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

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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 allo-specific 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.

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afe induction of Ag-specific long-term tolerance is essential to maintain immune 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/anergy-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 activity on alloantigen-specific effector T cells and the molecular mechanisms involved in such an effect. We also describe the therapeutic applicability of the VIP-treated T cells in a murine model of histoincompatible BMT.

Materials and Methods

Ags and reagents

VIP, Sp-8-Br-cAMP, H-89, and histone H1 were purchased from Calbiochem. The type 1 VIP receptor (VPAC2) antagonist (Ac-HisD-PheLys2-Arg2Leu23)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 pIRB, CD3, and IL-10 were obtained from BD Pharmingen. Human rIL-2 and rIL-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. Piggy 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 gradients (22). 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, -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 Biotec). CD4+CD25− and CD4+CD25+ T cell fractions were isolated (purity >96%) using the CD4+CD25+ T Regulatory Cell Isolation kit from Miltenyi Biotec. 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. APCs 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 APCs.

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 of medium in the presence or absence of VIP (10-7 M). Cells were pulsed with 0.5 μCi (0.0185 MBq)/well [3H]thymidine for the last 8 h of the culture and harvested onto membranes, and proliferation was determined by measuring [3H]thymidine incorporation. 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 × 105) were recovered by density gradient centrifugation with Lymphoprep (Nycosmed 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]Thymidine incorporation was determined at different time points of secondary MLC. When indicated, recovered viable cells (104/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 recuperate 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]thymidine uptake. Some cultures were performed in the presence of blocking anti-IL-10 (10 μg/ml), anti-TGF-β1 (10 μg/ml), and/or anti-CTLA4 (10 μg/ml) mAbs, or human rIL-2 (100 U/ml). To determine the cell-contact dependence of the suppressive response, we placed responder PBMCs (5 × 104) 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 × 105) 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 (106) 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 FACS-Calibur 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 first stained 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 P1H10 and 236E17) 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 106 cells/ml with PMA (10 ng/ml) plus ionomycin (50 ng/ml) for 6 h. Monensin (1.3 μM) was added for the last 4 h of culture. Cells were stained with PerCP anti-CD4 mAbs for 30 min at 4°C; washed; fixed/saponin permeabilized with Cytofix/Cytoperm solution; stained with 0.5 μg/sample FITC-conjugated anti-IL-2, anti-IFN-γ, anti-IL-4, anti-IL-17, anti-TGF-β, anti-TNF-α, or anti-IL-10 mAbs for 45 min at 4°C; and analyzed by flow cytometry.

Cytokine determination

Cytokine contents in the culture supernatants or sera were determined by specific sandwich ELISAs using capture/biotinylated detection Abs from BD Pharmingen.

Determination of gene expression

CTLA4 gene expression was determined by RT-PCR, as previously described (20). Primers used were designed to amplify the entire coding sequence of CTLA4 (5′-ATGCGTGCCTGATGGTCAAGAAGGACAAGG-3′ and 5′-TCTAATATTGAGGGAAATAATAAGGCCTATTGC-3′). PCR conditions were as follows: 94°C for 5 min, 30 cycles at 94°C for 30 s, 55°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 revealed bands was quantified by Image Quant 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 in the presence of syngeneic APCs in complete medium in the absence (Tcontrol) or presence of 10−7 M VIP (TIP) 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 (TCD-BM; 5 × 107) 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 × 106/mouse) isolated from C57BL/6 into recipient BALB/c mice lethally irradiated (10 Gy total body irradiation from a 200 Kvp 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 5 days following transplantation, and splenic donor H-2KdCD4+ and H-2KdCD8+ T cells were isolated by immunomagnetic selection, as described (22). Donor H-2KdCD4+ cells (5 × 105) were stimulated with allogeneic splenic APCs (H-2d, 5 × 106), 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-C57/7Rag1 transgenic (I-Ei) 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/B6, CD25, and anti-CD25 Ab-depleted mice. In all experiments, CD25 cells were depleted (in vivo depletion >98%) from PCCF TCR transgenic mice by treating mice i.v. with anti-CD25 Ab (clone PC61, 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 allogeneically 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 G₀/G₁ observed after stimulation (Fig. 1D), suggesting that the antiproliferative action of VIP is due to a sustained blocking in the transition from G₀/G₁ 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-γ, and TNF-α, 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-β1-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 allogeneic 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). This hyporesponsive state was partially reversed by the addition of IL-2 (Fig. 2A). Similar hyporesponsiveness of the VIP-treated cells was observed upon CD3/CD28 costimulation (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-γ, and TNF-α 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 (TVIP) or presence of VIP (TVIP) were added at different ratios to a new set of primary MLCs consisting of responder PBMCs and allogeic stimulator PBMCs. Whereas TVIP significantly contributed to the proliferative response of responder T cells, TVIP 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-induced 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 immunomagnetic 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 proliferation of anti-CD3/anti-CD28-coated beads, or restimulated in a secondary MLC with original allologeneic responder PBMCs in the absence or presence of IL-2. Proliferation was determined at different time points after initiation of the secondary MLC. Because PBMCs comprise both CD4+CD25+ Tregs, separation of T cells into Treg populations by flow cytometry was achieved by sorting three different cell subsets (CD25low, CD25int, and CD25high) (Fig. 3A). These results were confirmed in cocultures 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 T cells from responder T cells by a semipermeable membrane in Transwells significantly reversed the suppressive effect of VIP on alloantigen reactions (Fig. 3B), suggesting 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-dependent effect observed, neutralizing Abs for CTLA4, but not for IL-10 and TGF-β1, fully reversed the regulatory action of VIP on allostimulated responder T cells (Fig. 3C). As expected, the addition of IL-2 to cocultures bypassed their suppressor activity (Fig. 3C). Interestingly, VIP suppressed the proliferation of third party stimulated PBMCs and T cells, but only in the presence of the original allologeneic PBMCs or APCs used in the priming of VIP (Fig. 3D), suggesting that activation of VIP with the corresponding alloantigen is a requisite for their suppressive activity.

Flow cytometry analysis of the CD4+ T cells generated from allologeneic 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 expression of FoxP3 and CTLA4 as markers of Tregs and their functional activities. An elevated percentage of the CD25high population generated with VIP expressed FoxP3 and CTLA4+ FoxP3+ in comparison with the CD25− 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 cells showed an effector-memory phenotype characterized by expression of CD45RO, but not CD62L and CD27 (data not shown), making these cells suitable to act at a site of inflammation.

The CD25negative population mainly comprised ignorant alloantigen-unspecific CD4+ T cells because they did not respond to alloantigenic restimulation in the presence of IL-2, whereas the
VIP-induced CD25int population slightly proliferated in response to allogeneic PBMCs and the CD25high subset remained anergic (data not shown). The administration of the VIP-induced CD25high cells to allogeneic responder-stimulator PBMC cocultures efficiently inhibited the proliferation of the responder T cells (Fig. 4A). However, the CD25int 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 TVIP (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 FoxP3in allogeneic-activated CD4+CD25+ 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+CD25+ Treg generation (data not shown), or T cell suppression (Fig. 4D).

To further investigate whether VIP induced de novo Tregs, CD4+CD25+ 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-high (nondividing cells) and CFSE-low/medium (cycling cells). VIP treatment resulted in low numbers of cycling cells and confined Foxp3 and CD25high expression to the CFSE high nondividing CD4 cells.
(Fig. 5A). Moreover, VIP failed to increase FoxP3 expression in and to expand isolated CD4+CD25+ Tregs (Fig. 5B). However, IL-2 plus IL-15 stimulation (used as a positive control) resulted in a 6-fold expansion of CD4+CD25+ and FoxP3+ T cells (Fig. 5B).

Taken together, these results indicate that allogenic stimulation in the presence of VIP resulted in the new generation of an anergic CD4+CD25highFoxP3+CTLA4+ T cell population with regulatory functions.

**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 longterm and significantly increased the

**FIGURE 4.** VIP induces the generation of human 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 (CD25−, CD25int, and CD25high) 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 (5×10^4), and the proliferation during the secondary MLC was determined. n = 3 experiments performed in duplicate. B, 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 normalized by β2-microglobulin transcripts. 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. D, 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 (T_vip), 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 T_info or T_vip (4×10^4) 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 T_control. #, p < 0.001 vs T_vip.
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 (Fig. 6C). The CD4 T cells generated in the presence of VIP (T_VIP) showed potent suppressive functions on T cells activated with anti-CD3 and APCs (Fig. 6D). The regulatory activity of T_VIP seems to reside on the CD4⁺CD25⁺ cell population, because CD25⁺-depleted T_VIP failed to suppress T cell proliferation (Fig. 6D).

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<sub>VIP</sub> regulated the differentiation of GvHD-causing alloreactive T-effector cells in the grafted mice. We examined the subpopulations of transplanted H2-K<sup>b</sup> T cells and their ability to produce cytokines in T<sub>control</sub>- or T<sub>VIP</sub>-treated recipients (H-2<sup>b</sup>). Inoculation of T<sub>VIP</sub> decreased the number of H2-K<sup>b</sup>CD4<sup>+</sup> and H2-K<sup>b</sup>CD8<sup>+</sup> donor-derived T cells (Fig. 7D) and reduced the percentage of activated IFN-γ, IL-17, and IL-2-producing CD154<sup>+</sup> (CD40L) T cells in the H2-K<sup>b</sup>CD4<sup>+</sup> T cell population (Fig. 7E). At the same time, T<sub>VIP</sub> increased the percentage of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>CTLA4<sup>+</sup> T cells in spleen of the recipients (Fig. 7F). H2-K<sup>b</sup>CD4<sup>+</sup> T cells obtained from untreated or T<sub>control</sub>-treated mice that had received transplants responded vigorously to allogeneic PBMCs (H-2<sup>b</sup>), whereas H2-K<sup>b</sup>CD4<sup>+</sup> T cells from T<sub>VIP</sub>-treated recipients were hyporesponsive (Fig. 7G). T<sub>VIP</sub> treatment also reduced the levels of the proinflammatory cytokine TNF-α in the serum of grafted mice (Fig. 7H). These data indicate that the treatment of mice given transplants with VIP-tolerant T<sub>VIP</sub> reduced the number/activation of transplanted Th1, Th17, and CD8 cells; the inflammatory response against the recipient tissue; and the subsequent GvHD lethality, while increasing the number of CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs in the host.

VIP-tolerant T cells are arrested in G1 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-B1/IL-10 treatment and do not induce GvHD in vivo fail to progress into the late G1 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 G1 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 G1 phase (Fig. 8A). However, progression through the G1 restriction point to the late G1 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 G1 phase, and cyclin E, which is expressed in the G1 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 G1 phase, but fail to progress into the late G1 and enter the S phase of the cycle.
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,
PKA activation.

Human T cells were used. Activity of cyclins, cdk2, phosphorylated pRb, or actin. As a control, unstimulated human T cells were used. After 36 h of restimulation, cell lysates were subjected to Western blot analysis using Abs against CD28-coated beads for the indicated times.

Viable T cells were recovered and restimulated with anti-CD3/anti-CD28-coated beads for the indicated times. Viable T cells were recovered and restimulated with anti-CD3/anti-CD28-coated beads for the indicated times. Viable T cells were recovered and restimulated with anti-CD3/anti-CD28-coated beads for the indicated times. Viable T cells were recovered and restimulated with anti-CD3/anti-CD28-coated beads for the indicated times.

FIGURE 8. VIP-tolerant T cells are arrested at the early G1 phase of the cell cycle. Human PBMCs were stimulated in a primary MLC with allogeneic PBMCs in the absence (control) or presence of VIP (10^{-7} M) for 6 days. Viable T cells were recovered and restimulated with anti-CD3/anti-CD28-coated beads for the indicated times. A, After 48 h of restimulation, cell lysates were subjected to Western blot analysis using Abs against cyclins, cdk2, phosphorylated pRb, or actin. As a control, unstimulated human T cells were used. B, After 36 h of restimulation, cell lysates were immunoprecipitated (IP) with Abs against cyclin D2 or cdk4 (upper panels) or against cyclin E or cdk2 (lower panels). The cyclin D2-cdk4 and cyclin E-cdk2 interactions were assayed by Western blot analysis of precipitates with anti-cdk4 or anti-cdk2, respectively. Activities for cdk4/cyclin D2- and cdk2/cyclin E-associated kinases were determined in in vitro reactions using pRb-GST and histone H1 as substrates, respectively. C, After 48 h of restimulation, cell lysates were subjected to Western blot analysis using Abs against p27^{kip1} or actin. One representative experiment of three is shown.

FIGURE 9. VIP mediates the suppressive effect by elevating cAMP levels. Human PBMCs were stimulated with allogeneic stimulator PBMCs in primary MLCs for 6 days in the absence (control) or presence of VIP (10^{-7} M), 8-Br-cAMP (0.1 mM), or VIP (10^{-7} M) with or without H89 (50 ng/ml) or a VPAC1 antagonist (10^{-6} M). A, Cells were analyzed for FoxP3 and CTLA4 expression by real-time RT-PCR and flow cytometry, respectively. B, Cells were harvested, rested for 2 days, and restimulated in a secondary MLC with original allogeneic responder PBMCs. Proliferation was determined 3 days after initiation of the secondary culture. C, Recovered cells (5 \times 10^{5}) from the primary MLC were added to newly set-up primary MLCs consisting of responder PBMCs (10^{5}) and allogeneic stimulator PBMCs (10^{5}). Proliferation of responder T cells was determined after 4 days of coculture. D, Recovered cells from the primary MLC were restimulated with anti-CD3/anti-CD28-coated beads, and after 36-48 h culture, cell lysates were subjected to Western blot analysis using Abs against cyclins, p27^{kip1}, or actin. Cyclin D2/cdk4-associated kinase activity was determined using pRb-GST as substrate. As a control, unstimulated human T cells were used. n = 3-4 experiments performed in duplicate. *, p < 0.001 vs control. #, p < 0.01 vs VIP treatment.

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^{+}CD25^{high} 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 repertoire 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 initial 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 CD25high 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-ckd4 and cyclinE-ckd2 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 p27kip1. Previous studies have shown that the cell cycle inhibitor p27kip1 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 p27kip1 is dissociated from the cdk2-cyclin E/A complexes. This is in general achieved by ubiquitination and degradation of p27kip1, which is preceded by phosphorylation of p27kip1 (41, 42). Indeed, anergic and tolerant T cells are characterized by impaired degradation of p27kip1 and other tolerance strategies pointed out to p27kip1 as a critical target of anergy (24–26). The increase of p27kip1 by VIP could be due to impaired p27kip1 degradation and/or to increased p27kip1 synthesis. Recent results from our laboratory indicate that VIP prevents CD3/CD28-induced p27kip1 phosphorylation while increasing total p27kip1 levels (our unpublished observations). Various pathways are involved in the phosphorylation of p27kip1, 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 study demonstrates 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 p27kip1.

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 p27kip1, 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 t₁/₂ time in vivo. Therefore, its potential use as an immunosuppressive drug in 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⁺CD25high Tregs or ex vivo manipulated CD4⁺ T
cell lines containing induced regulatory T 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 allogeneic hematopoietic stem cells. These effects are mediated by impairing allogeneic 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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