Vasoactive intestinal peptide induces regulatory dendritic cells that prevent acute graft-versus-host disease while maintaining the graft-versus-tumor response

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Acute graft-versus-host disease (GVHD) is a major cause of morbidity and mortality in patients undergoing allogeneic bone marrow transplantation (BMT) for the treatment of leukemia and other immunogenetic disorders. The use of tolerogenic dendritic cells (DCs) that induce the generation/activation of regulatory T (Tr) cells for the treatment of acute GVHD following allogeneic BMT has been recently established. Therefore, the identification of factors that contribute to the development of tolerogenic DCs is highly relevant. We report on the use of the known immunosuppressive neuropeptide, the vasoactive intestinal peptide (VIP), as a new approach to induce tolerogenic DCs with the capacity to prevent acute GVHD. DCs differentiated in the presence of VIP impair allogeneic haplotype-specific responses of donor CD4+ cells in mice given transplants by inducing the generation of Tr cells in the graft. VIP-induced tolerogenic DCs did not abrogate the graft-versus-leukemia response presumably by not affecting the cytotoxicity of transplanted T cells against the leukemic cells. Therefore, the inclusion of VIP-induced tolerogenic DCs in future therapeutic regimens may minimize the dependence on nonspecific immunosuppressive drugs used currently as antirejection therapy, and facilitate the successful transplantation from mismatched donors, by reducing the deleterious consequences of acute GVHD and extending the applicability of BMT. (Blood. 2006;107:3787-3794)

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Materials and methods

Animals

C57Bl/6 (B6) (H-2b), Balb/c (H-2d), DBA/2 (H2b), [B6 × DBA/2] F1 (H2bxd). 5- to 9-week-old female mice were obtained from Iffa Credo...
(L’Arbresle, France) and Jackson Laboratories (Bar Harbor, ME). All animal protocols were approved by the Committee on Use and Care of Laboratory Animals at Rutgers University and CSIC.

**Cell preparation**

BM-DCs were generated as previously described. Briefly, BM cells (2 × 10^6) obtained from Balb/c (H-2b), C57Bl/6 (H-2d), or DBA/1 (H2b) mice were incubated in complete medium (RPMI 1640 supplemented with 100 U/mL penicillin/streptomycin, 2 mM l-glutamine, 50 mM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum) containing 20 ng/mL GM-CSF (PreproTech, Rocky Hill, NJ) in the presence or absence of VIP (10^-8 M; Calbiochem, San Diego, CA). At day 6, nonadherent cells were collected (containing 80%-90% CD11c<sup>+</sup> cells) and stimulated for 48 hours with LPS (1 μg/mL) to induce activation/maturity.

Allogeneic T cells, naive CD4<sup>+</sup>CD62L<sup>+</sup> and CD8<sup>+</sup> cells were purified from spleen mononuclear cells obtained from normal mice (H-2b or H-2d) by positive immunomagnetic selection following the manufacturer’s instructions (magnetic-activated cell sorting [MACS], Miltenyi Biotech, Auburn, CA). Purified naive CD4<sup>+</sup> and CD8<sup>+</sup> cells (5 × 10<sup>5</sup>) were exposed to allogeneic DC<sub>controls</sub> or DC<sub>VIP</sub> (10<sup>5</sup>). After 3 days of culture, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were recovered by immunodepletion of CD11c<sup>+</sup> DCs, rested for 3 days in complete medium supplemented with IL-2 (20 U/mL), and used as potential CD8<sub>Tr</sub> and CD8<sub>Tr</sub> cells in mice given transplants as indicated in “Models for acute GVHD and GVT response.”

**Models for acute GVHD and GVT response**

Allogeneic transplantation was performed by a single intravenous injection of T cell-depleted BM cells supplemented with 1.5 × 10<sup>6</sup> spleen mononuclear cells (BMs; 1.5 × 10<sup>6</sup> cells/mouse) isolated from C57Bl/6 (H-2b) into irradiated Balb/c (H-2b) mice lethally irradiated (10 Gy total body irradiation [TBI]) from a 200-KeV x-ray source. In addition, GVHD was induced in irradiated recipient C57Bl/6 mice by allogeneic transplantation of Balb/c BMs (1.5 × 10<sup>6</sup> cells/mouse). GVHD was also induced in [B6 × DBA/2] F1 (H-2<sup>b</sup>) recipients by transplantation of BM (1.5 × 10<sup>5</sup> cells/mouse) isolated from C57Bl/6 (H-2b), GVH was induced by injecting intravenously A20 leukemia cells (derived from Balb/c mice, 10<sup>5</sup> cells) or P815 mastocytoma cells (derived from DBA/2 mice, 10<sup>5</sup> cells) into irradiated Balb/c mice at the time of BM transplantation (H-2<sup>b</sup> BMs, supplemented with different numbers of spleen mononuclear cells: 1.5 × 10<sup>6</sup>, 5 × 10<sup>5</sup>, or 1 × 10<sup>5</sup> cells). Recipients received single or repetitive intravenous injections of different numbers of host-matched or host-mismatched DC<sub>controls</sub> or DC<sub>VIP</sub> (H-2<sup>b</sup>, H-2<sup>d</sup>, or H-2<sup>q</sup>) or DC<sub>Tr</sub> (H-2<sup>b</sup> or H-2<sup>d</sup>) at the time of BM transplantation. Alternatively, recipients received different numbers of T<sub>control</sub> or T<sub>VIP</sub> cells (H-2<sup>b</sup> or H-2<sup>d</sup>) at the time of BM transplantation. T<sub>control</sub> or T<sub>VIP</sub> cells were removed once every day from the day of transplantation until they died naturally of GVHD or tumor burden to determine survival time and body weight. Tumor growth/elimination was assessed by the presence of A20 cells in blood detected by coexpression of B220 and H-2K<sub>d</sub> and by their large size using flow cytometry. In other experiments, serum and spleen cells were harvested at different times following transplantation. For mice bearing P815 tumor cells the liver and spleen were weighed at the time of death or 60 days after transplantation.

Where mentioned, the transplant recipients received intravenous injections of anti-CD25 monoclonal antibody (mAb), neutralizing anti–IL-10 polyclonal antibody (Ab), neutralizing anti-TGFβ mAb, or preimmune rat IgG (500 μg Ab/mouse) every other day up to 15 days after transplantation. To trace the injected cells in vivo, DC<sub>controls</sub> and DC<sub>VIP</sub> were labeled with 5,6-carboxy-succinimidyl-fluorescein-isothiocyanate (CFSE; Molecular Probes, Eugene, OR) and injected intravenously, and their presence was determined in spleen by flow cytometry.

In vivo priming and characterization of grafted T cells in mice given transplants

Following transplantation of H-2<sup>b</sup> recipients with H-2<sup>b</sup> BM plus BMS cells, we isolated splenic I-<sup>K</sup>-CD4<sup>+</sup> and I-<sup>K</sup>-CD8<sup>+</sup> T cells by immunomagnetic selection as described. Briefly, spleen mononuclear cells obtained 5 days after transplantation were depleted of recipient cells by incubation with anti-I-<sup>K</sup>-mAb followed by goat anti–mouse IgG mAb-conjugated immunomagnetic beads. Donor-derived CD4<sup>+</sup> and CD8<sup>+</sup> T cells were selected from the isolated donor-I-<sup>K</sup>-cells by using anti-CD8 mAb or anti-CD4 mAb (BD PharMingen, San Diego, CA), followed by sheep anti–rat IgG mAb-conjugated immunomagnetic beads. The T-cell preparations were typically more than 98% pure as indicated by flow cytometry. Donor I-<sup>K</sup>-DC4<sup>+</sup> cells (5 × 10<sup>5</sup>) were cultured with medium alone (none) or with mature DCs (H-2<sup>q</sup>) (5 × 10<sup>5</sup>) in the presence or absence of IL-2 (20 U/mL), and the proliferative response was determined by using a cell proliferation assay (BrdU) from Roche Diagnostics (Mannheim, Germany). Cytokine contents in the culture supernatants were determined by specific sandwich enzyme-linked immunosorbent assays (ELISAs) using capture/biotinylated-detection Abs from BD PharMingen. Donor I-<sup>K</sup>-CD8<sup>+</sup> cells were assayed for cytotoxicity against tumor cells as described in “Cytotoxicity assay.”

**Flow cytometry**

Cells were incubated with various PerCP-, FITC-, and PE-labeled mAbs (BD PharMingen) diluted at optimal concentration for immunostaining, fixed in 1% paraformaldehyde, and analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). We used isotype-matched Abs as controls and IgG block (Sigma, St Louis, MO) to avoid nonspecific binding to Fc-receptors. For analysis of intracellular CTLA4, cells were stained first for surface CD4 with PerCP–anti-CD4, fixed with Cytofix/Cytopermt solution (BD PharMingen), incubated with PE-anti-CTL4a mAb diluted in 0.5% saponin, and analyzed by flow cytometry. For intracellular cytokine analysis isolated CD4 T cells (10<sup>6</sup> cells/mouse) were stimulated with PMA (1 ng/mL) plus ionomycin (20 ng/mL) for 8 hours. Monensin (1.3 μmol/mL) was added for the last 4 hours of culture. Cells were stained with PerCP–anti-CD4 mAbs for 30 minutes at 4°C; washed, fixed/saponin permeabliized with Cytofix/Cytoperm, and stained with 0.5 μg/sample of FITC- and PE-conjugated anti–IL-2, anti–IFN-γ, and anti–IL-4, or anti–IL-10–specific mAbs for 45 minutes at 4°C. Cells were analyzed by flow cytometry.

**Cytotoxicity assay**

In vivo primed CD8 T cells were cultured with Na<sup>2</sup>51CrO<sub>4</sub>-labeled (100 μCi [3.7 MBq/10<sup>6</sup> cells, NEN Life Science Products, Boston, MA]) P815, EL4, or A20 cells (10<sup>4</sup>) at 4 hours at various effector-to-target cell ratios (E/T ratios). The radioactivity released in the supernatants was measured, and the percent specific lysis was calculated.

**Statistical analysis**

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 the 2-tailed Student t test. A P value below .01 was considered significant.

**Results**

DC<sub>VIP</sub> protect from acute GVHD

We reported recently that the presence of VIP in the early differentiation stages of murine BM-derived DCs and of human monocyte-derived DCs results in the generation of DCs with a regulatory/tolerogenic phenotype. DCs differentiated in the presence of VIP (DC<sub>VIP</sub>) are CD11c<sup>+</sup>CD45RB<sup>+</sup>CD<sup>−</sup>CD8<sup>−</sup>B220<sup>−</sup>, do not up-regulate CD40, CD80, and CD86, and do not express TNF-α and IL-12 following LPS or other inflammatory stimulation (not shown). In contrast to DC<sub>controls</sub>, DC<sub>VIP</sub> secreted high amounts of IL-10 and exhibited a very poor stimulatory activity for allogeneic CD4 T cells (not shown). In addition, murine CD4 T cells primed with allogeneic DC<sub>VIP</sub> exhibit a Tr1-like phenotype, characterized by IL-10 and TGF-β, but not IL-2 and IFN-γ.
production, and efficiently suppress the proliferative response of syngeneic responder CD4+ T cells cocultured with allogeneic mDCs. Therefore, we examined the potential therapeutic effect of DCVIPs in acute GVHD following allogeneic BMT. We transplanted either T cell-depleted BM cells or T-depleted BMS from C57Bl/6 (H-2b) into lethally irradiated Balb/c recipients (H-2d). Following transplantation, we injected DCcontrols or DCVIPs, generated from Balb/c BM cells. Mice given T cell-depleted BM appeared healthy, and 100% of the animals survived for at least 75 days (not shown). Mice that received BMS developed severe signs of GVHD, including weight loss, reduced mobility, hunched posture, diarrhea, and ruffled fur, and died within 25 days (Figure 1A). Whereas treatment with DCcontrols enhanced GVHD lethality, the administration of host major histocompatibility complex (MHC)-matched DCVIPs (H-2d) protected from lethal GVHD, and more than 70% of the mice survived for more than 75 days (Figure 1A). The therapeutic effect was dose-dependent for recipients receiving a single injection with DCVIPs, and repetitive injections of lower doses of DCVIPs (2 and 5 days after transplantation) enhanced the survival rate (Figure 1B). In contrast to the therapeutic effect of the host MHC-matched DCVIPs, the injection of host-mismatched DCVIPs following BMT failed to protect from acute GVHD (Figure 1C). The window of opportunity for DCVIPs administration (2 × 10^6 cells/mouse) was apparently short, however, because delaying this injection further until day 5 after BMT reduced the therapeutic effect (10% survival). Higher levels of protection (60% survival) were obtained with 6 × 10^6 DCVIPs, administered on day 5 (not shown). The therapeutic window of DCVIPs was significantly increased with a haploidentical model of GVHD, a model closer to human applications. In this model, lethally irradiated (B6 × DBA/2)F1 (H-2b/H-2d) mice were given transplants of B6 BMS (H-2d) and treated with DCcontrols or DCVIPs at different times after BMT. DCVIPs were more efficient preventing lethality in this model than in a full H-2 mismatch transplant and showed significant therapeutic effect when administered 10 days after BMT (Figure 1D).

DCVIPs impair allogeneic antigen-specific responses of donor CD4+ T cells in mice that have received transplants by inducing the generation of Tr cells in the graft

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. We therefore investigated whether DCVIPs regulate the differentiation of GVHD-causing alloreactive T-effector cells in the grafted mice. First we examined the subpopulations of transplanted I-Kb T cells and their ability to produce cytokines in DCcontrols or DCVIPs-treated recipients (H-2d) (Figure 2A). Inoculation of DCVIPs decreased the number of CD3+, CD4+, and CD8+ I-Kb donor-derived T cells, reduced the percentage of activated IL-2/IFN-γ-producing CD154+ (CD40L) Th1 cells, and increased the number of regulatory IL-10–producing CTLA4+ T cells in the I-Kb CD4+ T-cell population (Figure 2A). I-Kb CD4+ T cells obtained from untreated or DCcontrols-treated mice that had received transplants responded vigorously to allogeneic mDCs (H-2d). In contrast, I-Kb CD4+ T cells from DCVIPs-treated recipients were hyporesponsive, and the addition of IL-2 partially restored this response (Figure 2B). We further examined the serum levels of inflammatory cytokines. DCVIPs treatment reduced the levels of the proinflammatory cytokines IFN-γ, TNF-α, and IL-12 in the serum of grafted mice (Figure 2C). These data indicate that the treatment of mice given transplants with DCVIPs reduced the number/activation of transplanted Th1 cells, the inflammatory response against the recipient tissue, and the subsequent GVHD lethality, and could be involved in the generation of Tr cells. This correlates with the fact that DCVIPs induce in vitro the generation of IL-10/TGF-β–producing regulatory CD4+CD25+CTLA4+ cells. Therefore, we further examined the role of Tr cells in the therapeutic effect of DCVIPs on acute GVHD. In vivo blockade experiments showed that treatment with anti-CD25, anti–IL-10, or anti–TGF-β Abs significantly decreased survival rates, and treatment with all 3 Abs abrogated the survival effect exerted by DCVIPs (Figure 2D).

To better understand the half-life, stability, and trafficking of the infused DCVIPs, we injected CFSE-labeled DCs into mice given transplants. As previously described for other tolerogenic DCs, we detected the inoculated DCVIPs in the spleen of the recipients the next day after transplantation, with a half-life of about 17 days and some degree of proliferation (Figure 3A). In addition, DCVIPs...
GVHD and marked body weight loss, although leukemic cells could not be detected in blood and developing tumors at the site of injection and hepatosplenomegaly were not observed, attesting to an efficient GVT effect (Figure 4). In contrast, most of the leukemia-bearing mice given transplants with DC<sub>VIP</sub>s were still alive on day 70 (Figure 4A). Compared to the 2 control groups, the presence of DC<sub>VIP</sub>s protected mice from lethal GVHD, whereas the GVL activity was maintained. Indeed, leukemic cells could not be detected in these mice, except for one animal that died at day 40. A20-bearing mice given transplants with BMS and with host-mismatched DC<sub>VIP</sub>s succumbed to GVHD, whereas GVT response was maintained intact (not shown), indicating the requirement for the haplotype-specificity of DC<sub>VIP</sub>s. Treatment of leukemia-bearing mice with host-matched DC<sub>VIP</sub>s and no BMS or with T cell-depleted BM cells did not affect tumor growth (not shown), indicating that graft allogeneic T cells are responsible for GVT effect. Interestingly, although acute GVHD was completely prevented, DC<sub>VIP</sub>s were less efficient in maintaining GVT response in mastocytoma-bearing mice compared to A20 leukemia, as assessed by a significant hepatosplenomegaly and body weight loss in the P815 recipients (Figure 4B). However, GVT maintenance by DC<sub>VIP</sub>s was not exclusive for A20 cells because a similar GVT response was observed in a BCL1 lymphoma model (not shown).

**Treatment with DC<sub>VIP</sub>s does not abrogate the cytotoxicity of transplanted T cells against leukemic cells**

To investigate the mechanisms through which DC<sub>VIP</sub>s prevent GVHD in mice receiving transplants while maintaining an effective GVT response, we first determined whether the DC<sub>VIP</sub>s treatment affects the cytotoxicity of transplanted T cells against tumor cells. I-K<sup>â€”</sup>CD8<sup>+</sup> T cells isolated from DC<sub>VIP</sub>-treated recipients exhibit potent lytic activity against A20 cells (H-2<sup>d</sup>), similar to BMS (H-2<sup>b</sup>) together with A20 or P815 cells, and treated with or without DC<sub>controls</sub> (H-2<sup>d</sup>), died with clinical signs characteristic of GVHD and marked body weight loss, although leukemic cells could not be detected in blood and developing tumors at the site of injection and hepatosplenomegaly were not observed, attesting to an efficient GVT effect (Figure 4). In contrast, most of the leukemia-bearing mice given transplants with DC<sub>VIP</sub>s were still alive on day 70 (Figure 4A). Compared to the 2 control groups, the presence of DC<sub>VIP</sub>s protected mice from lethal GVHD, whereas the GVL activity was maintained. Indeed, leukemic cells could not be detected in these mice, except for one animal that died at day 40. A20-bearing mice given transplants with BMS and with host-mismatched DC<sub>VIP</sub>s succumbed to GVHD, whereas GVT response was maintained intact (not shown), indicating the requirement for the haplotype-specificity of DC<sub>VIP</sub>s. Treatment of leukemia-bearing mice with host-matched DC<sub>VIP</sub>s and no BMS or with T cell-depleted BM cells did not affect tumor growth (not shown), indicating that graft allogeneic T cells are responsible for GVT effect. Interestingly, although acute GVHD was completely prevented, DC<sub>VIP</sub>s were less efficient in maintaining GVT response in mastocytoma-bearing mice compared to A20 leukemia, as assessed by a significant hepatosplenomegaly and body weight loss in the P815 recipients (Figure 4B). However, GVT maintenance by DC<sub>VIP</sub>s was not exclusive for A20 cells because a similar GVT response was observed in a BCL1 lymphoma model (not shown).

**GVL activity is maintained in the presence of DC<sub>VIP</sub>s**

The current preclinical immunosuppressive therapies for the prevention of GVHD fail to control the balance between the anti-GVHD effect and the beneficial GVT activity of the allogeneic BM transplants. We therefore tested whether the GVT effects in the recipients could be maintained in the presence of DC<sub>VIP</sub>s. Balb/c mice (H-2<sup>b</sup>) receiving leukemia (H-2<sup>b</sup>) A20 cells died within 20 days from leukemia, as attested by the presence of leukemic cells in the blood (Figure 4A). Similarly, Balb/c mice (H-2<sup>b</sup>) receiving P815 mastocytoma (H-2<sup>c</sup>) cells died within 30 days, as attested by the presence of growing tumors at the site of injection and hepatosplenomegaly (Figure 4B). Mice given transplants with BMS (H-2<sup>d</sup>) together with A20 or P815 cells, and treated with or without DC<sub>controls</sub> (H-2<sup>d</sup>), died with clinical signs characteristic of GVHD and marked body weight loss, although leukemic cells could not be detected in blood and developing tumors at the site of injection and hepatosplenomegaly were not observed, attesting to an efficient GVT effect (Figure 4). In contrast, most of the leukemia-bearing mice given transplants with DC<sub>VIP</sub>s were still alive on day 70 (Figure 4A). Compared to the 2 control groups, the presence of DC<sub>VIP</sub>s protected mice from lethal GVHD, whereas the GVL activity was maintained. Indeed, leukemic cells could not be detected in these mice, except for one animal that died at day 40. A20-bearing mice given transplants with BMS and with host-mismatched DC<sub>VIP</sub>s succumbed to GVHD, whereas GVT response was maintained intact (not shown), indicating the requirement for the haplotype-specificity of DC<sub>VIP</sub>s. Treatment of leukemia-bearing mice with host-matched DC<sub>VIP</sub>s and no BMS or with T cell-depleted BM cells did not affect tumor growth (not shown), indicating that graft allogeneic T cells are responsible for GVT effect. Interestingly, although acute GVHD was completely prevented, DC<sub>VIP</sub>s were less efficient in maintaining GVT response in mastocytoma-bearing mice compared to A20 leukemia, as assessed by a significant hepatosplenomegaly and body weight loss in the P815 recipients (Figure 4B). However, GVT maintenance by DC<sub>VIP</sub>s was not exclusive for A20 cells because a similar GVT response was observed in a BCL1 lymphoma model (not shown).

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the CD8 T cells obtained from untreated or DC controls-treated recipients (Figure 5A). In contrast, I-KbCD8 T cells isolated from DCVIPs-treated recipients showed reduced cytotoxic activity against the mastocytoma P815 cells (H-2d) (Figure 5A). In addition, there was no lytic activity of the I-KbCD8 T cells against EL4 cells (H-2b), indicating their H-2d-specific cytotoxicity (not shown).

Recently it has been reported that DC-activated CD8+CD44high T cells are poor inducers of acute GVHD, whereas CD8+CD44lowCD62Llow T cells are extremely potent. We investigated whether DCVIPs induce the generation of CD8+CD44high or CD44low T cells. The donor I-KbCD8 T cells isolated from mice that received transplants treated with DC controls showed a high percentage of CD44low cells similar to untreated animals (Figure 5B). Treatment with DCVIPs significantly increased the number of CD44highCD62Llow cells in the grafted CD8+ T cells (Figure 5B). Therefore, DCVIPs could be inducing/activating CD8+CD44highCD62Llow T cells in the graft that fail to generate GVHD while maintaining effective GVT activity.

DCVIPs-induced Tr cells prevent acute GVHD while maintaining GVL response

In certain circumstances, the successful suppression of an allogeneic response might require high numbers of Tr cells, and the in vivo administration of DCVIPs might be sufficient for a complete and rapid suppression. Therefore, we decided to generate DCVIPs-induced Tr cells in vitro and to determine their suppressive capacity in vivo both in a model of BMT. We generated CD4TrVIPs through the stimulation of H-2b CD4 T cells with H-2d DCVIPs. CD4Trcontrols were generated in the same manner with DC controls. Administration of CD4TrVIPs, but not CD4Trcontrols, to H-2d mice given transplants with H-2d allogeneic BMS significantly increased survival by preventing GVHD (Figure 6A left panel). The effect was haplotype-specific because mice (H-2b) given transplants with BMS (H-2d) and treated with CD4TrVIPs (H-2b) succumbed to GVHD (Figure 6A right panel). The therapeutic effect of CD4TrVIPs was dose-dependent (Figure 6B) and mediated mainly through TGF-β and IL-10 because in vivo administration of anti–IL-10 or anti–TGF-β Abs abrogated the protective effect (Figure 6C). These findings suggest that DCVIPs induce haplotype-specific CD4Tr cells, which suppress the deleterious antithost activity of the grafted allogeneic T cells.

We have recently demonstrated that VIP-differentiated human tolerogenic DCs generate CD8Tr cells, which regulate the function of allogeneic Th1 cells. Therefore, the generation of regulatory CD8 T cells could also be involved in the DCVIPs prevention of GVHD. We investigated the effect of murine CD8 T cells (H-2d) exposed in vitro to allogeneic DC controls or DCVIPs (H-2d) in the prevention of GVHD. Whereas CD8Trcontrols did not show any beneficial effect, the treatment of BMS (H-2d) transplanted mice (H-2d) with CD8TrVIPs (H-2b) significantly prevented lethal GVHD (Figure 6D). Similar to CD4TrVIPs, this effect was haplotype-specific because CD8TrVIPs (H-2d) showed little or no effect (not shown).

We investigated next whether DCVIPs-induced CD4 and CD8 Tr cells can prevent GVHD while maintaining GVT response. The tumor-bearing control group receiving neither irradiation nor undergoing allogeneic BMT died from progressive leukemia (Figure 7A none). After cotransplantation of BMS and CD4Trcontrols, all animals died within 20 days from severe GVHD without signs of tumor relapse before their GVHD death (Figure 7A). In contrast, when leukemic-bearing mice were given cotransplants with allogeneic BMS together with CD4TrVIPs, recipients were protected from GVHD, and none of them had a relapse from A20 leukemia (Figure 7A left and middle panels). Similar results were obtained in the...
BCL1 model (not shown). Transplanted allogeneic T cells are responsible for both GVHD and GVT effect. In the absence of T cells (T-depleted BM transplants) there is no GVHD but no elimination of tumor cells either, even in the presence of DC4TrVIPs (not shown). Also, if the graft contains a limited number of allogeneic T cells, CD4 TrVIPs suppress GVHD but tumor relapses occur in most animals (Figure 7A, right panel). CD8 TrVIPs behave similar to CD4TrVIPs (Figure 7B).

These results suggest that CD4/CD8 TrVIPs inhibit haplo-type-matched mature T cells present in the BM graft from initiating an alloreactive response against the host, probably by suppressing the massive expansion of alloreactive T cells, thereby permitting the cotransplantation of sufficient numbers of T cells for tumor eradication. We investigated next whether CD4TrVIPs affect CD4/CD8 cell expansion and CD8 lytic activity. In contrast to CD4Trcontrol, CD4TrVIPs inhibit the proliferation of T cells in response to allogeneic mDCs, affecting the expansion of both effector CD4 and CD8 T cells (Figure 7C). However, CD4TrVIPs did not suppress the cytotoxic activity of the CD8 T cells against tumor cells (Figure 7C).

**Discussion**

Allogeneic BMT is the treatment of choice for many hematologic malignancies and primary immunodeficiencies. GVHD is a life-threatening and frequent complication of allogeneic BMT, due to mature donor T cells present in the transplant. It has been proposed recently to use tolerogenic DCs as a therapeutic strategy to limit the pathologic effect of donor-alloreactive T cells. Although underlying mechanisms are not fully elucidated, the capacity to induced T cells is an important property of tolerogenic/regulatory DCs. In this study, we established a novel immunotherapeutic approach using the neuropeptide VIP to induce tolerogenic DCs that prevent acute GVHD while maintaining GVT effect. We previously showed that the VIP presence during the early stages of DC differentiation from human blood monocytes or from mouse BM cells leads to the generation of DCs that cannot mature following inflammatory stimuli. The DCVIPs exhibit a tolerogenic phenotype characterized by low expression of costimulatory molecules, low production of proinflammatory cytokines, and increased production of IL-10 and TGF-β, which are characteristic markers of Tr cells and mediators of their regulatory functions. The fact that in vivo TGF-β blockade and IL-10 administration reversed the therapeutic effect of DCVIPs in vivo. DCVIPs treatment of mice receiving BM transplants reduced lethal GVHD, even when administered after disease onset. Sequential administration of DCVIPs enhances their therapeutic efficacy. A hallmark of acute GVHD is the alloreactive T-cell expansion in the inflammatory environment. DCVIPs retain their capacity to induce Tr in vivo under inflammatory conditions, including autoimmune diseases, allograft rejection and acute GVHD. The in vivo efficacy of DCVIPs depends on their compatibility with the host MHC antigens. We found that DCVIPs directly inhibit CD4+CD154+ Th1 effectors, whereas increasing the levels of CD4+CD25+ CTLA4+IL-10+ Tr1-like cells within the grafted T-cell population. CTLA4, TGF-β, and IL-10 are characteristic markers of Tr cells and mediators of their regulatory functions. The fact that in vivo TGF-β/IL-10 blockade and CD25+ cell deletion reversed the therapeutic effect of DCVIPs, in...
GVHD confirmed the partial involvement of newly generated Tr cells. In agreement with this hypothesis, DCVIPs, in vitro generated TrVIPs, express high levels of CTLA4, IL-10, and membrane-bound TGF-β. In addition, our study demonstrates that treatment with TrVIPs abrogates acute GVHD in a haplotype- and TGF-β/IL-10-dependent manner. GVHD was prevented only when the administered TrVIPs were of the same haplotype as the BMT cells.

Achieving reduced GVHD lethality without sacrificing a high level of donor engraftment or an effective GVT response underscores the importance of being able to control the progression of antihost-specific T cells following transplantation. Recent publications demonstrated that the use of tolerogenic DCs or of freshly isolated Tr cells allows the control of GVHD without affecting the GVT activity against leukemic cells and lymphomas.10,11,25-27 Our results show that both host-matched DCVIPs and host-mismatched TrVIPs did not interfere with long-lasting BM engraftment, preventing lethal GVHD while permitting GVT responses. GVHD progression seems to be rather due to an excessive donor T-cell expansion and inflammatory cytokine production, a process controlled by Tr, whereas GVT activity is mediated mainly by transplanted CD8 through perforin-dependent cytotoxicity, which is not controlled by Tr cells.25 This suggests that allogeneic DC VIPs are tolerogenic primarily for CD4 T cells. Several findings support this hypothesis. Donor CD8 T cells isolated from DCVIPS-treated recipients maintain cytotoxicity against leukemic cells. In addition, TrVIPs generated with allogeneic DC VIPs reduced the expansion of CD8 T cells without affecting their lytic activity against tumor cells. As expected, GVT activity in TrVIPs-treated recipients required transplantation of sufficient numbers of alloreactive T cells containing CD8+ T cells. DCVIPs induced the expansion/activation of graft CD8+CD44highCD62Llow T cells, which represent effector/memory T cells that are defective in mediating acute GVHD but retain GVT activity.28 Preliminary experiments show that DCVIPs induce memory CD8+CD44high T cells from naive CD8+CD44low T cells in vitro, arguing against an expansion of the already existing CD8+CD44high T cells by DCVIPs (M.D., E.G.-R., A.C., unpublished results, May 2005). Finally, DCVIPs are able to induce allogeneic regulatory CD8 T cells capable of preventing acute GVHD while permitting tumor eradication.

Despite the involvement of these redundant mechanisms, the maintenance of the GVT effect by DCVIPs or TrVIPs depends on the tumor model. Whereas GVT activity was maintained for tumors such as A20 leukemia, which infiltrates BM, and BCL1 lymphoma, which primarily invades the liver and spleen, DC VIPs did not maintain the GVTS response against P815 mastocytoma. Similar results have been obtained by others for the same tumor model.25-26 Although Sato and colleagues were able to generate regulatory DCs with the capacity to maintain GVT response against P815 cells,11 because the GVTS activity against P815 mastocytoma strongly depends on alloreactive donor T cells,29 the TrVIPs-mediated decrease in donor T-cell alloreactivity could explain the abrogation of GVT against P815 cells. Indeed, CD8+ T cells isolated from transplanted mice treated with DCVIPs showed decreased cytotoxicity against P815, but not A20 cells.

Immunosuppressive therapies, traditionally focused on lymphocytes, have been revolutionized by targeting the development and key functions of DCs, and the generation of “designer” DCs using specific cytokines, or immunologic and pharmacologic reagents, such as vitamin D₃, IL-10, TGF-β, glucocorticoids, and N-acetyl-L-cysteine, alone or in combinations, has become the focus of new therapies.30 Our data demonstrate that VIP is a very efficient agent for the induction of regulatory DCs, and we propose that VIP addition to cocktails of immunomodulatory agents will increase their efficiency. The possibility of generating tolerogenic DCVIPs opens new therapeutic perspectives for the treatment of allogeneic BMT. In vitro pulsing of
tolerogenic DCViPs with alloantigens, followed by in vivo administration, leads to the differentiation of haplotype-specific T cells in the graft. Therefore, the inclusion of tolerogenic DCViPs in future therapeutic regimens may minimize the dependence on nonspecific immunosuppressive drugs used currently as antirejection therapy, facilitate the successful transplantation from mismatched donors, reduce the deleterious consequences of acute GVHD, and extend the applicability of BMT.

References

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Vasoactive intestinal peptide induces regulatory dendritic cells that prevent acute graft-versus-host disease while maintaining the graft-versus-tumor response

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