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

David Pozo,* Per Anderson,† and Elena Gonzalez-Rey2†‡

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.

S
teful 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).

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 costimulatory 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...
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 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-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-His-D-Phe-Lys-glu-Leu-glu-Val)VIP(3–7)-GRF(8 –27) was previously described (18). FITC-, PerCP-, and PE-conjugated Abs against CTLA4, TGF-β, IFN-γ, IL-10, IL-17, IL-2, IL-4, Vβ3 (clone KJ25), CD154, CD25, and CD4, and Abs against pRb, CD3, and IL-10 were obtained from BD Pharmingen. Human rIL-2 and rIL-15 were obtained from Roche BioMedience. Protein A/G agarose, and Abs against cyclin E, cyclin D, and Abs against cyclin A, cyclin-dependent kinase cdk2, cyclin D2, cyclin D3, cdk4, cdk6, and p27kip1 were purchased from Santa Cruz Biotechnology. FITC- and Alexa-conjugated anti-mouse FoxP3 Ab (clone FKJ-16s) and anti-human FoxP3 Abs (clones PCH101 and 236A/E7) were obtained from eBioscience. Pigcondependent kinase (PCCF) was synthesized and purified in our laboratories.

**Cell isolation**

PBMCs were isolated from buffy coat preparations derived from the whole blood of healthy volunteers by density sedimentation on Ficoll-Hypaque gradient (Biocytex). 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 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 (19). 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%), nonsensel 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 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]Thymidine 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 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 ng/ml), anti-TGF-β1 (10 ng/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 × 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 × 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 (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 E13E7 and 236A/47) 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 10^6 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-CD4 mAbs for 30 min at 4°C; washed; fixed/saponin permeabilized with Cytofix/Cytoperm; stained with 0.5 µg/sample FITC-conjugated anti-IL-2, -IFN-γ, -anti-IL-4, -anti-IL-17, -anti-TGF-β1, -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'-ATGGCTTGGCTTGGATTTCAGCGGCACA AOG-3' and 5'-TCACTAATGTTGAAAATATAGGCTATTGC-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 resealed bands was quantified by ImageQuaNT software (Amersham Biosciences), normalized to those of β2-microglobulin, and expressed as arbitrary units. Human FoxP3 gene expression was quantified 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). The amount of FoxP3 mRNA expression was normalized with the human β-actin and calculated according to the comparative cycle threshold method. FoxP3 primers and probe were as follows: forward primer, 5'-GCGACCTTCCAAATCCAGT-3'; reverse primer, 5'-GGCCACTTG CAGCACGTTTGSATG-3'; and probe, 5'-CCAGAAAAGCACGACTTTCGCG-3'. Mouse FoxP3 gene expression was determined by RT-PCR, as described previously (21), by using the following primers: FoxP3, 5'-CAGCTGCTTACTCCAGCTAGC-3' and 5'-GATGTTGAGAAGTGCAGTGAGC-3'; and FoxP3 probe, 5'-GATGTTGAGAAGTGCAGTGAGCG-3'.

Western blot analysis

Whole-cell lysates were prepared by lysing 10^6 cells in lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM EDTA, 50 mM glycerophosphate, 1% Triton X-100, 50% glycerol, 1 mM DTT, 5 mM benzamidine, 2 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM PMSF, 5 mM NaF, 10 mM Na2HPO4, and 1 mM Na3VO4) for 30 min on ice and mixing gently. Insoluble fragments were pelleted by centrifugation for 30 min at 10,000 × g. Proteins from whole extracts (60–80 µg/sample) were separated by SDS-PAGE (12%; analysis of pRb: 8%) and blotted onto polyvinylidene difluoride membranes (Millipore) using a semidyve system. Membranes were blocked with TBS/Tween 20%/3% nonfat milk for 1 h at room temperature and subsequently probed with the indicated primary Ab overnight at 4°C. Immunodetection was performed by incubation with HRP-conjugated Abs and visualized by ECL (Amersham Pharmacia). Equal protein loading was controlled by reprobing with anti-β-actin Abs. Cdk-associated kinase activities were determined, as described below.

Determination of cdk-associated kinase activity

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, Amersham Biosciences), and 5 µg of histone H1 for cdk4 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 keV x-ray source) and reconstituted i.v. with allogeneic T cell-depleted bone marrow cells (TCD-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 keV 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 10−6 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 H2-KB-CD4+ and H2-KbCD8+ T cells were isolated by immunomagnetic selection, as described (22). Donor H2-KbCD4+ cells (5 × 105) were stimulated with allogeneic splenic APCs (H-2b, 5 × 104), 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-C57/1Rag1 transgenic (I-Eb) mice (PCFFC TCR transgenic) were obtained from Taconic Farms. PCFFC TCR transgenic mice were injected i.p. on days 0 and +2 with Ag (PCFFC, 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 obtained and analyzed by flow cytometry. In some experiments, CD25− and CTLA4− T cells in the spleen of recipient mice were determined by flow cytometry, as described above.

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 y-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 alloimmune 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 allologically primed in the absence (Tcontrol) or presence of VIP (TVIP) were added at different ratios to a new set of primary MLCs consisting of responder PBMCs and allogeneic 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 responder T cells in medium alone, restimulated with anti-CD3/anti-CD28-coated beads, or restimulated in a secondary MLC with original allogenic responder PBMCs in the absence or presence of IL-2. Proliferation was determined at different time points after initiation of the secondary MLC.

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<sub>VIP</sub> from responder T cells by a semipermeable membrane in Transwells significantly reversed the suppressive effect of T<sub>VIP</sub> 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-β, fully reversed the regulatory action of T<sub>VIP</sub> on allostimulated responder T cells (Fig. 3C). As expected, the addition of IL-2 to cocultures bypassed their suppressor activity (Fig. 3C). Interestingly, T<sub>VIP</sub> suppressed the proliferation of third-party stimulated PBMCs and T cells, but only in the presence of the original allogenic PBMCs or APCs used in the priming of T<sub>VIP</sub> (Fig. 3D), suggesting that activation of T<sub>VIP</sub> with the corresponding alloantigen is a requisite for their suppressive activity.

Flow cytometry analysis of the CD4<sup>+</sup> T cells generated from allogeneic primed CD4<sup>+</sup>CD25<sup>−</sup> T cells in the presence of VIP revealed three different cell subsets based on the level of CD25 expression (CD25<sup>negative</sup>, CD25<sup>int</sup>, and CD25<sup>high</sup>) (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 CD25<sup>high</sup> population generated with VIP expressed FoxP3 and CTLA4 high, in comparison with the CD25<sup>int</sup> and CD25<sup>negative</sup> populations (data not shown), suggesting that activation of T<sub>VIP</sub> with the corresponding alloantigen is a requisite for their suppressive activity.

The CD25<sup>negative</sup> population mainly comprised ignorant alloantigen-unspecific CD4<sup>+</sup> T cells because they did not respond to alloantigenic restimulation in the presence of IL-2, whereas the

**FIGURE 2.** VIP generates anergic T cells with suppressive functions after allogeneic stimulation. A, Human PBMCs were stimulated with allogeneic stimulator PBMCs in primary MLCs for 6 days in the absence (control) or presence of VIP (10<sup>−7</sup> M). Cells were harvested and rested for 2 days, and the recovered viable cells were incubated in medium in the absence or presence of IL-2. Proliferation was determined at different time points after initiation of the secondary MLC. B, Human PBMCs were stimulated with allogeneic stimulator PBMCs in primary MLCs for 6 days in the absence (T<sub>control</sub>) or presence of VIP (T<sub>VIP</sub>). Cells were harvested and rested for 2 days, and viable T<sub>control</sub> or T<sub>VIP</sub> were added at different numbers to newly set up primary MLCs consisting of responder PBMCs (10<sup>5</sup>) and allogeneic stimulator PBMCs (10<sup>5</sup>). Proliferation of responder T cells was determined after 4 days of coculture. PBMCs (10<sup>5</sup>) cultured with syngeneic PBMCs (10<sup>5</sup>) were used as controls of the background stimulation (medium). n = 3–4 experiments performed in duplicate. *p < 0.001 vs control or T<sub>control</sub>.
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 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 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>low</sup>/low (cycling cells) cells. VIP treatment resulted in low numbers of cycling cells and confined FoxP3 and CD25 expression to the CFSE<sup>high</sup> nondividing CD4 cells.
CD4 in the presence of VIP resulted in the new generation of anergic /H11003 determined. CFSE-labeled PBMCs (2 a 6-fold expansion of CD4 IL-2 plus IL-15 stimulation (used as a positive control) resulted in or control IgG (10 /H9262 normalized by /H11003 expressed as the mean fluorescence intensity (MFI), and mRNA expression of CTLA4 was determined by quantitative PCR and expressed as arbitrary units performed in duplicate. Human CD4 restimulated at different cell ratios with allogeneic PBMCs (5 /H11003 for FoxP3 (results with clone PCH101 are shown, and were confirmed with clone 236A) and CTLA4 expression. Expression of FoxP3 and CD25 in the background stimulation (first bar).

**FIGURE 4.** VIP induces the generation of human CD4 CD25 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 (CD25negative, CD25mid, 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 x 106), 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 /beta;2-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 /mug/ml), anti-TGF-beta, or control IgG (10 /mug/ml). After 4 days, FoxP3 expression was quantitated by real-time PCR and normalized to the /beta;2-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 (Tvip), with or without anti-CTLA4 (10 /mug/ml), anti-TGF-beta, or control IgG (10 /mug/ml) for 6 days. After the 2-day resting period, the recovered Tcontrol or Tvip (4 x 106) were added to a coculture of responder CFSE-labeled PBMCs (2 x 105 from donor B) and allogeneic stimulator PBMCs (2 x 106 from donor C). After 96-h culture, total number of cycling cells (percentage of CFSE-positive cells that had divided x the total number) was determined. CFSE-labeled PBMCs (2 x 105 from donor B) cultured with syngeneic PBMCs (2 x 106 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.

(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 allogeneic stimulation in the presence of VIP resulted in the new generation of an anergic CD4 CD25FoxP3 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 T 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 T 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<sup>+</sup>FoxP3<sup>+</sup> cells (Fig. 6B). We observed similar results by using CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from nontransgenic C57BL/6 mice (Fig. 6C). In addition, VIP increased the mRNA expression of FoxP3 in the CD4<sup>+</sup>CD25<sup>+</sup> cell population, but it failed to significantly increase FoxP3 in isolated CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup> cells was determined by flow cytometry. The percentage of CD25<sup>+</sup> cells in the CFSE<sup>high</sup> cycling cells was determined by flow cytometry, n = 3–4 experiments performed in duplicate. *p < 0.001 vs unstimulated cells.

**Protective effect of VIP-tolerant CD4<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> T cells (T<sub>control</sub>) from C57BL/6 mice (H2<sup>b</sup>) to irradiated BALB/c mice (H2<sup>b</sup>) 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<sup>+</sup>CD25<sup>+</sup> T cells stimulated in the presence of VIP (T<sub>VIP</sub>) died as a consequence of acute GvHD (Fig. 7A), reflecting the hypersensitivity of the T<sub>VIP</sub> in vivo. More importantly, H2<sup>b</sup> CD4<sup>+</sup>CD25<sup>+</sup> T cells primed with allogeneic H2<sup>d</sup> spleen cells in the presence of VIP (T<sub>VIP</sub>) significantly prevented the mortality that occurred as a consequence of the GvHD in animals receiving allogeneic BMT (Fig. 7B). This suggests that T<sub>VIP</sub> could impair allogeneic Ag-specific responses of donor CD4 T cells in mice that have received transplants. This suppressive activity belongs to the CD4<sup>+</sup>CD25<sup>+</sup> population, because when the T<sub>VIP</sub> were fractionated as CD25<sup>hi</sup> and CD25<sup>lo</sup> populations before transfer, only the CD4<sup>+</sup>CD25<sup>hi</sup> fraction mimicked the protective effect of T<sub>VIP</sub> 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<sup>+</sup> and CD8<sup>+</sup> 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_{VIP} regulated the differentiation of GvHD-causing alloreactive T-effector cells in the grafted mice. We examined the subsets of populations of transplanted H2-K^b T cells and their ability to produce cytokines in T_{control}- or T_{VIP}-treated recipients (H-2^d). Inoculation of T_{VIP} decreased the number of H2-K^bCD4^+ and H2-K^bCD8^+ donor-derived T cells (Fig. 7D) and reduced the percentage of activated IFN-γ, IL-17, and IL-2-producing CD154^+ (CD40L) T cells in the H2-K^bCD4^+ T cell population (Fig. 7E). At the same time, T_{VIP} increased the percentage of CD4^+ CD25^+FoxP3^+ and CD4^+ CTLA4^+ T cells in spleen of the recipients (Fig. 7F). H2-K^bCD4^+ T cells obtained from untreated or T_{control}-treated mice that had received transplants responded vigorously to allogeneic PBMCs (H-2^d), whereas H2-K^bCD4^+ T cells from T_{VIP}-treated recipients were hyporesponsive (Fig. 7G). T_{VIP} 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_{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^+FoxP3^+ Tregs in the host.

VIP-tolerant T cells are arrested in G_{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 G_{1} phase of the cell cycle (24). Moreover, alloreactive mouse T cell clones that have been rendered tolerant by CD40 blockade or TGF-β1/IL-10 treatment and do not induce GvHD in vivo fail to progress into the late G_{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_{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 D-associated cdk4 and cdk6 upon CD3/CD28 restimulation, indicating that they could enter in the G_{1} phase (Fig. 8A). However, progression through the G_{1} restriction point to the late G_{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_{1} phase, and cyclin E, which is expressed in the G_{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_{1} phase, but fail to progress into the late G_{1} and enter the S phase of the cycle.

FIGURE 6. VIP converts mouse CD4^+CD25^+ T cells on CD4^+CD25^+FoxP3^+ T cells with regulatory functions. A, PCCF transgenic Rag^-/- mice (six 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 the percentages of CD25^+FoxP3^+ regulatory Rag^-/- mice. Percentage and total numbers of spleen CD25^+ T cells were determined by flow cytometry. B, CD4^+CD25^+ T cells isolated from C57BL/6 mice were cultured with anti-CD3 and APCs in the absence or presence of 10^{-7} M VIP for different times, and analyzed for the percentages of CD25^+FoxP3^+ regulatory Rag^-/- mice. Sorted cells were analyzed for FoxP3 expression by flow cytometry before T control, or presence of 10^{-7} M VIP for 4-5 days and analyzed for the percentages of FoxP3^+ and CD4^+ CTLA4^+ 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. C, CD4^+CD25^+ and CD4^+CD25^- T cells isolated from C57BL/6 mice were cultured with anti-CD3 and APCs in the absence or presence of 10^{-7} M VIP for 4-5 days and analyzed for the percentages of FoxP3^+ and CD4^+ CTLA4^- T cells. The expression of FoxP3 was determined by realtime RT-PCR and normalized using hypoxanthine phosphoribosyltransferase expression. Unstimulated cells were used as basal controls. D, CD4^+CD25^- T cells isolated from C57BL/6 mice were cultured with anti-CD3 and APCs in the absence or presence of 10^{-7} M VIP for 4-5 days. Viable T cells were stained with FITC anti-CD25 mAb, and the resultant CD25^+ and CD25^- populations were sorted by flow cytometry. Sorted cells (2 × 10^6) were added to freshly isolated CD4^+CD25^- responder T cells (5 × 10^5) stimulated with anti-CD3 and APCs. Proliferation was determined after 72-h culture. Dashed line represents the proliferative response of CD4^+CD25^- T cells in the absence of any stimulus. Sorted cells were analyzed for FoxP3 expression by flow cytometry before T cells were added to responder T cells, and showed the following percentage of FoxP3^+ cells: <0.4% in CD25^- T control, 2.3% in CD25^- T_{VIP}, 1.1% in CD25^+ T_{control}, and 24% in CD25^+ T_{VIP}. n = 3 experiments performed in duplicate. *p < 0.01 vs CD3/APC-stimulated cells.
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. 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

![Figure 8](http://www.jimmunol.org/) 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, cdkks, 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 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 p27kip1 or actin. One representative experiment of three is shown.

![Figure 9](http://www.jimmunol.org/) 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, rest 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 × 10⁶) from the primary MLC were added to newly set-up primary MLCs consisting of responder PBMCs (10⁵) and allogeneic stimulator PBMCs (10⁵). 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- to 48-h culture, cell lysates were subjected to Western blot analysis using Abs against cyclins, p27kip1, 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.
These common biochemical analyses may provide a powerful tool.
cell lines containing induced regulatory Tr1 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 allogeneic-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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