects of a VPAC₁ antagonist, H89 (a PKA inhibitor), and 8-Br-cAMP (a cell-permeable cAMP analog). The effects of VIP on the increase of FoxP3 and CTLA4 expression (Fig. 9A), the induction of T cell anergy (Fig. 9B), the generation of T cell-suppressive activity (Fig. 9C), and the modulation of cyclin levels, cdk4 kinase activity, and expression of p27kip1 (Fig. 9D) were reversed by the PKA inhibitor and the VPAC₁ antagonist. In agreement with this, 8-Br-cAMP mimicked the effects of VIP in these events (Fig. 9). These findings indicate that the regulatory effect of VIP on T cells is mainly mediated by its binding to VPAC₁ and the subsequent increase of intracellular cAMP and PKA activation.

Discussion

Cellular therapy with in vitro induced/expanded Tregs is considered a feasible approach to modulate effector T cells responsible for causing pathology in autoimmune diseases, allergies, allograft rejection or GvHD, and inflammatory diseases (44, 45). The ability to translate preclinical studies with Tregs into the clinic requires an increasing effort to identify immune factors that regulate the tolerance/anergy state mediated by this cell population (45, 46). In this study, we have investigated the potential of the anti-inflammatory neuropeptide VIP to promote immune tolerance toward alloantigens. We focused on the effects of VIP on CD4⁺ T cells given the fact that these cells play an established role in allograft rejection as well as in immune regulation. Our data show that VIP treatment of human CD4⁺CD25⁻ T cells during in vitro stimulation induces an anergic CD4⁺CD25high T cell subset with regulatory activity. The phenotype of the Tregs induced by VIP was further characterized by sustained expression of FoxP3 and CTLA4, both markers being associated with regulatory activity of T cells (2, 3, 5, 47), and other markers characteristic of an effector-memory phenotype. Although the mechanisms involved in the generation of this Treg population are not fully understood, our data indicate that VIP directly programs the CD4⁺CD25⁻ T cell repertory toward a regulatory phenotype in the absence of naturally occurring CD4⁺CD25⁺ Tregs. Some in vivo evidence supports this hypothesis. VIP administration prevented disease progression in CD25-depleted mice with experimental autoimmune encephalomyelitis and arthritis by inducing the new emergence of
peripheral CD4^+CD25^+ Tregs (16, 17). Similarly, in this study we show that VIP injection in TCR transgenic mice increased the numbers of Ag-specific CD25^+FoxP3^+ T cells in lymphoid organs, even in the absence of CD25^+ T cells. Moreover, in vitro data show that VIP failed to expand isolated CD4^+CD25^+ T cells and to increase FoxP3 expression in these cells. Whether in our system the effect of VIP is uniform on all T cells, or only on a subset that differentiates into FoxP3^+CD4^+CD25^+ Tregs and subsequently mediates anergy induction to the remaining T cells, needs to be further analyzed. In this sense, it has been described that certain CD25^+ regulatory cells that reside within the total CD4^+CD25^- T cell repertoire up-regulate CD25 and FoxP3 upon TCR triggering (48, 49). VIP could simply facilitate the expansion of this Treg-committed CD4^+CD25^+ T cell population. Our results show that VIP promotes the expression of CD25^{high} and FoxP3 in the nondividing CD4^+CD25^- cells, supporting the hypothesis of de novo conversion of Tregs from CD4^+CD25^- cells.

Although VIP-induced Tregs produce IL-10 and some TGF-β1 upon restimulation, none of these cytokines seem to play a major role in their suppressive action. In this sense, the VIP-tolerated T cells clearly differ from the Tr1 cells generated in the presence of IL-10 (50, 51), which suppress T cell activation through the soluble factors IL-10 and TGF-β1. However, a cell-to-cell contact-dependent mechanism mediated by the suppressive molecule CTLA4 seems to be critically involved in the effect of VIP-tolerant T cells. CTLA4 has been widely identified as a membrane-bound molecule with potent immunosuppressive effects that acts directly on T cells and indirectly on APCs interfering with the costimulatory signaling (2, 3, 5). Most importantly, a rapid and sustained induction of CTLA4 by VIP seems to be involved in the generation of Tregs by the neuropeptide. Induction of CTLA4 seems to be a prerequisite for VIP to generate CD4^+FoxP3^- T cells with regulatory functions. Zheng et al. (34) also demonstrated that TGF-β requires CTLA4 early after activation to induce FoxP3 and generate adaptive mouse CD4^+CD25^- Tregs. Considerable controversy exists regarding the regulation of FoxP3 expression in human T cells, and some studies have suggested that TCR stimulation alone is sufficient to induce FoxP3 expression, at least transiently, and that the TGF-β produced by activated T cells and the TGF-β present in the serum are critically involved in such induction (19, 49, 52, 53). FoxP3 expression could easily be induced in most naive T cells by the addition of exogenous TGF-β. However, in contrast to mouse CD4^+CD25^- naive T cells that are converted by TGF-β to CD4^-CD25^- Tregs with suppressive activities, the human FoxP3^- T cells induced with TGF-β in a single round of stimulation were neither anergic nor suppressive (19, 21, 49, 52, 53). Our data show that the VIP-tolerant CD4^+CD25^- FoxP3^+ T cells are anergic and suppressive in both mouse and human systems. Whether FoxP3 induction is critical in the generation of VIP-tolerant T cells and in their suppressive function remains unknown, but we know that both generation and function are mostly TGF-β independent and CTLA4 dependent. In contrast, a secondary mechanism that could contribute to the suppressive activity of the VIP-tolerant T cells is the consumption of IL-2 produced by the naive responders in the MLRs through the high levels of CD25 expressed by these cells.

Cell cycle arrest and anergy seem to be critically related to the generation of Tregs (21, 24–33). The VIP-tolerant T cells share a number of biochemical characteristics with anergic T cells generated following other approaches, including the blockade of CD28 costimulation or CD40L/CD40 interactions, the treatment with IL-10 and TGF-β1, or the treatment with synthetic immunosuppressive agents typically used in transplantation (21, 24–26, 36). These common biochemical analyses may provide a powerful tool in quantifying the degree of tolerance induction in individual patients who receive T cells tolerized by different strategies. Our results and the aforementioned works indicate that one of the most critical events that occur during induction of T cell anergy following allostimulation is the alteration in the control of the expression and activation of regulatory molecules of the cell cycle. The association of cyclins with specific cdks leads to activation of holoenzymes that regulate the progression through the different phases of the cell cycle. Our data indicate that VIP-tolerant T cells are capable of entering the G1 phase, but do not progress through the G1 restriction point to the late G1 and S phases. In agreement with this, both cyclin D-cdk4 and cyclinE-cdk2 activities are impaired in VIP-tolerant T cells, due to a reduced expression of cyclins D3 and E and up-regulation of the cdk inhibitor p27^{kip1}. Previous studies have shown that the cell cycle inhibitor p27^{kip1} acts during the late G1 phase by binding and inhibiting cdk2-cyclin E/A complexes (41). Upon stimulation, T cells can only progress through the cell cycle when p27^{kip1} is dissociated from the cdk2-cyclin E/A complexes. This is in general achieved by ubiquitination and degradation of p27^{kip1}, which is preceded by phosphorylation of p27^{kip1} (41, 42). Indeed, anergic and tolerant T cells are characterized by impaired degradation of p27^{kip1} and other tolerance strategies pointed out to p27^{kip1} as a critical target of anergy (24–26). The increase of p27^{kip1} by VIP could be due to impaired p27^{kip1} degradation and/or to increased p27^{kip1} synthesis. Recent results from our laboratory indicate that VIP prevents CD3/CD28-induced p27^{kip1} phosphorylation while increasing total p27^{kip1} levels (our unpublished observations). Various pathways are involved in the phosphorylation of p27^{kip1}, including our own formation of cdk2/cyclin E enzymatic complex and the activation of the Ras-MAPK and PI3K-Akt pathways (41, 42, 54, 55). Our data demonstrate that VIP decreases the expression of cyclin E, and another recent study demonstrated the inhibition of the Ras-ERK1/2 and PI3K-Akt pathways by VIP in human activated T cells (our unpublished observations). These mechanisms are most likely responsible for the inability of the VIP-tolerant T cells to downregulate p27^{kip1}.

Our results show that VIP's tolerizing effect on T cells is mediated by its binding to the VPAC1 receptor and subsequent increase in the intracellular levels of cAMP and activation of PKA. Indeed, some of the effects described for VIP in this study had previously been mimicked by other CAMP-inducing agents, e.g., T cell cycle arrest, up-regulation of p27^{kip1}, and increase of CTLA4 and FoxP3 expression (20, 25, 56–60). Moreover, Gavin et al. (61) described that the suppressive action of Tregs correlated with elevated intracellular cAMP levels, as a consequence of a diminished expression of phosphodiesterase 3 (an enzyme that hydrolyzes cAMP) in Tregs. The findings we report in this study have important therapeutic implications. Due to its peptidic nature, VIP is very unstable and possesses a very short t_{1/2} time in vivo. Therefore, its potential use as an immunosuppressive drug in clinical transplantation may be limited. The identification of VPAC1 as a potential target for the screening of more stable nonpeptidic agonists by drug discovery programs should overcome this limitation and extend the therapeutic use of VIP-based drugs in clinical transplantation.

It is worth noting that the strategies described in this work generate Tregs with direct alloantigen specificity. These cells may be of particular benefit for patients receiving an HLA-mismatched stem cell graft, in which alloantigen reactivity is important in GVHD. Although no effects have to date been reported, some clinical trials on Treg immunotherapy have been recently initiated on bone marrow-transplanted patients by infusing either CliniMACS-isolated CD4^-CD25^{high} Tregs or ex vivo manipulated CD4^+ T cells.
cell lines containing induced regulatory T regulatory (Treg) cells (44, 62). Our study indicates that the infusion of VIP-tolerant T cells together with the graft significantly reduces the clinical signs and mortality rate typical of the acute GvHD response in mice reconstituted with alllogeneic hematopoietic stem cells. These effects are mediated by impairing alllogeneic haplotype-specific responses of donor CD4+ cells in the transplanted animals. Therefore, the inclusion of VIP-generated alloantigen-specific Tregs ex vivo in future therapeutic regimens may be a valuable aid in the applicability of BMT to minimize the dependence on nonspecific immunosuppressive drugs currently used, to facilitate the successful transplantation from mismatched donors, and to reduce the deleterious consequences of acute GvHD.

**Disclosures**

The authors have no financial conflict of interest.

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