Modulation of Immune Checkpoints and Graft-versus-Leukemia in Allogeneic Transplants by Antagonizing Vasoactive Intestinal Peptide Signaling

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Abstract

The goal of allogeneic bone marrow transplantation (allo-BMT) is elimination of leukemia cells through the graft-versus-leukemia (GvL) activity of donor cells, while limiting graft-versus-host disease (GvHD). Immune checkpoint pathways regulate GvL and GvHD activities, but blocking antibodies or genetic inactivation of these pathways can cause lethal GVHD. Vasoactive intestinal peptide (VIP) is an immunosuppressive neuropeptide that regulates coinhibitory pathways; its role in allo-BMT has not been studied. We found VIP transiently expressed in donor NK, NK-T, dendritic cells, and T cells after allo transplant, as well as host leukocytes. A peptide antagonist of VIP signaling (VIPhyb) increased T-cell proliferation in vitro and reduced IL10 expression in donor T cells. Treatment of allo-BMT recipients with VIPhyb, or transplanting donor grafts lacking VIP (VIP-KO), activated donor T-cells in lymphoid organs, reduced T-cell homing to GvHD target organs, and enhanced GvL without increasing GvHD in multiple allo-BMT models. Genetic or ex vivo depletion of donor NK cells or CD8^{+} T cells from allografts abrogated the VIPhyb-enhanced GvL activity. VIPhyb treatment led to downregulation of PD-1 and PD-L1 expression on donor immune cells, increased effector molecule expression, and expanded oligoclonal CD8^{+} T cells that protected secondary allo transplant recipients from leukemia. Blocking VIP signaling thus represents a novel pharmacologic approach to separate GvL from GvHD and enhance adaptive T-cell responses to leukemia-associated antigens in allo-BMT. *Cancer Res; 76(23); 6802–15. ©2016 AACR.*

Introduction

A long-standing goal in allogeneic bone marrow transplantation (allo-BMT) has been to separate the beneficial GvL activity of donor T cells from the detrimental effects of GvHD (1). Temporal and tissue-specific modulation of costimulatory and coinhibitory pathways that regulate donor T-cell activation in response to leukemia-specific antigens and antigens expressed on host epithelial GvHD-target cells offers a strategy to activate GvL-specific T cells while limiting GvHD. VIP is an immunosuppressive neuropeptide secreted by lymphocytes and non-lymphoid cells (2, 3) that binds to G-coupled protein receptors: VPAC1 and VPAC2 expressed on T cells and dendritic cells (DC; ref. 2). T-cell activation leads to enhanced expression of VPAC2, and downregulation of VPAC1 (4). VIP activates multiple signaling pathways including cAMP-protein kinase

A (5), PI3K/PKC, and MAPK/p38 (3, 6). VIP signaling in T cells induces CD152 expression (7), and promotes Treg development (8). VIP signaling also downregulates expression of CD80/86 on DC during inflammation (9), and induces tolerogenic DCs in vitro and in vivo (10).

We have previously shown inhibition of VIP signaling expands antigen-specific T cells and improves survival after murine cytomegalovirus (mCMV) infection (11–13). Allo-BMT from VIP-KO mice lacking both the VIP gene and the related peptide histidine isoleucine (PHI) gene (14), or treating allo-BMT recipients of wild-type grafts with VIPhyb augmented adaptive donor T-cell responses to mCMV infection and a mCMV vaccine, enhancing viral clearance and survival (11, 12). VIPhyb treatment decreased PD-L1 expression on DC and PD-1 expression on CD8^{+} T cells following mCMV infection (12, 13), leading to the hypothesis that interfering with VIP signaling might improve anticancer immunity (15, 16). However, interference with PD-1/PD-L1 signaling may cause severe autoimmune disease (17), and the role for VIP signaling in allo-immune responses is unclear, as previous reports indicated that ex vivo treatment of bone marrow cells with VIP induced tolerogenic DC (18).

We report herein VIP production by donor immune cells is dynamically regulated after allo-BMT, and that transplanting VIP-KO cells, or daily treatment with VIPhyb (12, 13, 19), significantly enhanced survival of leukemia-bearing transplant recipients via a CD8^{+} T-cell–dependent GvL effect without increased GvHD in murine models of MHC mismatched allo-BMT.

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Materials and Methods

Cell lines and mice

Drs. B. R. Blazar and Y. Lu (Winship Cancer Institute, Emory University, Atlanta, GA) provided C1498-luciferase[+] myeloid and LBRM-luciferase[+] T-lymphocytic leukemia cell lines, respectively (20, 21). C57BL/6 (H-2K[b]), B6 CD45.1, B6 albino, B6 CD4-KO, B6 CD8 KO, B6 Beige, B10.BR (H-2K[k]), VERT-X, and BALB/c (H-2K[d]) mice were purchased from Jackson Laboratory. B6 TEa mice, developed by Dr. A. Rudensky (Memorial Sloan Kettering Cancer Center, New York, NY; ref. 22), were obtained from Dr. Bromberg at Mount Sinai University (New York, NY). B6 VIP/PHI KO mice (14) and wild-type littermates were obtained from Dr. Waschek at University of California, Los Angeles (Los Angeles, CA). VIP-GFP mice [MMRRC strain #31009, FVB/N-Crl:CD1(ICR)] were purchased from MMRRC, University of California, Davis (Davis, CA), and backcrossed to C57BL/6 10 generations. PCR screening identified the presence of the VIP-GFP transgene with VIP (Vip-31009 F1 5-GCTAGACCCTCTGAAATGTTGCCAA-3) and GFP (GS eGFP R3 5-GGTCGGGGTAGCGGCTGAA-3) primers and PCR. B6 GFP mice (23) and congenic CD90.1 B10.BR mice were bred at Emory University (Atlanta, GA). Male donor and recipient mice were 6–8 and 8–10 weeks old, respectively.

T-cell activation and proliferation in culture

Splenocytes (1×10^6/mL) harvested from VERT-X (IL10-GFP) or B6 mice were stimulated with PMA (10 ng, Sigma) + ionomycin (500 ng, Sigma) plus Golgi plug (BD Biosciences) with the addition of 3 µmol/L VIPhyb for 6 hours, and then stained for T-cell markers (CD3, CD4, and CD8), intracellular cytokines (IFNγ, IL4, and IL17) and analyzed by flow cytometry. Splenocytes (4×10^5) from B6 wild-type mice, VIP-KO mice, or 4×10^5 MACS column–enriched splenic T cells from luciferase[+] B6 mice were cultured with 4×10^5 irradiated (20 Gy) splenocytes from B10.BR or FVB mice in 96-well plates. VIP and/or VIPhyb (0.3–10 µmol/L) were added daily. After 3 days, T-cell proliferation was assessed by adding 0.3 µg luciferin and analyzing bioluminensence using an IVIS Spectrum instrument and Living Image Software (PerkinElmer). Antigen-specific T-cell proliferation was assessed by culturing 2.5×10^5 MACS column–enriched splenic T cells from TEa transgenic mice with 5×10^4 FACS-sorted plasmacytoid dendritic cells [pDC, lineage (CD3, NK1.1, TER119, Ig M)[-], CD11b^{-}, PDCA-1^{+}, CD11c^{lo}] or classical DC [cDC, lineage^{-}, B220^{-}, PDCA-1^{-}, CD11c^{hi}] purified from bone marrow or spleens of VIP-KO or wild-type B6 mice plus 10 nmol/L Ea 52-68 peptide (EaP, ASFEAQGALANIAVDKA).

Donor cell preparation and bone marrow transplantation

Bone marrow cells and splenocytes were harvested by flushing with sterile RPMI1640 containing 1% heat-inactivated FCS. On day 2, recipient mice were irradiated with two 5.5 Gy fractions (24). On day 1, mice were injected intravenously with 2×10^6 LBRM or 1×10^5 C1498. A total of 5×10^6 MACS T-cell–depleted bone marrow cells (TCD-BM) either from wild-type, CD4-KO, CD8-KO, or beige mice plus 0, 0.5, 1, or 3×10^6 splenocytes from wild-type B6 donors, VIP-KO splenocytes, or splenocytes that were CD4, CD8, or NK1.1 MACS-depleted were injected on day 0. Mice were monitored for GvHD using published scoring methods (25). Growth of luciferase[+] C1498 or LBRM was assessed by bioluminescent imaging (BLI) after mice were anesthetized, injected intraperitoneally with firefly luciferin

substrate (15 µg/g mouse), and imaged using an IVIS imaging system. Donor leukocyte chimerism was typically ≥ 98% beyond day +20. B6 recipient mice >60 days posttransplant without evidence of luciferase[+] C1498 were either rechallenged with 2×10^5 C1498, or euthanized and their splenocytes combined with TCD-BM from naïve mice and retransplanted into secondary recipients. B10.BR mice surviving allo-BMT >140 days from the initial transplant were rechallenged with 3.6×10^7 LBRM.

VIPhyb administration

Wild-type mice were treated with daily subcutaneous injections of VIPhyb (H2N-KPRRPYTDNYTRLRKQMAVKKYLNSILN-amide, New England Peptide; 10 µg/mouse) for 1 week (19, 26) starting either day −1 or day +6 post-BMT.

VIP expression after transplantation

Congenic B10.BR mice (H-2K[k], CD90.1) were irradiated (11 Gy), inoculated with 2×10^6 LBRM, and transplanted with 5×10^6 BM and 1×10^6 or 3×10^6 splenocytes from B6 VIP-GFP[+] mice. Control recipient mice were engrafted with T cells from B6 VIP-GFP–negative littermates. B6 syngeneic recipients were transplanted with splenocytes from VIP-GFP[+] mice and wild-type bone marrow. To enhance the GFP signal, splenocytes were cultured with PMA (10 ng, Sigma) plus ionomycin (500 ng, Sigma) and Golgi plug (BD Biosciences) for 6 hours in RPMI1640 complete media in 24-well plates. Cells were stained with mAbs against CD45.2 or H-2K[b] (donor markers), lymphoid markers, and analyzed by flow cytometry. Classical DCs and pDCs were isolated by gating CD3^{-}, NK1.1^{-}, CD11c^{hi}, B220^{-} (cDC) or CD3^{-}, NK1.1^{-}, CD11b^{-}, CD11c^{lo}, B220^{+}, PDCA-1^{+} (pDC).

Analysis of peripheral blood and spleen samples

Leukocytes in blood and spleen were counted using a Beckman Coulter device, red cell–depleted by ammonium chloride lysis, washed twice, and stained with antibodies to CD3, CD4, CD8, CD62L, CD25, CD44, PD-1, Lineage (CD3, NK1.1, IgM, TER119), CD11c, CD11b, B220, PD-L1, α4β7, CXCR3, CCR5, and CCR6 (Pharmingen) and antibodies specific for donor strain H-2K[b] or H-2K[k] as described previously (11, 12, 20, 27). Samples were acquired on a FACSAria (Beckon Dickinson) and analyzed using FlowJo software (Tree Star, Inc.).

CTL activity

B6→B10.BR transplants recipients were euthanized on day +7 following rechallenge with LBRM. Splenic T cells were isolated by MACS-negative selection using Abs to CD11b, DX5, B220, and TER119 (Pharmigen) and cultured with splenocytes from B10.BR, BALB/c mice, or LBRM tumor cells in RPMI1640 plus 10% FCS. Cytotoxic activity of 2×10^6 effector T-cells against 2×10^5 surface-labeled target cells targets was measured with the CyToxiLux PLUS kit (OncoImmunin) after incubation with the caspase substrate for 30 minutes, and the percentage of apoptotic cells was calculated using flow cytometric methods (27).

TCR deep sequencing and genotyping

Splenocytes were harvested from tumor-bearing B10.BR→B6 recipients, donor-type B10.BR mice, or B10.BR→B6 mice with clinical GvHD on day +17 following transplantation of 3×10^6 donor splenocytes. T_{CM} CD8^{+} T cells CD62L^{+}, CD44^{hi} were isolated by FACS sorting (FACSAria, BD Biosciences) and

sequencing of TCR-β CDR3 V and J sequences performed by Adaptive Biotechnologies.

Statistical analysis

Data were analyzed using SPSS version 22 for Mac and are presented as mean ± SD of all evaluable samples if not otherwise specified. Survival differences among groups were calculated with the Kaplan–Meier log-rank test in a pairwise fashion. Other data were compared using Student t test, one-way ANOVA, and non-parametric tests (Mann–Whitney U or Kruskal–Wallis H test). A P value of < 0.05 was considered significant.

Results

VIP signaling regulates T-cell proliferation induced by allo antigen

We first examined the effect of VIPhyb on cytokine production of PMA/ionomycin stimulated T cells. IL10 expression in CD4 T cells was significantly reduced by VIPhyb compared with control cultures (Fig. 1A and B). To explore the role of VIP signaling in allo-immune responses, we performed one-way mixed lymphocyte reactions (MLR) containing VIP and/or the antagonistic VIPhyb peptide. Addition of VIP decreased luciferase[+] T-cell proliferation in a dose-dependent manner, while adding VIPhyb increased T-cell proliferation (Fig. 1C–E). VIPhyb reversed the suppressive effect of VIP in MLR, restoring T-cell proliferation to higher levels than control cultures. To examine antigen specific allo-immune responses, we measured proliferation of transgenic B6 TEa CD4^{+} T-cells (recognizing a H-2K[d] MHC-II peptide presented on H-2K[b] MHC-II) cultured with peptide-pulsed B6 pDC or cDC from wild-type or VIP-KO mice. TEa T-cell proliferation was greater with VIP-KO pDC compared with control pDC (Fig. 1F and G), but did not vary between peptide-pulsed VIP-KO and wild-type cDC (Fig. 1H). These in vitro data are consistent with an immunosuppressive role of VIP, and suggest that VIPhyb antagonizes the effect of local synthesis of VIP by pDC during initial antigen presentation.

VIP expression is upregulated in donor immune cells following allo-BMT

Given the very short half-life of native VIP in vivo (28, 29), we measured GFP expression in donor VIP-GFP transgenic cells in which the GFP gene is downstream of the VIP promoter (30) in two BMT models: syngeneic (H-2K[b] CD45.2→H-2K[b] CD45.1) and allogeneic (H-2K[b]→H-2K[k]; Fig. 2A–C). There was minimal GFP expression in donor B cells and CD4^{+} T-cells in both allo- and syngeneic-BMT recipients (Fig. 2B and C). Transient GFP expression was seen in donor NK cells, NKT cells, CD11b^{+} myeloid cells, and pDC in the first week post-BMT, and in CD8^{+} T-cells on day +20 post-BMT. Donor NK cells and pDC in leukemia-bearing mice had somewhat higher levels of GFP expression compared with the same cell populations from nonleukemic allo-BMT recipients (Fig. 2B and C). In vitro experiments using one-way MLR with VIP-GFP splenocytes as responding cells confirmed that the VIP gene is activated in DC and NK T cells during inflammation (Fig. 2D).

To determine the cellular source of VIP, we transplanted FACS-purified LKS HSC, unfractionated bone marrow, or splenocytes cells from VIP-GFP donors admixed with complementary populations of splenocytes, bone marrow, or HSC from VIP-KO mice. Donor pDCs from the bone marrow or splenocyte graft, as well as donor-derived pDCs from sorted LKS HSC produced VIP on day +7 after transplant, with less VIP produced by donor T cells, B cells, cDCs, and CD11b^{+} non-DC subpopulations (data not shown).

To distinguish VIP produced by host versus donor cells, we performed allogeneic transplants from wild-type mice into VIP-GFP recipients, and found that rare CD45^{+} VIP-GFP^{+} host-type cells were seen in the liver on day +7 after transplant with larger numbers of VIP-GFP^{+} host-type leukocytes in the spleen (data not shown). These data indicate that production of VIP by both donor and host leukocytes may be important in regulating of donor T-cell allo-reactivity.

Reducing VIP signaling enhanced the GvL effect of allo-BMT without increasing GvHD

As VIP is immunosuppressive (2, 3) and the GvL effect of allo-BMT is predominantly mediated by donor T cells (1), we next determined the effect of inhibiting VIP signaling on GvL and GvHD in murine allo-BMT models. We treated recipients of wild-type B6 splenocytes and TCD-BM with seven daily injections of VIPhyb or transplanted VIP-KO B6 splenocytes with wild-type B6 and TCD-BM in the B6→B10.BR +LBRM (T-cell lymphoblastic leukemia) model. All mice transplanted with TCD-BM alone died from progressive growth of luciferase[+] leukemia within 60 days (Fig. 3A and D). Transplanting low numbers of wild-type donor T-cells (0.5 × 10^{6} splenocytes) resulted in >60% survival in VIPhyb-treated mice, versus 20% survival in recipients of VIP-KO grafts, and 17% survival in PBS-treated mice (Fig. 3A, D and E). Transplanting an intermediate T-cell dose (1 × 10^{6} splenocytes) resulted in 60% survival in VIPhyb-treated mice, 90% survival in recipients of VIP-KO grafts and 20% survival in PBS-treated recipients of wild-type grafts (P < 0.001, Fig. 3B and D). Transplanting high-dose splenocytes (3 × 10^{6}) caused early GvHD-related mortality in all treatment groups (Fig. 3C and D).

To study the effect of VIhyb on GvHD, we used the same B6→B10.BR model without LBRM leukemia and found equivalent survival and GVHD clinical scores across a range of donor T-cell doses comparing VIPhyb-treated and PBS-treated mice and recipients of wild-type or VIP-KO splenocytes (Supplementary Fig. S1A–S1F). Treating nonleukemia bearing B6→B10.BR recipients of 5 × 10^{6} bone marrow and 3 × 10^{6} splenocytes from either wild-type or VIP-KO donors with 7 daily doses of VIP or VIPhyb starting from one day before transplant or starting 6 days after transplant led to no significant differences in survival or GvHD clinical scores (Supplementary Fig. S1G–S1I).

Measuring tumor burden by post-transplant BLI showed fewer mice with detectable luciferase[+] LBRM, and slower leukemia growth in VIPhyb-treated mice and in recipients of VIP-KO grafts compared with saline-treated mice (Fig. 3D and E). Of note, VIPhyb had no direct antiproliferative activity against LBRM in vitro using a range of concentrations that exceeded the predicted peak in vivo concentration of VIPhyb (0.6 µmol/L, Supplementary Fig. S2).

We next performed B10.BR→B6 albino transplants and measured GvL against C1498, a B6 myeloid leukemia cell line (21) to test whether the effect of VIPhyb treatment on GvL was generalizable across transplant models. VIPhyb was administered early (days −1 to +5) or late post-transplant
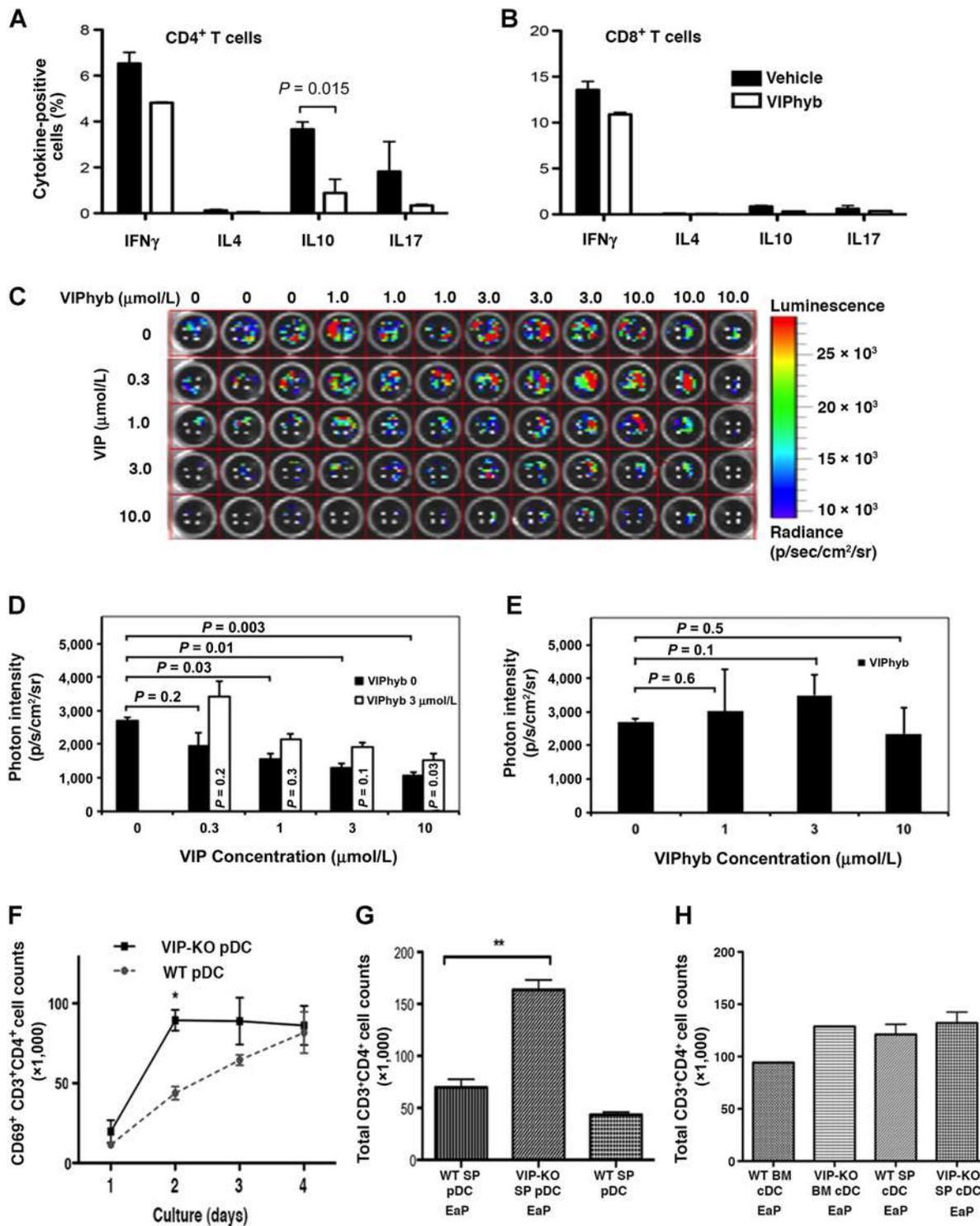
Figure 1.
VIPhyb treatment augmented *in vitro* T-cell proliferation stimulated by alloantigen. Cytokine expression in CD4 (**A**) and CD8 (**B**) T cells measured by flow cytometry after 6 hours stimulation of 1×10^6 VERT-X splenocytes/mL with PMA and ionomycin, with or without addition of VIPhyb (3 μmol/L). **C–E,** Proliferation of luciferase[+] B6 splenic T cells cultured for 3 days in MLR with irradiated FVB splenocytes, with daily addition of VIP and/or VIPhyb to achieve the concentrations shown (0–10 μmol/L). MLRs were initiated with 2×10^6 cells/mL for both cell types, and BLI measured on day 3. **F–H,** Numbers of activated CD69^{+} transgenic TEa T cells (**F**) and total CD4^{+} T cells (day 3; **G** and **H**) cultured with pDCs (**G**) or cDCs (**H**) purified from bone marrow (BM) or spleen of VIP-KO or wild-type mice and loaded with 10 nmol/L EaP peptide, specific for TEa CD4 T cells. Data are representative of three replicate experiments. Mean values and SD from triplicate samples at each time point are shown.
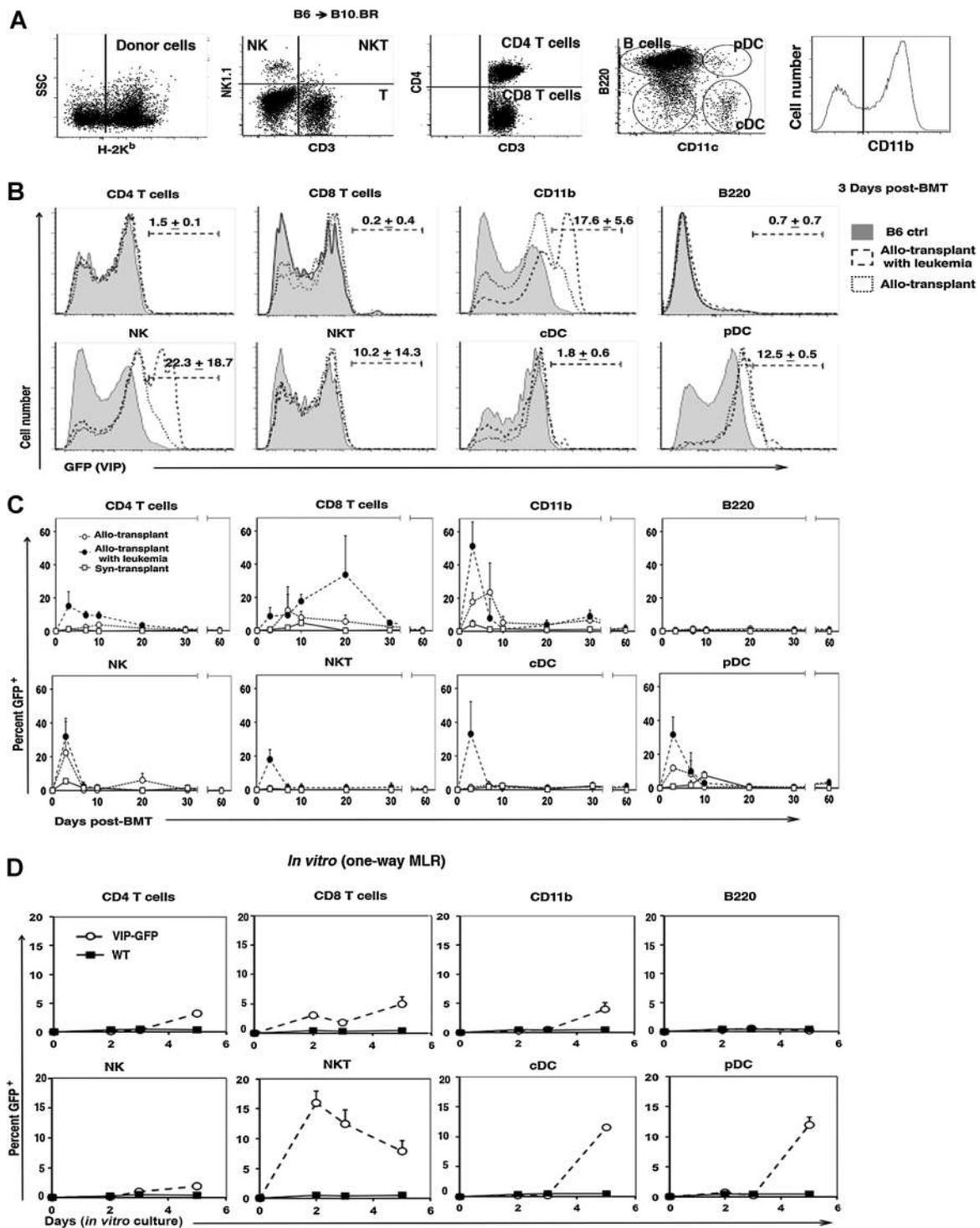
Figure 2.
VIP expression is induced in donor immune cells after allo-BMT. B10.BR congenic (CD90.1, H2K^K) mice received 11 Gy irradiation on day −2 and were transplanted with 5×10^6 bone marrow cells plus 1×10^6 splenocytes from allogeneic B6 VIP-GFP$^+$ or VIP-GFP negative littermates, with LBRM cells injected on day −1. Syngeneic B6 CD45.2→CD45.1 transplants were also performed. Splenocytes from recipients were analyzed by flow cytometry after a 6-hour incubation with 10 ng/mL PMA, 500 ng/mL ionomycin and Golgi plug. **A,** Gating strategy of allogeneic donor (H-2K^b) immune cells NK, NKT, CD4$^+$ T cells, CD8$^+$ T cells, B cells, pDCs, cDCs, and CD11b$^+$ myeloid cells. **B,** VIP-GFP expression by donor-derived cells 3 days post-BMT. **C,** Kinetics of VIP-GFP$^+$ expression in donor cells comparing syngeneic-BMT recipients and allo-BMT recipients ± LBRM. **D,** VIP-GFP expression from 3 day one-way MLR. A total of 2×10^6 VIP-GFP$^+$ B6 splenocytes or WT control B6 splenocytes cultured with equal numbers of irradiated B10.BR splenocytes. Data are mean values from a pool of four mice and are representative of four replicate experiments.
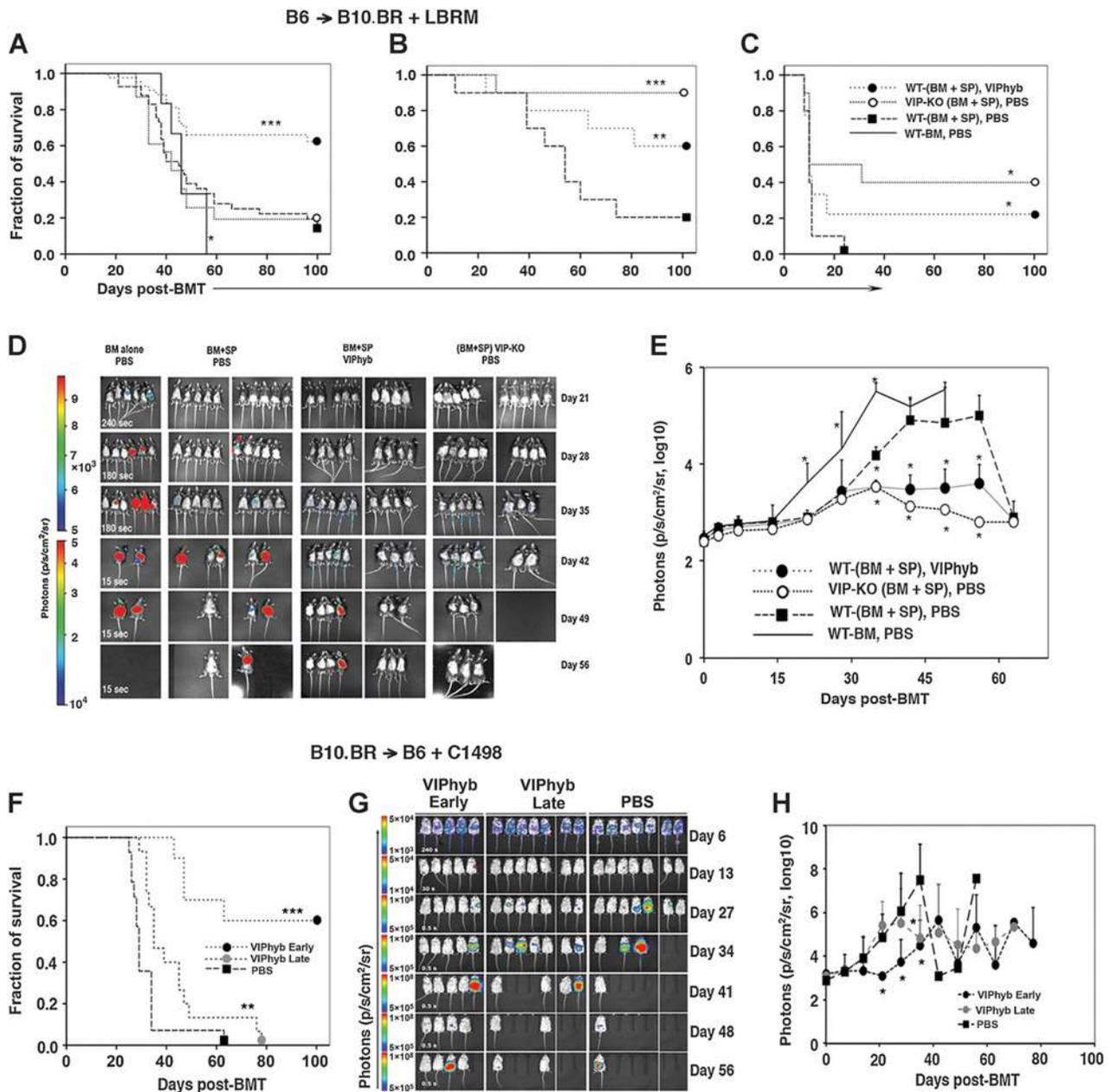
Figure 3.
Reduced VIP signaling enhanced the GvL effect of allo-BMT against lymphoblastic leukemia. B10.BR mice and B6 albino mice were irradiated on day −2, injected with luciferase[+] LBRM and luciferase[+] C1498, respectively, and transplanted as described in Materials and Methods. **A,** B10.BR recipients transplanted with 5×10^6 B6 TCD-BM plus 0 or 0.5×10^6 splenocytes, [bone marrow (BM) alone, $n = 6$; 0.5×10^6 splenocytes: VIPhyb-treated, $n = 43$; PBS-treated, $n = 41$; VIP-KO, $n = 23$). **B,** B10.BR recipients transplanted with 1×10^6 splenocytes (VIPhyb-treated, $n = 20$; PBS-treated, $n = 40$; VIP-KO, $n = 10$). **C,** B10.BR recipients transplanted with 3×10^6 splenocytes, (VIPhyb-treated, $n = 9$; PBS-treated, $n = 10$; VIP-KO, $n = 10$). Data are from two or three replicate experiments. **D,** BLI from a representative experiment using bone marrow (BM) alone or 0.5×10^6 splenocytes. BLI was measured for 3 minutes up to day +28 (scale 0.5 to 1×10^4 p/s/cm²/sr), or for 1 minute subsequently (scale 1 to 5×10^4 p/sec/cm²/sr). **E,** Mean photon counts (p/sec/cm²/sr) of abdomen from three replicate BLI experiments. **F,** Survival of B6 albino mice transplanted with 5×10^6 TCD-BM plus 1×10^6 splenocytes from B10.BR donors. Data were pooled from two replicate experiments of VIPhyb early treatment (day−1 to day +5), $n = 10$; late treatment (day +7 to day +13), $n = 15$; or PBS treatment, $n = 15$. **G,** BLI from one of two replicate experiments. **H,** Mean photon counts (p/sec/cm²/sr, log) from BLI of 2 B10.BR→B6 albino replicate experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, comparing with recipients of wild-type donors treated with saline.

(days +7 to +13; ref. 31). Both groups of VIPhyb-treated mice had significantly better survival compared with saline-treated recipients, with 60% tumor-free survival in mice treated early with VIPhyb, ($P < 0.001$); and a median survival time of 35 days in mice treated later with VIPhyb versus 29 days in saline-treated controls ($P = 0.005$; Fig. 3F). BLI imaging of

luciferase[+] C1498 in the B10.BR→B6 transplant model showed less leukemia-BLI signal in VIPhyb-treated groups compared with saline-treated control mice (Fig. 3G and H). Recipients treated early with VIPhyb had durable leukemia-free survival, further suggesting that blocking VIP signaling early during donor T-cell activation led to elimination of residual leukemia cells.

VIPhyb treatment reduced homing of donor T cells to GvHD target organs

To explore why interference with VIP signaling augmented GvL activity of donor cells without increasing GvHD, we next measured the effects of VIPhyb treatment on homing and expansion of donor T cells in mice transplanted with luciferase[+] donor T cells combined with wild-type TCD-BM cells. Whole-animal BLI showed no significant differences between VIPhyb-treated mice (Fig. 4A and B) and PBS-treated controls on day +7 after transplant. Ex vivo analysis of GvHD-target organs at necropsy showed increased BLI signals in Peyer patches and mesenteric lymph nodes in saline-treated animals compared with VIPhyb-treated mice (Supplementary Fig. S3A). VIPhyb treatment led to initial lower BLI signals in the gut and liver, and increased signals in the spleen of transplant recipients compared with saline-treated controls (Supplementary Fig. S3B). Similar BLI signals of donor T cells in isolated organs were seen on day +14 after transplant, comparing VIPhyb- and saline-treated animals (Supplementary Fig. S3B), with increased numbers of donor-derived cells in spleen, and reduced numbers in the liver of VIPhyb-treated recipients (Supplementary Fig. S3C and S3D). To explore the basis for differences in donor T-cell homing in VIPhyb-treated mice, donor CD8^{+} T cells were harvested from the spleen and liver on days +7 and +14 after transplant and chemokine receptor expression was measured by flow cytometry. T cells from VIPhyb-treated mice had higher levels of α4β7, CXCR3, CCR5, and CCR6 on donor CD4^{+} and CD8^{+} T-cells in the spleen, and lower levels of α4β7, CXCR3, CCR5, and CCR6 expression in the liver compared with T cells from saline-treated mice (Fig. 4C). Taken together, these data indicate that VIPhyb treatment altered chemokine receptor expression to retain activated donor T cells in lymphoid organs and reduce homing to GvHD target organs (32).

VIPhyb treatment reduced immune checkpoint and enhanced effector molecule expression

To characterize the effect of VIPhyb on immune effector mechanisms, we measured immune checkpoint molecules, intracellular cytokines, costimulatory ligands, and receptors in donor T cells and DC in VIPhyb- and saline-treated mice in the B6→B10.BR allo-BMT model. Splenic CD4^{+} and CD8^{+} T cells from leukemia-bearing mice treated with early VIPhyb (day −1 to day +5) expressed lower levels of PD-1 compared with T-cells from saline-treated mice at most time points after transplant (Fig. 5A and B). Conventional DCs and pDCs from VIPhyb-treated mice had reduced expression of PD-L1 compared with saline-treated control mice (Fig. 5C and D). Splenic CD8^{+} T cells from leukemia-bearing mice treated with VIPhyb expressed higher levels of ICOS, IFNγ, TNFα, and granzyme B, and lower levels of CD152, Tim-3, and PD-L1 compared with T cells from saline-treated mice (Supplementary Fig. S4A). Both cDCs and pDCs from VIPhyb-treated mice had increased expression of CD80, CD86, ICOS-L, and MHC-II (Supple-

mentary Fig. S4B). These data indicate that VIPhyb treatment suppressed upregulation of immune checkpoint molecules in donor DC and T cells and enhanced expression of Th1 cytokines and effector molecules in allo-BMT recipients.

Donor CD8^{+} T cells and NK cells mediated anti-leukemia activity following VIPhyb treatment

To identify the donor cells responsible for the increased GvL activity observed with VIPhyb treatment, we depleted CD4^{+} T cells, CD8^{+} T cells, or NK cells from donor splenocytes using magnetic-activated cell sorting (MACS) and transplanted the remaining splenocytes in combination with TCD-BM in the B10.BR→B6+C1498 leukemia. VIPhyb-treated recipients transplanted with CD4-depleted splenocytes had 86% survival, comparable with 80% survival in VIPhyb-treated recipients transplanted with unfractionated splenocytes (Fig. 6A). In contrast, MACS removal of CD8^{+} cells or NK cells from donor splenocytes resulted in a significant decrease in the GvL effect (29% and 33% survival, respectively), comparable with survival in control mice that received unfractionated splenocytes and no VIPhyb treatment (Fig. 6A and B).

To confirm the role of donor CD8^{+} T cells on the GvL-promoting effect of VIPhyb, we repeated GvL experiments in the B6→B10.BR +LBRM allo-BMT model using CD4-KO, CD8-KO, Beige, and B6 wild-type mice as donors. VIPhyb-treated recipients engrafted with cells from CD4-KO donors had similar survival and weight gain as VIPhyb-treated recipients of wild-type cells, while VIPhyb-treated recipients of grafts from Beige (NK-cell deficient) or CD8-KO donors had lower survival compared with recipients of wild-type splenocytes ($P = 0.12$ and $P < 0.001$, respectively) (Fig. 6C–E). Taken together, these data indicate that donor CD8^{+} and NK1.1^{+} T cells are necessary to manifest the full GvL-promoting effects of VIPhyb treatment.

VIPhyb treatment led to expansion of anti-leukemia CD8^{+} effector T cells

To address the effects of treatment with VIPhyb on memory CD8^{+} T cells with GvL activity, B6→B10.BR transplant recipients in remission by BLI imaging following initial treatment with VIPhyb or saline (as in Fig. 4A and B) were rechallenged with an 18-fold higher dose of LBRM (3.6×10^7) 140 days post-BMT. Seven days later, the cytotoxicity of splenocytes against B10.BR splenocytes (recipient), BALB/c splenocytes (third party), or cultured LBRM cells was measured. LBRM-specific lytic activity, but not lytic activity against recipient-type or third-party targets, was significantly enhanced in splenocytes harvested from VIPhyb-treated recipients (Fig. 7A). The remaining VIPhyb-treated mice had 100% survival following rechallenge with luciferase[+] LBRM (no detectable bioluminescence signals) compared with 40% survival among transplant recipients initially treated with saline then rechallenged with high-dose LBRM (Supplementary Fig. S5A and S5B). To confirm these results in a different BMT model, we rechallenged tumor-free B10.BR→B6 recipients 60 days after transplant with a 2-fold higher dose of C1498. VIPhyb-treated recipients had higher survival (72%, Supplementary Fig. S5C) than PBS-treated recipients (≤20%, Fig. 3F and A).

We then assessed whether VIPhyb treatment led to expansion of memory T cells with long-term GvL activity. Splenocytes were harvested on day +60 from tumor-free B10.BR→B6 +C1498 transplant recipients initially treated with VIPhyb
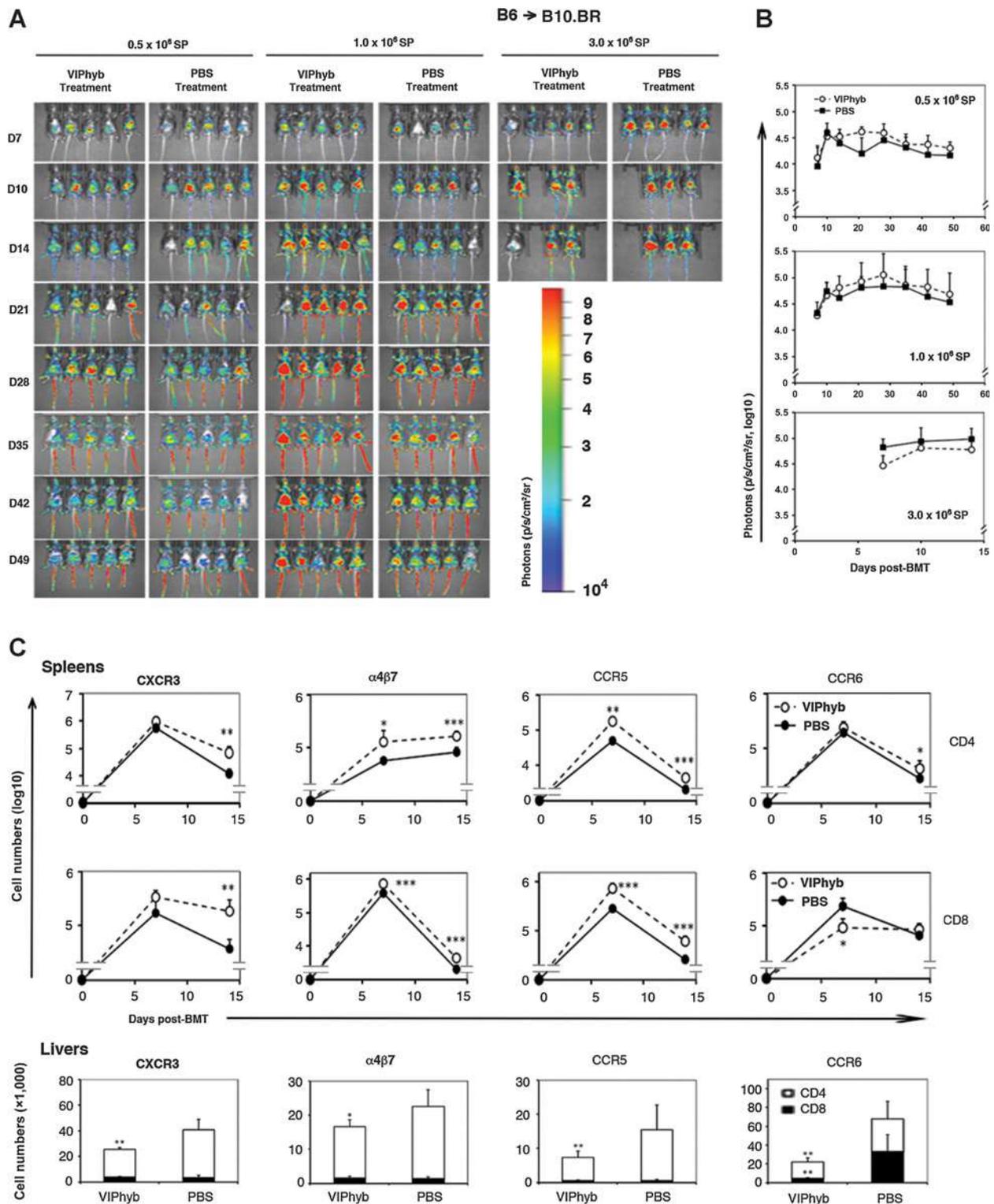
Figure 4.
Limited GvHD activity after VIPhyb treatment associated with redirected T-cell homing. B10.BR congenic (CD90.1, H2K^K) mice were transplanted with 5 × 10^6 B6 BM (CD45.1, H2K^b) plus 0.5, 1, or 3 × 10^6 splenocytes from B6 luciferase$^+$ mice (CD45.2, H2K^b) and treated with VIPhyb or PBS as described in Materials and Methods. **A,** BLI of mice. Data are representative of two experiments with 5 mice at each time point per group. **B,** Photon emission rates from BLI in the abdomen. **C,** α4β7, CXCR3, CCR5, and CCR6 expression on donor CD4$^+$ and CD8$^+$ T cells in the spleens and the livers of transplant recipients 7 and 14 days post-BMT. Data are pooled from four mice at each time point per group and are representative of two experiments. Data are shown on a log scale with the day 0 time point representing absence of donor cells in the spleen prior to transplantation. *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with PBS control.
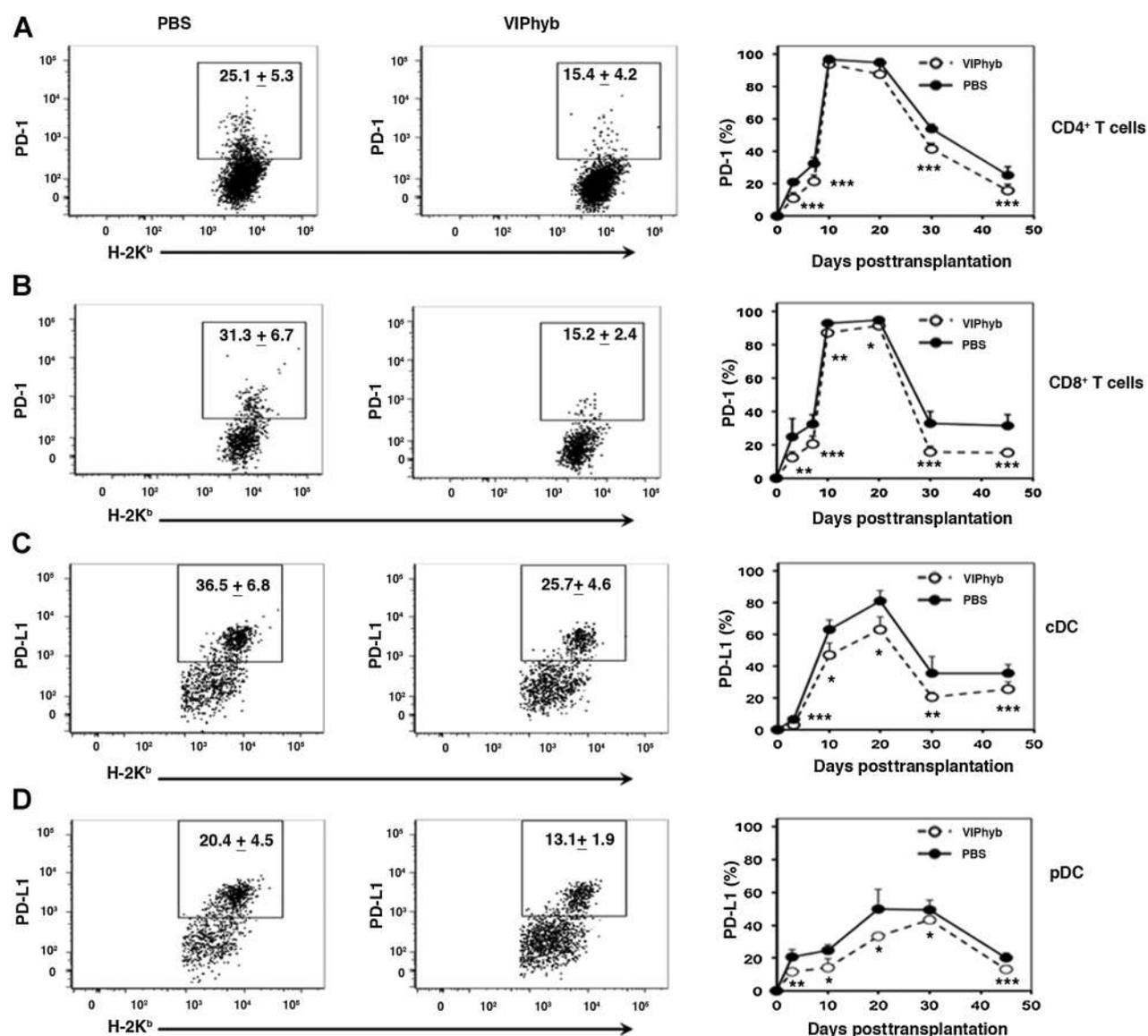
Figure 5.
VIPhyb treatment reduced expression of coinhibitory molecules on donor immune cells. B10.BR mice were irradiated, inoculated with LBRM, transplanted with 5×10^6 BM plus 1×10^6 splenocytes from B6 wild-type donors, and treated with VIPhyb or saline (day −1 to day +5) as described in Materials and Methods. Splenocytes were harvested from euthanized mice post-BMT and analyzed by flow cytometry, $n = 8$ per time point per group. Data were pooled from two replicate experiments and mean values are presented. In flow cytometry dot plots, expression of PD-1 on CD4$^+$ and CD8$^+$ T cells, and PD-L1 on cDCs and pDCs are shown on donor H-2K^b–gated cells. **A** and **B,** Expression of PD-1 on activated (CD25$^+$, CD69$^+$) donor CD4$^+$ and CD8$^+$ T cells. **C** and **D,** PD-L1 expression on donor cDCs and pDCs. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, comparing cells from VIPhyb-treated recipients and cells from saline-treated recipients.

and transplanted with B10.BR TCD-BM into secondary transplant recipients. Control groups received donor splenocytes from immunologically naïve B10.BR donors in combination with TCD-BM. Secondary transplant recipients challenged with $2\times$ dose of C1498 had higher survival (70%) compared with mice transplanted with immunologically naïve B10.BR splenocytes and bone marrow (8% survival, $P = 0.001$; Supplementary Fig. S5D).

T cells recovered from VIPhyb-treated transplant recipients 7 days following leukemia rechallenge expressed higher levels of CD107a and granzyme B, and lower levels of PD-1 compared with T cells from transplant recipients initially treated with saline (Supplementary Fig. S6A–S6C) while cDCs and pDCs had increased expression of MHC-II and reduced expression of PD-L1 (Supplementary Fig. S6D and S6E). Prior VIPhyb treatment did not affect the numbers of Treg, but increased the frequency of donor-derived CD8$^+$ memory T cells (Supplementary Fig. S7A–S7C).

Deep sequencing of CDR3 in the TCR-β gene characterized the CD8$^+$ T-cell anti-leukemia response in VIPhyb-treated
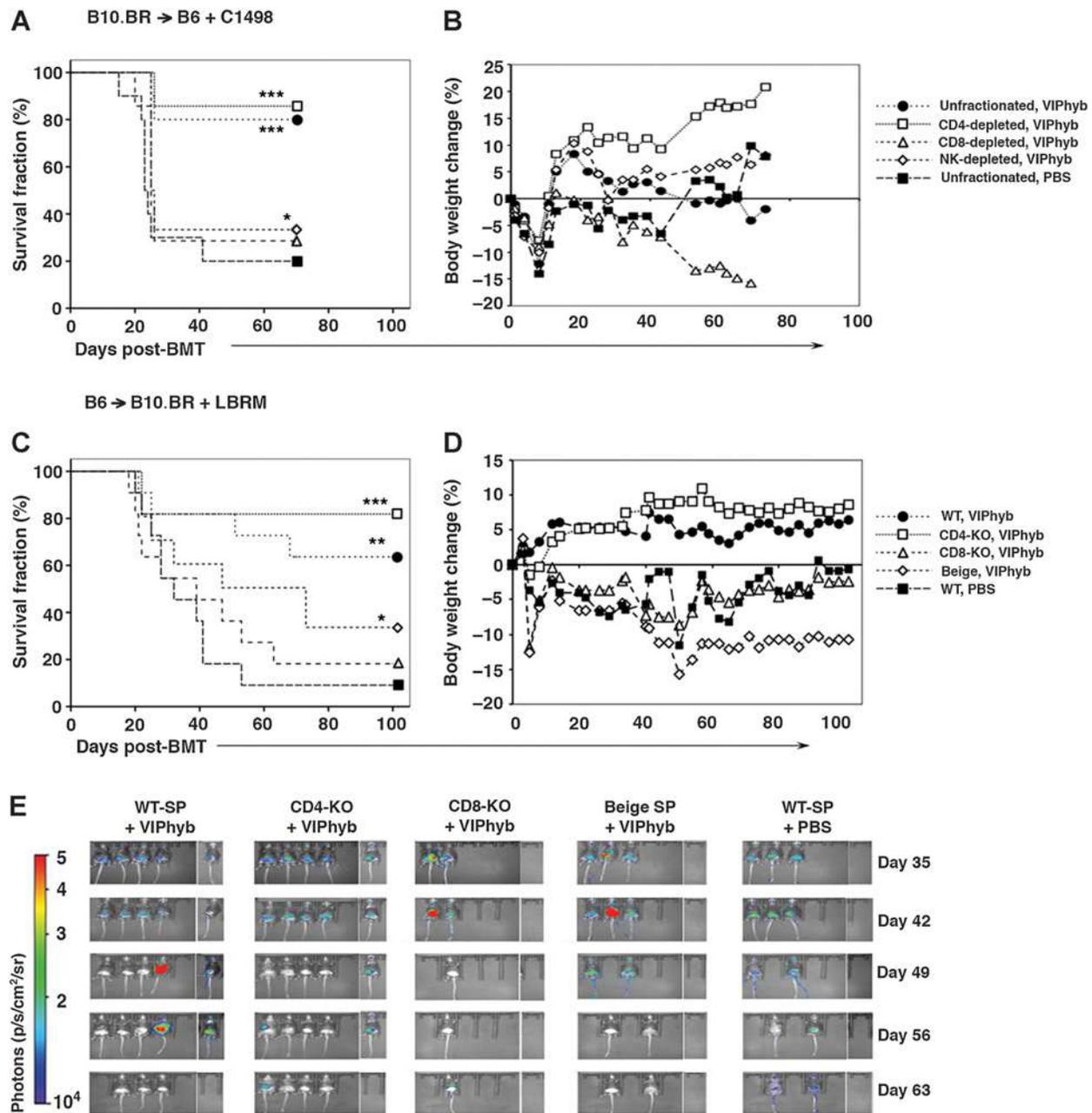
Figure 6.
Donor CD8$^+$ T-cells and NK cells are critical to VIPhyb-enhanced GvL activity in allo-BMT. B6 and B10.BR transplant recipients were irradiated, treated with VIPhyb or with saline for 7 days, inoculated with C1498 or LBRM, and transplanted with B10.BR or B6 donor cells as described in Materials and Methods. **A** and **B,** Survival and body weight change for groups using the B10.BR→B6 + C1498 model. B6 mice received bone marrow cells plus splenocytes from B10.BR donors that were unfractionated or MACS-depleted of CD4$^+$ T cells, $n = 11$; CD8$^+$T cells, $n = 11$; or NK cells, $n = 11$. Data were pooled from two replicate experiments. **C** and **D,** Survival and body weight change for groups using the B6→B10.BR + LBRM model. B10.BR mice were transplanted with 5×10^6 MACS TCD-BM cells and 1×10^6 of the corresponding splenocytes from either wild-type, $n = 10$; CD4-KO, $n = 7$; CD8-KO, $n = 6$; or beige, mice, $n = 7$. **E,** BLI from one of two replicate experiments using B6→B10.BR + Luc$^+$-LBRM. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$; comparing recipients of VIPhyb-treated grafts to recipients of saline-treated unmanipulated wild-type grafts.

B10.BR→B6 transplant recipients. CD8$^+$ T$_{CM}$ cell subsets were sorted from the spleens of leukemia-free transplant recipients 7 days after day +60 rechallenge with C1498, from B10. BR→B6 mice with clinical GvHD on day +14 (no leukemia), and from immunologically naïve B10.BR splenocytes (Fig. 7B).

The pattern of Vβ and Jβ TCR genes expressed in FACS-sorted CD8$^+$ T$_{CM}$ from GvL, GvHD, and donor mice were strikingly different (Fig. 7C and D). Recipient mice with GvHD shared 4 (40%) of the 10 most frequent TCR-β clones with T cells from donor mice, while T$_{CM}$ from VIPhyb-treated GvL mice
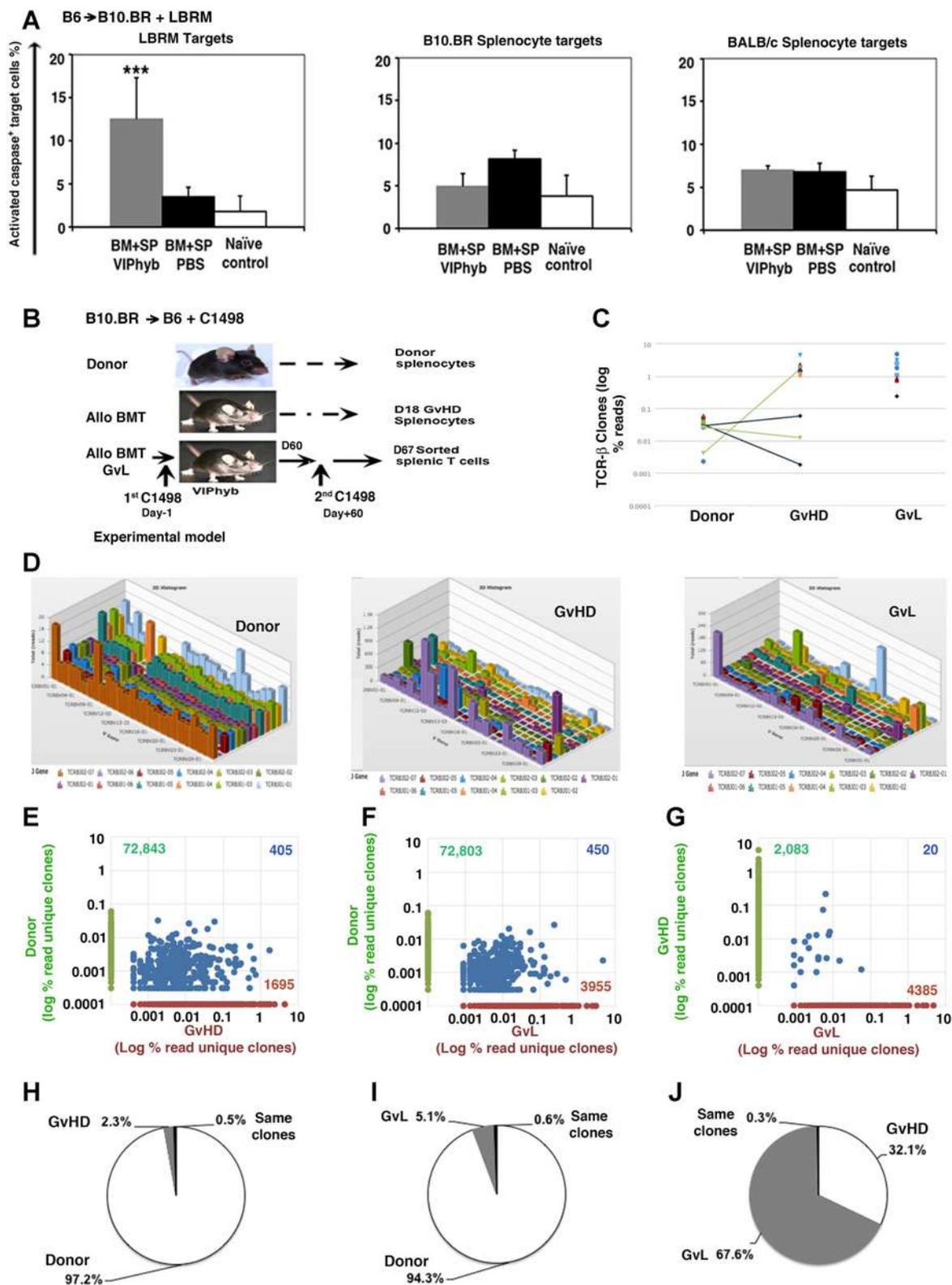
Figure 7.
VIPhyb treatment increased anti-leukemia cytotoxicity and expansion of oligoclonal T cells. Leukemia-free B10.BR and B6 transplant recipients were rechallenged with 3.6×10^7 LBRM cells on day +140 or 2×10^5 C1498 on day +60 post-BMT, respectively, and splenocytes harvested 7 days later. CD62L^{−}, CD44^{hi} CD8 T cells were isolated by FACS from B10.BR→B6 mice. (*Continued on the following page.*)

had unique Vβ and J sequences (Fig. 7C, E–J; Supplementary Table S1). Oligoclonality was significantly higher in T cells from mice with GvHD or GvL compared with T cells from donor mice (Fig. 7E–J). Novel TCR-β clones from T cells of GvL recipients were twice as frequent as unique T-cell TCR-β sequences from T cells of mice with severe GvHD (Fig. 7I and J).

Discussion

Previous studies have shown that VIP suppresses T-cell–mediated immunity (7, 10). We found VIP transiently expressed by donor immune cells in allogeneic but not syngeneic BMT recipients. Short-term treatment with a VIP antagonist during the first week post-transplant, or transplantation of VIP-KO splenocytes, markedly increased the anti-leukemic activity of donor T cells and led to durable leukemia-free survival in allo-BMT recipients without increased GvHD. VIPhyb-treated mice had similar donor T-cell chimerism compared with saline-treated mice, but had increased numbers of donor CD8$^+$ T cells in the spleen and reduced numbers of donor T cells in liver and gut, consistent with altered chemokine receptor expression changing donor T-cell homing. VIPhyb treatment led to persistent changes in the balance between costimulatory and coinhibitory signals, including decreased PD-L1 and increased CD80/86 levels on donor pDCs; decreased levels of PD-1 and PD-L1 on donor T cells; and increased expression of IFNγ, TNFα, granzyme B, and ICOS on donor T cells. These data indicate that short-term blockade of VIP signaling in the peritransplant period reprogrammed adaptive immunity and improved the anti-leukemia therapeutic index of donor T cells (33).

Experiments using donor splenocytes depleted of or lacking specific T-cell subsets indicated that CD8$^+$ T cells were critical for the enhanced GvL activity seen following VIPhyb treatment. Donor CD4$^+$ T cells were dispensable for enhanced antitumor activity seen with VIPhyb treatment, and recipients of donor CD4-KO grafts and CD4-depleted splenocytes had slightly better survival than VIPhyb-treated recipients of nondepleted wild-type grafts, suggesting that removal of CD4$^+$ Treg/Th2 from the graft (or transplanting relatively more CD8$^+$ T cells) may further enhance the anti-leukemia effect of VIPhyb treatment. Removal of NK1.1$^+$ cells using MACS and transplanting donor cells from beige mice resulted in significantly decreased GvL activity in VIPhyb-treated transplant recipients. As VIP suppresses NK-cell activity (34), these data are consistent with VIPhyb treatment enhancing the GvL activity of donor NK cells and donor CD8$^+$ T cells (35) by antagonizing the effect of paracrine VIP (Fig. 2).

Activation of anti-leukemic donor CD8$^+$ T cells entails priming by DC cross-presenting leukemia-associated antigens

(36, 37) and upregulation of chemokine receptor expression (38). Blocking VIP signaling reduced homing of donor T cells to the liver and gut, especially CCR5$^+$ cells in the liver. Mechanisms for how VIP blockade changes T-cell homing patterns are likely complex. VIP decreases Th1 chemokine expression (CCR5 ligands, CCL3, CCL4, and CCL5 expression; ref. 3) and deactivates CCR5 signaling by dephosphorylation of CCR5 (39), consistent with our observation of increased retention of CCR5$^+$ donor T cells in lymphoid organs of VIPhyb-treated mice. Furthermore, changes in splanchnic circulation induced by VIP may be relevant, as VIP reduces blood flow to the spleen (40, 41). Thus VIPhyb treatment led to retention of activated T cells in lymphoid organs, and reduced their homing to GvHD target organs (42).

VIPhyb treatment led to increased levels of MHC-II, CD80, and CD86 expression, and reduced PD-L1 expression on DCs, with lower PD-1 expression on donor T cells. Of note, endogenous VIP expression was upregulated in NK cells and pDCs before VIPhyb treatment led to downregulated PD-1 and PD-L1 expression on T cells and DCs, suggesting that VIP is part of an immune checkpoint pathway that limits productive activation of memory CD8$^+$ T cells. This hypothesis is supported by in vitro data from cocultures of transgenic TEa T cells with peptide-pulsed pDCs that showed greater in vitro expansion of TEa T cells cocultured with pDCs from VIP-KO mice versus wild-type mice. Furthermore, delayed treatment with VIPhyb was less effective than early treatment in enhancing GvL activity, suggesting initial cross-presentation of peptide antigen by DCs is the critical event during which blocking VIP signaling enhances immune activation of T cells (43).

Previous reports of a direct antitumor effect of VIPhyb (19) were not seen with the two leukemia cell lines used in the current study. While an earlier report used ex vivo VIP treatment to induce tolerogenic DCs that were adoptively transferred to allo-BMT recipients (18), our experimental models utilized a short course of in vivo treatment with VIPhyb to enhance GvL activity of Th1 polarized CD8$^+$ central memory T cells (44) with TCR-β patterns distinct from those of CD8$^+$ T cells from transplant recipients with GvHD (45, 46). Deep-sequencing data showing expansion of individual T-cell clones to 5%–10% of total CD8$^+$ T cells suggest selection and expansion of T cells directed to immune-dominant leukemia-associated antigens. Of note, equivalent GvL activity in VIPhyb-treated mice was seen against the original C1498 and LBRM cell lines compared with their luciferase$^+$ counterparts, indicating that the GvL effect was not limited to the luciferase xenoantigen.

Previous reports showed that genetic loss of VIP and the related pituitary adenylate cyclase–activating polypeptide (PACAP) reduced T-cell infiltration into the CNS and conferred to resistance to experimental autoimmune encephalomyelitis (47), and that

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(*Continued.*) **A,** Cytotoxic activity of splenocytes from B6→B10.BR recipients assessed by caspase-3 activation in targets. Mean percentages (±SD) of caspase$^+$ targets following 30 minutes at an effector:tumor (E:T) ratio of 10:1 (3–5 mice per group). ***, $P < 0.001$, comparing VIPhyb-treated and saline-treated groups. **B,** Experimental model for GvL and GvHD mice. **C,** Top 10 unique TCR-β clones (V and J sequences) in donor splenocytes, splenocytes from mice with GvHD, and splenocytes from mice with GvL after VIPhyb-treatment and leukemia rechallenge. Different symbols represent the different top TCR-β clones. **D,** Numbers of unique V/J TCR-β reads from donor, GvHD, or GvL mice. **E** and **H,** Frequencies of unique V/J TCR-β clones from donor versus GvHD mice. **F** and **I,** Donor versus GvL mice. **G** and **J,** GvHD mice versus GvL mice. Points lying on the *x*-axis or *y*-axis represent nonshared clones; shared clones lie between the *x*-axis and *y*-axis.

VIP treatment reduces both autoimmune and inflammatory components of experimental arthritis (48). These data suggest distinct pathologic mechanisms for VIP in autoimmunity based upon differences in T-cell homing or tolerance. PACAP treatment also protected against EAE (49), and the pharmacologic activity of PACAP in VIP-KO mice was potentiated by upregulation or hypersensitization of VIP-related receptors PAC and VPAC (50). Thus, these data indicate a complex role for VIP in different models of autoimmune diseases.

In conclusion, VIPhyb treatment enhanced the anti-leukemia activity of CD8$^+$ T cells in allo-BMT through enhanced activation of donor T cells and DC and inhibition of immune checkpoint molecule expression. VIPhyb treatment thus represents a novel pharmacologic approach to regulate immune checkpoints by targeting the VIP–VPAC axis. VIP signaling blockade may be additive to or synergistic with other immunotherapy approaches that induce anticancer immunity; hypotheses that are currently under investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.-M. Li, C.T. Petersen, B.R. Blazar, E.K. Waller
Development of methodology: J.-M. Li, E.K. Waller

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