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Graft versus Host Disease and Donor T cell Activation Is Limited by Vasoactive Intestinal Peptide Synthesis by Host Non-hempatopoietic Cells

By

Yiwen Li

Master of Science

Graduate Division of Biological and Biomedical Sciences

Cancer Biology and Translational Oncology

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Yiwen Li
B.S., Emory University, 2017

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Abstract

Graft versus Host Disease and Donor T cell Activation Is Limited by Vasoactive Intestinal Peptide Synthesis by Host Non-hempatopoietic Cells

By

Yiwen Li

Vasoactive Intestinal peptide (VIP) is a neuropeptide expressed by both neural and lymphoid cells with pleiotropic effects on brain, immune, pulmonary, GI and cardiovascular systems. The potent immunomodulatory and anti-inflammatory activity of VIP make it an attractive therapeutic target to suppresses immune responses in conditions of deleterious inflammation. In allogeneic bone marrow transplantation (allo-BMT), graft-versus-host disease is mediated by activated allo-reactive T cells that also upregulate a variety of co-inhibitory pathway molecules, including up-regulation of VIP expression on T cells and dendritic cells. It is unclear whether VIP production by host cells in the transplant recipient influence the incidence or severity of GvHD and donor T cell activation. We examined the effect of endogenous VIP signaling in host tissues and organs in modulating the severity of GvHD in a murine allo-BMT model. We examined the effect of endogenous VIP signaling in host tissues and organs in modulating GvHD in a murine allo-BMT model in which VIP-knock-out C57BL/6 mice, genetically deficient for VIP and the related peptide histidine isoleucine (PHI), were transplanted with bone marrow and splenocytes from MHC mis-matched B10.BR donors and compared to wild-type C57BL/6 transplant recipients. Compared with WT recipient mice, VIP-KO recipients transplanted with allogeneic bone marrow and splenocytes had decreased survival, reduced co-inhibitory pathway molecule expression on T cells, and elevated Th1 and Th17 cytokine production. WT and VIP-KO mice treated with lethal doses of irradiation but not followed with allo-BMT showed no survival difference, suggesting VIP-KO mice are not intrinsically more susceptible to irradiation. Transplant experiments

using radiation chimeric mice recipients in which either the hematopoietic or non-hematopoietic compartment lacked expression of VIP further demonstrated that VIP production in non-hematopoietic cells is the key factor in limiting the GvHD activity of donor T cells. Finally, immunofluorescent imaging of transgenic mice in which GFP is regulated by the VIP promoter showed VIP production is highly expressed in efferent neurons innervating the lungs, and that host expression of VIP in lung tissues continues for at least 15 days post-transplant. Taken together, these data suggest that local production of VIP in the lung may be a key factor in the control of allo-reactive donor T cells and subsequent GVHD in epithelial target organs. Furthermore, local production of VIP by host neurons surrounding lung alveoli suggests a novel pathway for pharmacological modulation of allo-reactive T cells and control of GVHD.

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Introduction & Background

VIP Signaling and Immunosuppressive Property

Vasoactive intestinal polypeptide (VIP) is a 28 amino acid immunosuppressive neuropeptide with pleiotropic effects (Ganea et al, 2015). VIP is expressed on immune cells and nerve endings presented in thymus, lymph nodes and mucosal-associated lymphoid tissues, serving as a link between the CNS and the immune system (Bellinger et al, 1997). VIP binds to three G protein-coupled receptors, with high affinity binding to VPAC1, VPAC2 and lower affinity binding to PAC1. VPAC1 is constitutively expressed on lymphocytes and dendritic cells while VPAC2 is expressed on immune cells upon stimulation (Delgado et al, 2004). Endogenous VIP production promote Th2 subtype development and suppresses Th1 differentiation in T cells (Voice et al, 2014) and VIP signaling activates anti-inflammatory cAMP/PKA pathway during DC and T cell differentiation. In allogeneic bone marrow transplant (allo-BMT) settings, activation of donor T cells leading to graft-versus-host disease (GvHD) occurs via early post-transplant interactions between donor T cells and host antigen presenting cells and causes secondary upregulation of immune co-inhibitory pathways, including expression of VIP in T cells and dendritic cells. We have previously shown VIPhyb treatment favorably regulates the activation status and the graft-versus-leukemia (GvL) activities of donor T cells (Li et al., 2016). The role of the VIP produced by host cells in regulating GvHD in allo-BMT is unknown.

To understand the role of VIP in post-transplant alloreactivity and control of GvHD, we reviewed the VIP pathway in the context of what is known regarding the pathophysiology of GvHD and the role of other inflammatory and co-inhibitory pathways. Given the significant role of blocking VIP signaling in augmenting T cell activation and Th1 promotion, we are interested in whether endogenous VIP production in allo-BMT recipients have a role in regulating donor T cell activation and GvHD.

Allogeneic Stem Cell transplantation and GvHD

Allogeneic stem cell transplantation (allo-HSCT) can cure relapsed hematological malignancies through the graft versus leukemia (GvL) effect mediated by donor cells (Blazar, 2012). When multipotent hematopoietic stem cells and other immune cells are introduced to the recipient conditioned and lympho-depleted by chemoradiotherapy (Blazar 2012), transplanted naïve and bone marrow-derived donor T cells expand in the recipient and recognize and eliminate residual leukemia and other malignant cells them via adaptive immune pathways (Henden & Hill, 2015). The major clinical challenge in the more widespread clinical use of allo-HSCT is graft-versus-host-disease (GvHD), in which recipient alloantigen is presented to donor T cells that attack non-malignant epithelial tissues and organs leading to high morbidity and mortality (Ferrar *et al*, 2009). Transplantation of grafts depleted of T cell lead to reduced GvHD incidence but is associated with an increased incidence of leukemia relapse post-transplant (Marmont *et al*, 1999; Horowitz *et a,l* 1990) and opportunistic infections (Pachnio *et al,* 2006). As the presence of an alloantigen is sine qua non pre-requisite for T cell activation and both GvL and GvHD, decades of preclinical and clinical studies (Ryan CE *et al,* 2016; Kitko J *et al*, 2016) have focused on how to separate the beneficial GvL effect from the deleterious GvHD.

GvHD pathogenesis

Barnes initially characterized GvHD as a syndrome manifested in mice resulted from irradiation and introduction of foreign bone marrow graft (Barnes *et al,* 1962). Billingham further reported the pre-conditions for the development of GVHD including immunologically competent donor cells in the graft, differences in histocompability between donor and immunocompromised recipients, and a relatively immune-suppressed state of the recipient (Billingham et al, 1967). Acute GvHD (aGvHD) is separated from chronic GvHD (cGvHD) from the time of disease onset, with acute GVHD usually occurring during

the first three months (typically between 1 and 2 months post-transplant) while chronic GVHD typically presents more than 3 months post-transplant, with a usual time of onset of around 6 months post-transplant. In addition, aGvHD is differentiated from cGvHD by their distinctive histo-pathologies. Acute GvHD is characterized by epithelial cell apoptosis in GvHD-targeted organ (skin, lung, gut and liver that may be mediated by inflammatory cytokines as well as direct T cell mediated cytotoxicity, whereas the cardinal feature of cGvHD's is fibrosis and tissue damage (Henden & Hill, 2015) that is associated with dysregulated B cell development and the generation of auto-antibodies as well as alloreactive T cells (Sarantopoulos *et al*, 2015). The aGvHD cascade has been defined into four phases (Blazar *et al,* 2012; Villa *et al,* 2016). The initial step is initiated by epithelial tissue damage induced by the chemoradiotherapy conditioning regimen, with APC activation and transepithelial movement of gut bacteria from the intestine lumen into gut tissue and mesenteric lymph nodes, which further results in activation of innate immune system activation with PAMPs recognition and chemokine release. The result of these early stage of inflammation is further damage to the epithelial stem cells and synthesis and systemic expression of inflammatory cytokine termed "cytokine storm". The second phase is classified as antigen presentation, in which APCs present alloantigens to donor T cells. Recipient APC and host non-hematopoietic APCs also play important roles in augment GvHD in this stage. The third phase is enhanced secretion of inflammatory cytokines ("cytokine storm"), which promote the differentiation of naïve T-cells into alloreactive memory and effector Th1, Th2 and Th17 T cell subsets, and regulatory T cells. Finally, the fourth stage of aGvHD includes the infiltration of donor Teff, NK cells, macrophages into recipient tissues following their recruitment by proinflammatory cytokines resulting in further in end organ damage (Figure 1).

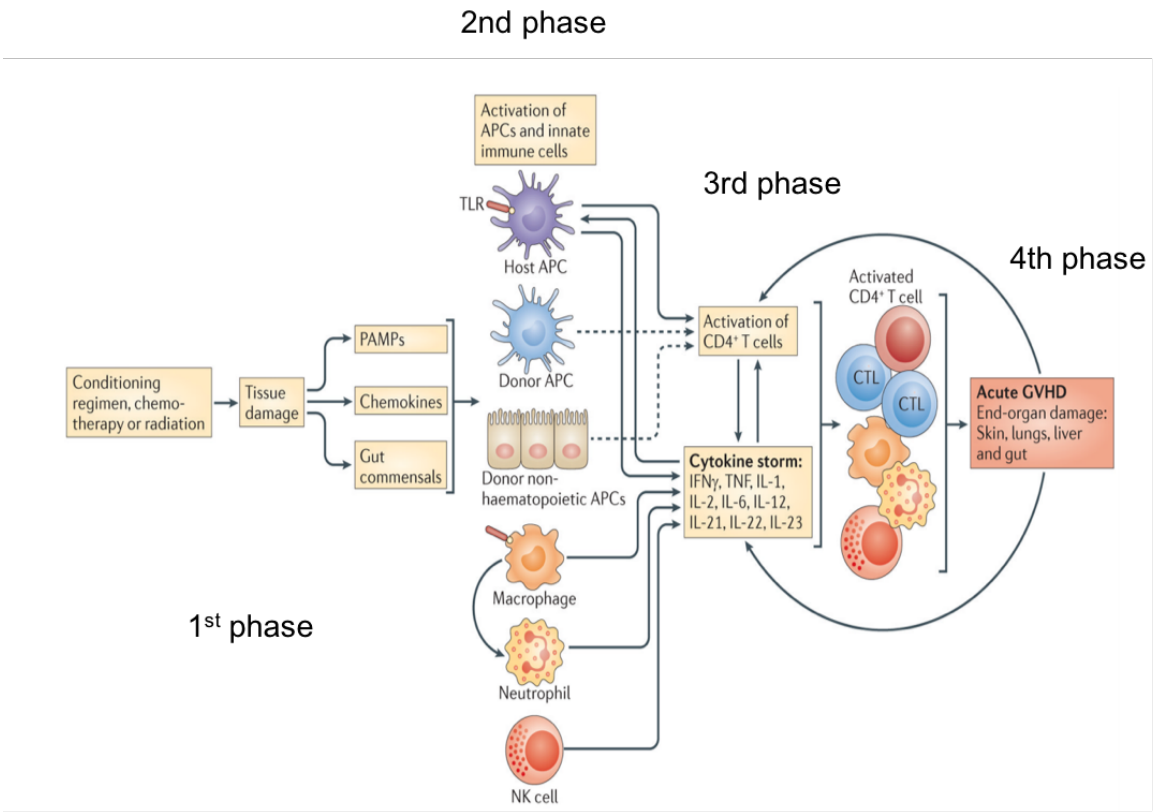


Figure 1. Acute GvHD Pathogenesis (adapted from Blazar et al, 2012). Understanding of the four signature phases of aGvHD cascade (tissue damage, innate and adaptive immune system activation, cytokine storm and infiltration of immune cell and cytokine into targeted organ) aids the development of immunotherapies against GvHD induced lethality.

Mouse models have been the primary preclinical model to understand GvHD pathogenesis and prevention due to the availability of the specific knockout and transgenic strains that present isolation of the role of specific molecules in the pathogenesis of GvHD and the availability of hematopoietic tumor lines that allows study of GvL effect (Blazar *et al*, 2012). The classical regimen in mouse aGvHD model usually involves lethal total body followed by transplantation of hematopoietic stem cells (HSCs) in combination with other donor immune cells into a recipient expressing different MHCI/II molecules or other minor histocompability antigens (mAg) from donor.

The interaction between host/recipient APC and donor T cells in GvHD initiation and maintenance has been extensively studied. Classically, antigen presentation has been categorized either direct or indirect antigen presentation. In direct presentation, recipient APC process and present alloantigens (self or non-self peptide) in the context recipient MHC to donor T cells; in indirect presentation, donor T cells recognize recipient alloantigen loaded on MHC of donor APCs (Koyama& Hill, 2018). Recent studies have discovered a third pathway in antigen presentation, namely semi-indirect pathway (Figure 2). Cross-dressed DCs have been identified in both C3H.SW → B6 and B6→ Balb/c transplant models , where donor derived DCs acquired relatively large portion of the host transmembrane, including MHC peptide complexes, and were capable of functionally priming T cells (Wang et al, 2011). In this context, MHCI/II molecules transferred from neighboring cells are capable of loading alloantigens subsequently initiate T cell-activation or suppression (Koyama& Hill, 2018).

GvHD can be classified as MHCI or MHC II mediated (CD8 or CD4 respectively) models. In the context of MHC I/CD8 T cell mediated GvHD, studies have shown APCs derived from hematopoietic cells play a more critical role than non-hematopoietic-derived APCs in aGvHD initiation (Toubi, 2012). In the context of MHCII/CD4 mediated GvHD, both hematopoietic and non-hematopoietic APCs are important for induction phase of aGvHD though non-hematopoietic APC population may be more critical (Koyama et al, 2011). Additionally, Matte demonstrated that recipient APCs were indispensable for a GvHD initiation in a murine model of GvHD and donor derived APC alone was unable to induce aGvHD (Matte et al, 2004).

Cytokine Production and aGvHD

Cytokine production drives maturation and differentiation of naïve T cells towards specific functional subset phenotypes associated with different patterns of cytokine synthesis that promote cytotoxic (Th1), humoral (Th2), inflammatory (Th17) phenotypes. In particular, Th1 and Th17 immune polarization may promote GvHD while Treg and Th2 immunity may limit GvHD. Advances in early recognition of the triggers of cytokine storm, the hallmark of third phase of aGvHD, has led to further

definition of the roles of Th1, Th2 and Th17 subset in aGvHD pathophysiology and potentially provide triggers for early (and potentially more effective) therapeutic interventions.

Th1 maturation and release of pro-inflammatory molecules including IL-1, IL-6, TNF-α and nitric oxide have been associated with aGvHD (Cooke KR, *et al,* 1998; Nestel FP *et al,* 1992). The production of Th1 associated cytokines (IFNγ, IL-2 and TNF-α) have been linked to GvHD exacerbation (Reddy *et al,* 2003), but other studies have shown that the timing of IFNγ production after allo-BMT is critical to the development of either cytotoxic vs immunosuppressive states (Brok HP *et al*, 1998). Early IFNγ production post-HSCT is associated with immunosuppression and absence of IFNγ production by donor cells is associated with more systemic inflammation and GvHD mortality (Lu & Waller, 2009).

Th2 differentiation has been classically considered as opposite to Th1 maturation. However, recent studies have shown timing in Th2-type cytokine productions (ie: IL-4) is critical. Another study showed donor T cells unable to secrete IL-4, IL-5, IL-9 and IL-13 have led to increased T cell proliferation and more GvHD lethality (Tawara I *et al*, 2008), suggesting protective effect of Th2-type cytokine against GvHD progression.

Th17 subsets have also demonstrated direct role in both aGvHD and cGvHD (in cutaneous tissues) induction and maintenance (Henden & Hill, 2016). IL-6 production induces Th17 differentiation (Steinman, 2007), and RORγt as the transcription factor, regulates Th17 expression (Fulton *et al*, 2012). IL-21 and IL-23 are also critical in Th17 proliferation (Serody & Hill, 2012). Lack of IL-17 production has led to impairment of CD4-dependent aGvHD (Yi T *et al*, 2008), but other studies have suggested enhanced IL-17 signaling can protect against aGvHD. Varelias and Hill have showed gut microbiota of mice genetically lacking IL-17RA/RC receptor complex on both hematopoietic and nonhematopoietic recipient cells are more susceptible to aGvHD (Varelias & Hill, 2018). Thus, more studies are required to elucidate the role of Th17 in GvHD induction.

Co-inhibitory Pathway

Improvements in our understanding of the underlying mechanisms of acute GvHD (aGvHD) pathogenesis led to the advances in GvHD prevention. Therapeutic interventions targeting co-stimulatory and co-inhibitory pathways have been developed to inhibit T cell activation and reduce inflammatory reactions (McDonald-Hyman et al, 2015). Signaling through programed death-1 (PD-1) limits T cell activation via recruitment of spore wall protein (SWP) phosphatase and persistently elevated PD1 expression on T cells has been associated with T cells exhaustion due to tonic signaling following by binding of the cognate ligands, PD-L1 and PD-L2. PDL1 is expressed on hematopoietic and non-hematopoietic cells (Blazar BR *et al* 2012; Freeman GJ *et al.* 2000; Latchman Y *et al* 2001; Yamazaki T *et al* 2002; Keir ME *et al* 2008) and PD-L2 expression is primarily restricted to resting monocytes and epithelial tissues that are targets for aGvHD ((Blazar BR *et al* 2012). Blockade of the PD1/PD-L1 pathway which antagonistic antibodies induced increased donor IFN-γ production which enhanced GvHD severity (Blazar *et al* 2003). However, in a different study, Saha and Blazar reported that absence of PD-L1 on donor T cells induced higher apoptosis, reduced proliferation and dampened Teff function in both CD4 and CD8 T cells, and remarkably reduced GvHD lethality after allo-BMT (Saha *et al* 2016). Antibodies blocking CTLA-4, i.e: ipilimumab, (Bashey *et al* 2009) and anti-PD-1 antibodies have been tested in clinic settings as strategies to enhance the graft-versus-tumor effect after HSCT, but studies have not identified such potent blocking reagents selectively prevent GvHD by targeting co-inhibitory pathway (Blazar *et al* 2012). Further studies on other molecules in the co-inhibitory pathway are necessary in identifying therapeutic targets for GvHD prevention.

Experimental Hypothesis

Based upon the role of VIP in suppressing inflammatory cytokines and the inflammatory pathways that lead to the initiation and amplification of acute GVHD described above, we tested the hypothesis that lack of VIP expression by recipient cells would enhance donor T cell activation and increase GvHD in allo-BMT recipients.

Materials & Methods

Mice

Female B10BR mice aged from six-to-eight weeks old were used as donors of allo-BMT and were purchased from Jackson Laboratory ((Bar Harbor, Maine). Six-to-eight weeks old male and female VIP-KO and WT littermates were bred by Emory Animal Breeding Service, and used as recipients of allo-BMT. VIP-GFP mice (MMRRC strain #31009, FVB/N-Crl:CD1(ICR)) were purchased from MMRRC, University of California, Davis, and backcrossed to C57BL/6 for 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. B6 VIP-GFP mice were bred at Emory University.All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Transplant Model

We used the B10BR→B6 MHC fully mismatched murine model of allogeneic BMT. Recipient wildtype (WT) and VIP knockout B6 (VIP-KO) mice were lethally irradiated with 11Gy in split fractions (5.5Gyx2) and injected i.v. with 5×10^6 T cell depleted (TCD) bone marrow cells (BM) plus 0, 1×10^6, or 3 $\times10^6$ splenocytes from WT B10BR donors. Fifteen mice (males and females) were assigned to each splenocytes dosage group. The survival of recipients after BMT was recorded daily and the average clinical GvHD scores were obtained by combining weight-loss, activity, posture, fur texture and skin integrity data following standard published procedures. All three groups of WT recipients had better survival than VIP-KO recipients.

Irradiation Sensitivity Experiment

To determine whether VIP-KO mice are more susceptible to irradiation compared to WT mice, both VIP-KO mice and their WT littermates were lethally irradiated with split dose 11Gy irradiation (5,5

Gy x 2 fractions separated by 3-4 hours) followed by no transplantation. The survival rates of the mice post-irradiation were recorded daily.

Flow Cytometry

Spleens were collected from allo-BMT recipient mice and dissociated in 2%FBS PBS media using a syringe plunger and passed through a 70μm strainer. Ammounium chloride buffer was used to lyse red blood cells. Samples were counted by Beckman Coulter Particle Coulter and then incubate with LIVE/DEAD ^{TM} V450 Fixable Stain (ThermoFisher) in PBS. The splenocytes were then washed and stained with CD16/32 Fc receptor-blocking antibody followed by surface staining (anti-CD3, anti-CD4, anti-CD8, anti- PD1, anti-Tim3, anti-CTLA4, anti-Tigit, anti-Lag3 and anti-CD25 antibodies), intra-nuclear staining (anti-FoxP3 and anti-RORγt antibodies), and cytokine staining (anti-IFN-γ , anti- IL-2, anti-IL-6, anti-TNF- α, anti-IL-4, anti-IL9, anti-IL-10, anti-IL-17, and anti-IL-22 antibodies). All samples were run on BD FACS Aria Sorter and analyzed with FlowJo software.

Confocal Imaging

Intestine, lung, liver, thymus and spleen specimens were collected in OCT on D15 post-BMT and frozen sections of the collected tissues were processed by the Pathology Core Laboratory at Winship Cancer Institute of Emory University. Leica SP8 Software were used to visualize VIP gene promoter activity as GFP expression in cells in recipient lymphoid tissues and GvHD target organs co-stained with DAPI and anti-CD45 (anti-leukocyte antibody). Selected lung frozen section slides were co-stained with DAPI and anti-MAP2 (anti-neuronal antibody).

Histology

Formalin-fixed and paraffin-embedded gut specimens were sectioned (5-10 μm) and stained with hemotoxylin and eosin by Emory Winship Pathology Core for histological assessment.

Statistical Analysis

Data were analyzed using Prism Version 7.0 for Mac and are presented as mean ± SD of all evaluable samples if not otherwise specified. Survival differences among WT and VIP-KO groups were calculated with the Kaplan-Meier log-rank test in a pair-wise fashion. Other data were compared using Student T-test, One-way analysis of variance, 2-way ANOVA and nonparametric tests (Mann-Whitney U or Kruskal-Wallis H test). A p-value of < 0.05 was considered significant.

Results

VIP-KO recipients had more GvHD-induced death after allogeneic BMT than wild-type mice.

We used the B10BR→B6 MHC fully mismatched murine model of allogeneic BMT. Both wildtype (WT) and VIP knockout B6 (VIP-KO) recipient mice received 11Gy split dose irradiation on day -1 and were transplanted i.v. on day 0 with 5×10^6 T cell depleted (TCD) bone marrow cells (BM) plus 0, 1×10^6, or 3×10^6 splenocytes from WT B10BR donors. All three groups of WT recipients had better survival than VIP-KO recipients. VIP-KO mice transplanted with TCD BM alone had only 33.3% survival at day 75 compared with 73.3% survival among the WT recipients. Chimerism studies on day 20 post-BMT showed equivalent levels of donor chimerism within each group that received the same dose of donor splenocytes. WT and VIP-KO recipients transplanted with BM only showed significantly lower donor chimerism compared to either WT or VIP-KO recipients received 1×10^6, or 3×10^6 splenocytes ($p<0.001$), indicating increased mortality in the WT and VIP-KO recipients transplanted with BM only was primarily due to early graft rejection. The addition of donor splenocytes to the graft resulted in significantly more GvHD-related mortality in VIP-KO recipients compared with WT recipients (Figure 1), with 80% vs 100% day 75 survival among recipients of $1x10^6$ splenocytes (p=NS) and 0% vs 40% survival ($p<0.01$) among recipients of $3x10^6$ donor splenocytes (Figure 2).

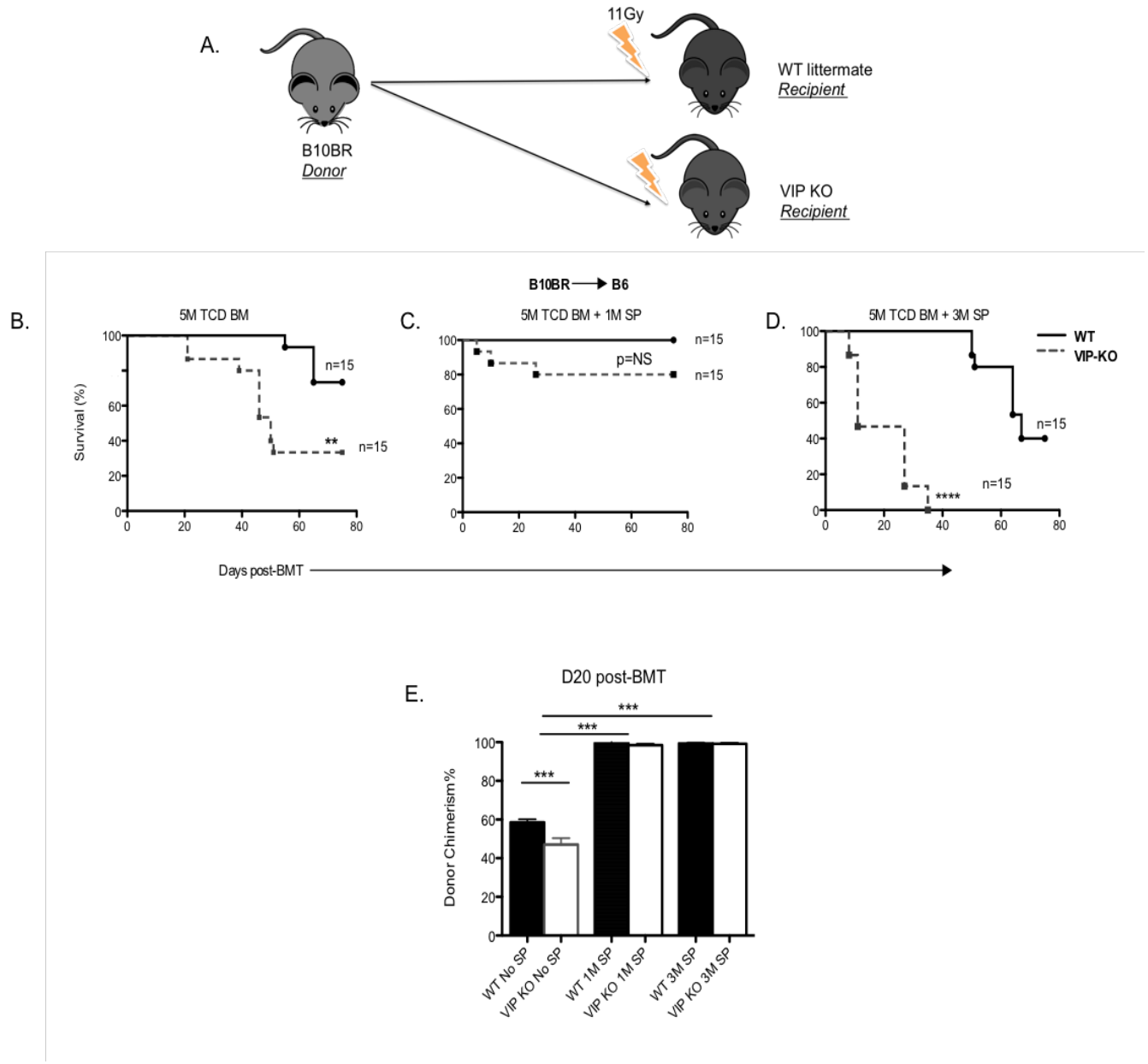


Figure 2. VIP-KO recipients resulted in more GvHD-induced mortality following allo- BMT compared to WT mice. Lethally irradiated B6 recipients were infused with 5M TCD BM plus 0, 1 or $3x10^6$ B10.BR splenocytes. (A) schematic illustration. (B-D) Kaplan-Meier survival plot pooled data from three independent experiments (n=15 per group; recipients infused with no splenocytes, WT vs VIP-KO $p<0.01$; recipients infused 1 $x10^6$ splenocytes, WT vs VIP-KO, p=NS; recipients infused with $3x10^6$ splenocytes, WT vs VIP-KO, $p<0.0001$). (E) Donor chimerism assessment on D20 post allo-BMT (recipients infused with no splenocytes, WT vs VIP-KO $p<0.001$; recipients infused 1 $x10^6$ splenocytes,

WT vs VIP-KO, p=NS; recipients infused with $3x10^6$ splenocytes, WT vs VIP-KO, p=NS; recipients insfused with no splenocytes vs 1M, p<0.0001; recipients insfused with no splenocytes vs 3M, p<0.0001). Data represent mean ± SD.

WT and VIP-KO recipients had equivalent radiation sensitivity

The outcomes after allo-BMT represent the synthesis of survival of epithelial tissues and organs damaging effects of ionizing irradiation, the successful engraftment of donor hematopoeituc stem cells, and the absence of lethal GvHD. To test whether the differences in survival between WT and VIP-KO MHC-mis-matched donor cells was caused by differences in radiation sensitivity, WT and VIP-KO mice (n=10 per group) received 11Gy split dose irradiation without transplantation of any donor cells. WT and VIP-KO irradiated mice had equivalent survival post-irradiation (p=NS) (Figure 3), indicating the survival differences post-BMT from previous experiment was not due to differences in differential susceptibility towards irradiation between WT and VIP-KO groups.

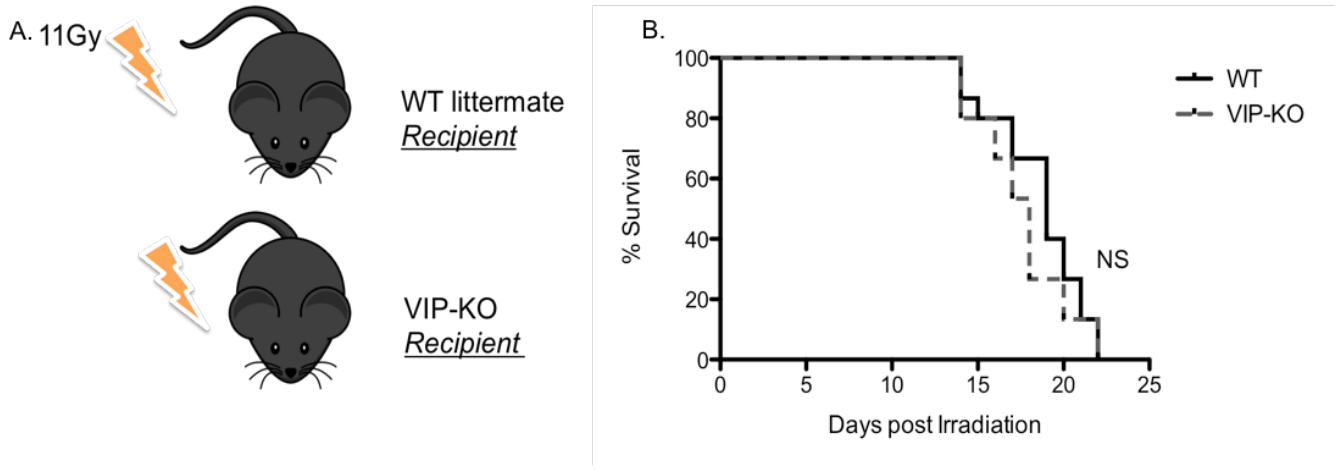


Figure 3. WT and VIP-KO recipients displayed no difference in irradiation sensitivity. WT B6 and VIP-KO mice were lethally irradiated with 11Gy in two equal splits 3-4 hrs apart. (A) schematic illustration. (B) Kaplan-Meier survival plot (n_{WT}=16; n_{VIP-KO} =15; p=NS).

VIP-KO recipients had elevated cytokine and lower co-inhibitory molecule production post-BMT

To determine how the absence of VIP production in KO recipients modulates immune effector mechanisms that contributes to increased GvHD after allo-BMT, we analyzed the numbers and phenotypes of donor-derived lymphocytes in the spleen of transplant recipients received 5M TCD BM plus 1×10^6 or 3×10^6 splenocytes (SP) from WT B10BR donors following a time course, with flow cytometry. Among WT and VIP-KO recipients transplanted with 5M TCD BM plus 1×10^6 splenocytes, there were no significant differences in expression levels of co-inhibitory molecules on donor CD4+, CD8+ T cells or Treg cell proliferation after transplant comparing WT and VIP-KO recipients (Figure 4). Notably, levels of donor CD4+ and CD8+ IFNγ and TNF-α in WT and VIP-KO recipients peaked on D6 post allo-BMT, consistent with the initiation phase of GvHD characterized by cytokine storm. The percentage and absolute number of donor CD4+ T cells expressing IFN-γ and TNF-α on D6 post allo-BMT, and CD8+ TNF-α on D13 post allo-BMT were significantly higher in VIP-KO recipients compared to WT recipients received 5M TCD BM plus 1×10^6 splenocytes ($p<0.0001$) (Figure 5). Among WT and VIP-KO recipients transplanted with 5M TCD BM plus 3×10^6 splenocytes, WT recipients had significantly increased expression of CD4+ Lag3 ($p<0.001$) on D6, elevated expressions of CD8+ PD1 ($p<0.01$) and CD4+ BTLA ($p<0.05$) on D13, higher frequencies of CD4+ Tim3 ($p<0.05$) on D19, and increased level of CD4+ Tim3 ($p<0.001$), CD4+ PD1 ($p<0.05$), CD4+BTLA ($p<0.01$) and CD8+ PD1 ($p<0.001$) on D25 post allo-BMT compared to VIP-KO recipients (Figure 6). In addition, VIP-KO recipients transplanted with 5M TCD BM plus 3×10^6 splenocytes had significantly higher expression levels of selected Th1 and Th17 cytokines. Specifically, we observed significantly higher frequencies of CD4+ IFN-γ ($p<0.001$) and CD4+ IL2 ($p<0.01$) on D4, significantly elevated levels of CD4+ TNF-α ($p<0.05$) and CD8+ TNF-α ($p<0.001$), on D19 and significantly increased CD8+ TNF-α ($p<0.05$) on D25 post allo-BMT compared to WT recipients. Consistent with the elevation of Th17 transcriptional factor RORγt in VIP-KO recipients on D13 ($p<0.01$) and D19 ($p<0.001$) post allo-BMT, significantly higher levels of IL17 production were detected in both CD4+ ($p<0.0001$) and CD8+ ($p<0.0001$) donor T cell population (Figure 7). Additionally, significantly more Th2 cytokine IL4 production by donor CD8+ T cells was observed in WT recipients ($p<0.01$) (Figure 7).

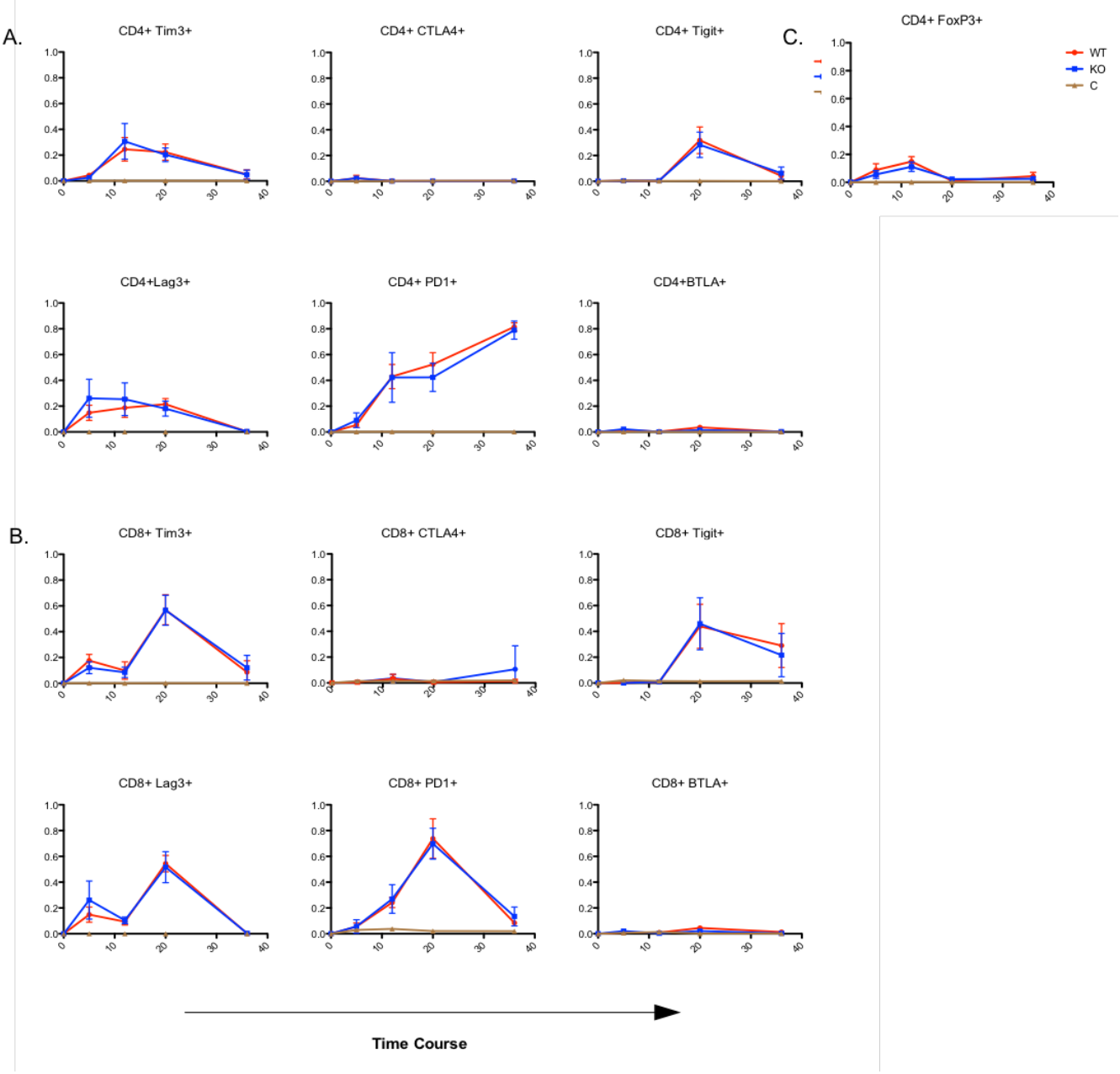


Figure 4. WT and VIP-KO recipients transplanted with low-dose splenocytes in allo-BMT had no significant differences in expression of surface co-inhibitory molecule expression on CD4+ or CD8 + T cells or numbers of CD4 Tregs. Lethally irradiated B6 recipients were infused with 5M TCD BM plus 1 x10^6 B10. BR splenocytes. Mice (n=3 to 6 per group) were sacrificed on D5, 12, 20 and 36, and donor CD4 and CD8 T cells in spleen were analyzed by flow cytometry. (A-B) Tim3, CTLA-4, Tigit, Lag-3, PD-1, BTLA. (C) CD4+CD25+FoxP3 Tregs (p=NS).

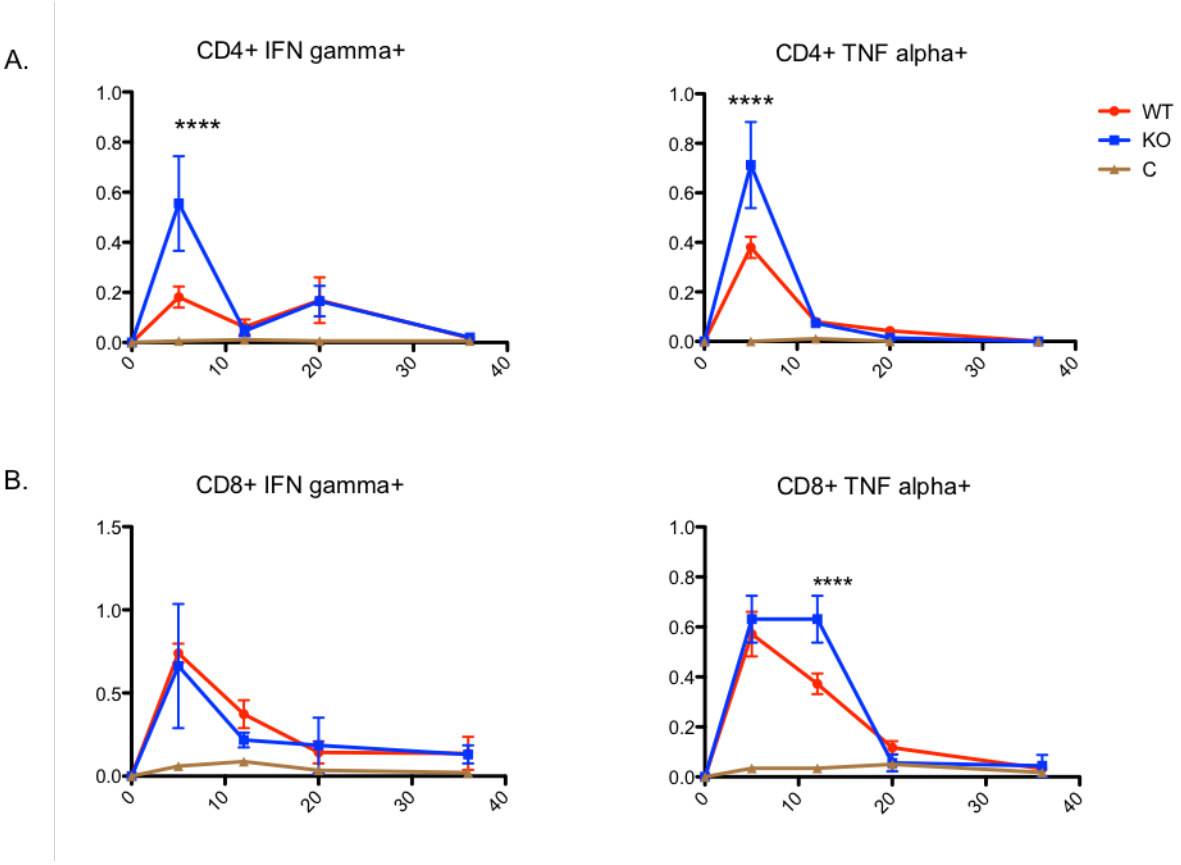


Figure 5. Higher levels of Th1 cytokine expression in VIP-KO recipients transplanted with 1M donor splenocytes in allo-BMT

Lethally irradiated B6 recipients were infused with 5M TCD BM plus $1x10^6$ B10.BR splenocytes. Mice (n=3 to 5 per group) were sacrificed on D5, 12, 20 and 36, and donor CD4 and CD8 T cells in spleen were analyzed by flow cytometry. (A-B) IFNγ and TNF-α. Data represent mean ± SD, and p-values were calculated by 2 way- ANOVA.*p<0.05, **p<0.01,***p<0.001, ****p<0.0001

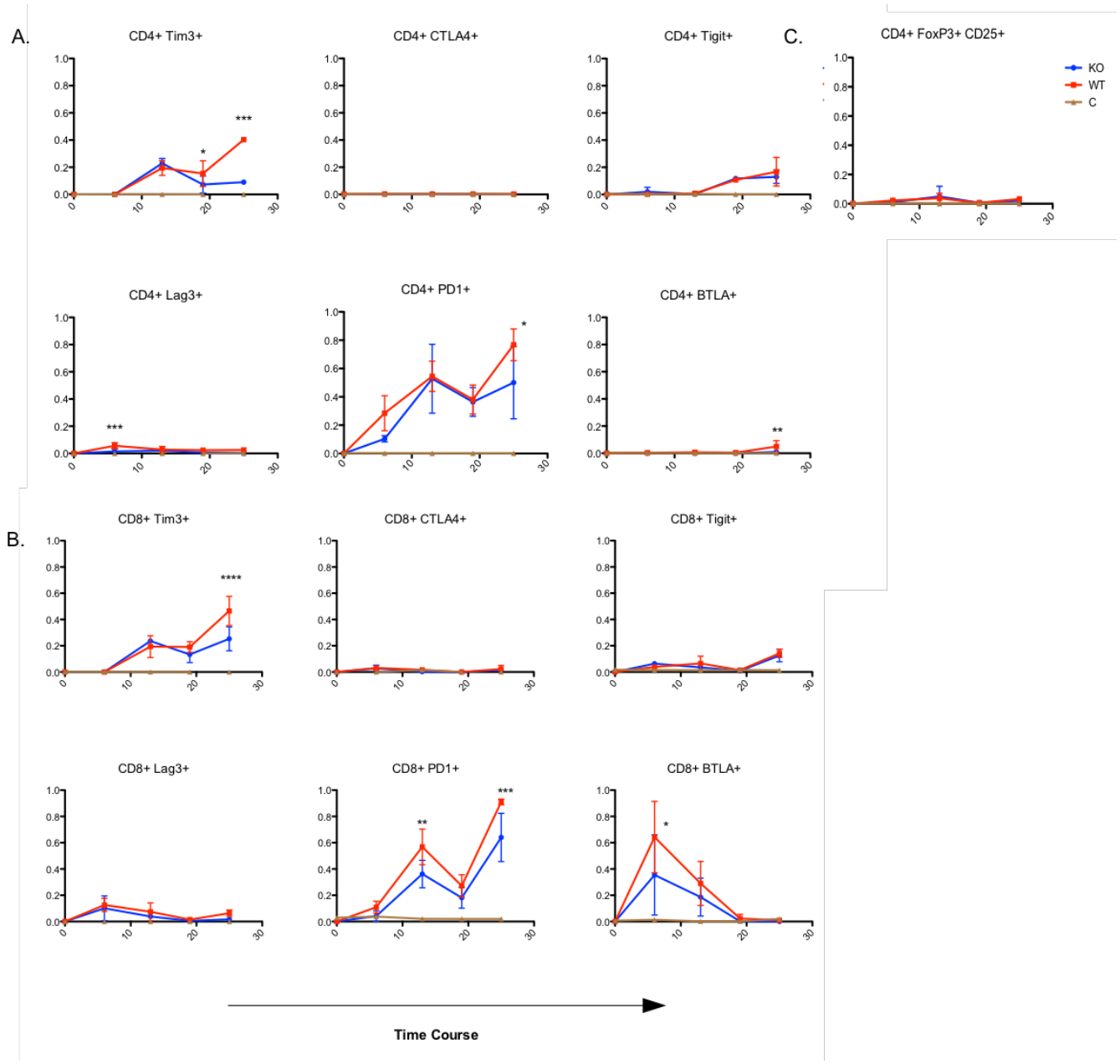
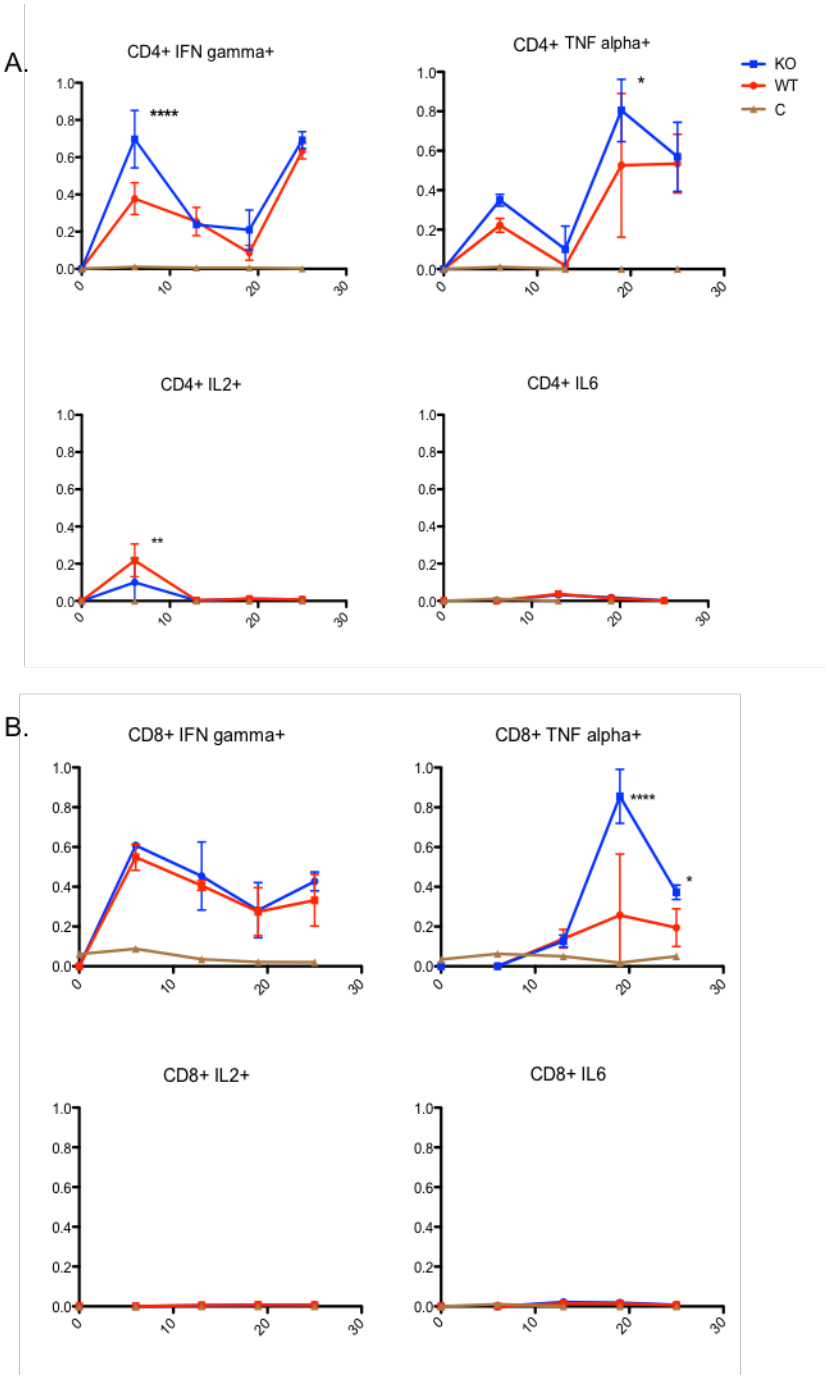


Figure 6. VIP-KO recipients transplanted with high-dose donor splenocytes in allo-BMT had lower levels of surface co-inhibitory molecules expressed on CD4+ or CD8 + Tcells.

Lethally irradiated B6 recipients were infused with 5M TCD BM plus 3 x10^6 B10.BR splenocytes. Mice (n=3 to 6 per group) were sacrificed on D6, 13, 19 and 25, and donor CD4 and CD8 T cells in spleen were analyzed by flow cytometry. (A-B) Tim3, CTLA-4, Tigit, Lag-3, PD-1, BTLA. (C)

CD4+CD25+FoxP3 Tregs (p=NS). Data represent mean ± SD, and p-values were calculated by 2 way-ANOVA.*p<0.05, **p<0.01,***p<0.001, ****p<0.0001



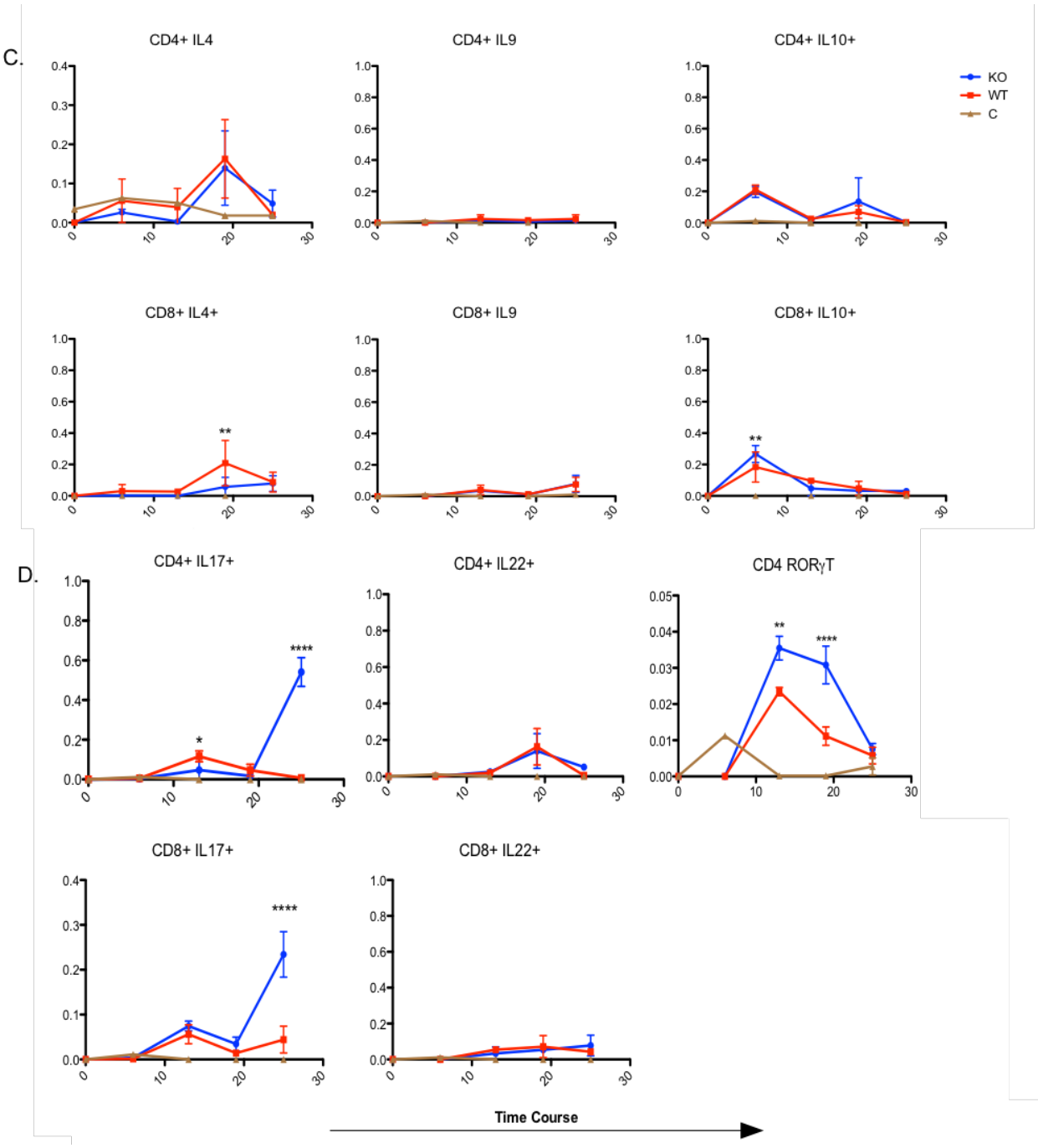


Figure 7. VIP-KO recipients transplanted with 3M donor splenocytes in allo-BMT had enhanced levels of Th1, Th2 and Th17 cytokines compared with WT recipients.

Lethally irradiated B6 recipients were infused with 5M TCD BM plus $3x10^6$ B10.BR splenocytes. Mice (n=3 to 5 per group) were sacrificed on D6, 13, 19 and 25, and donor CD4 and CD8 T cells in spleen were analyzed by flow cytometry. (A-B) IFNγ, IL-2, IL-6, and TNF-α. (C) IL-4, IL-9 and IL-10. (D) IL-17, IL-22, and RORγt. Data represent mean ± SD, and p-values were calculated by 2 way-ANOVA.*p<0.05, **p<0.01,***p<0.001, ****p<0.0001

VIP Production in Non-Hematopoietic Compartment post allo-BMT limits GvHD

As VIP production is not restricted to the immune system, it is important to investigate the role of VIP signaling exclusively mediated by recipient non-hematopoietic compartment (i.e., peripheral neurons). We made radiation chimeras via syngeneic transplants, where either the hematopoietic or non-hematopoietic compartment of recipients lacked VIP expression. Chimerism studies on day 45 post-BMT showed equivalent levels of donor chimerism within each group that received the same dose of donor bone marrow and splenocytes (Figure 8). We next used B10BR mice as donors to perform a second allogeneic transplant on the radiation chimeras. Consistent survival differences were observed in chimeric recipients received 5M TCD BM plus 1×10^6 or 3×10^6 SP. Recipients that lacked VIP expression in both hematopoietic or non-hematopoietic compartments (VIP KO → VIP KO) had 0% survival at D25 post allo-BMT, whereas chimeric recipients that lacked VIP expression in hematopoietic compartment (VIP-KO→PepBoy) had significantly better survival than chimeric recipients lacked VIP expression in non-hematopoietic compartment (PepBoy →VIP-KO) ($p<0.05$). Notably, 100% survival was observed in recipients not lacked VIP production in neither hematopoietic nor non-hematopoietic compartments (WT C57 Bl/6→PepBoy).

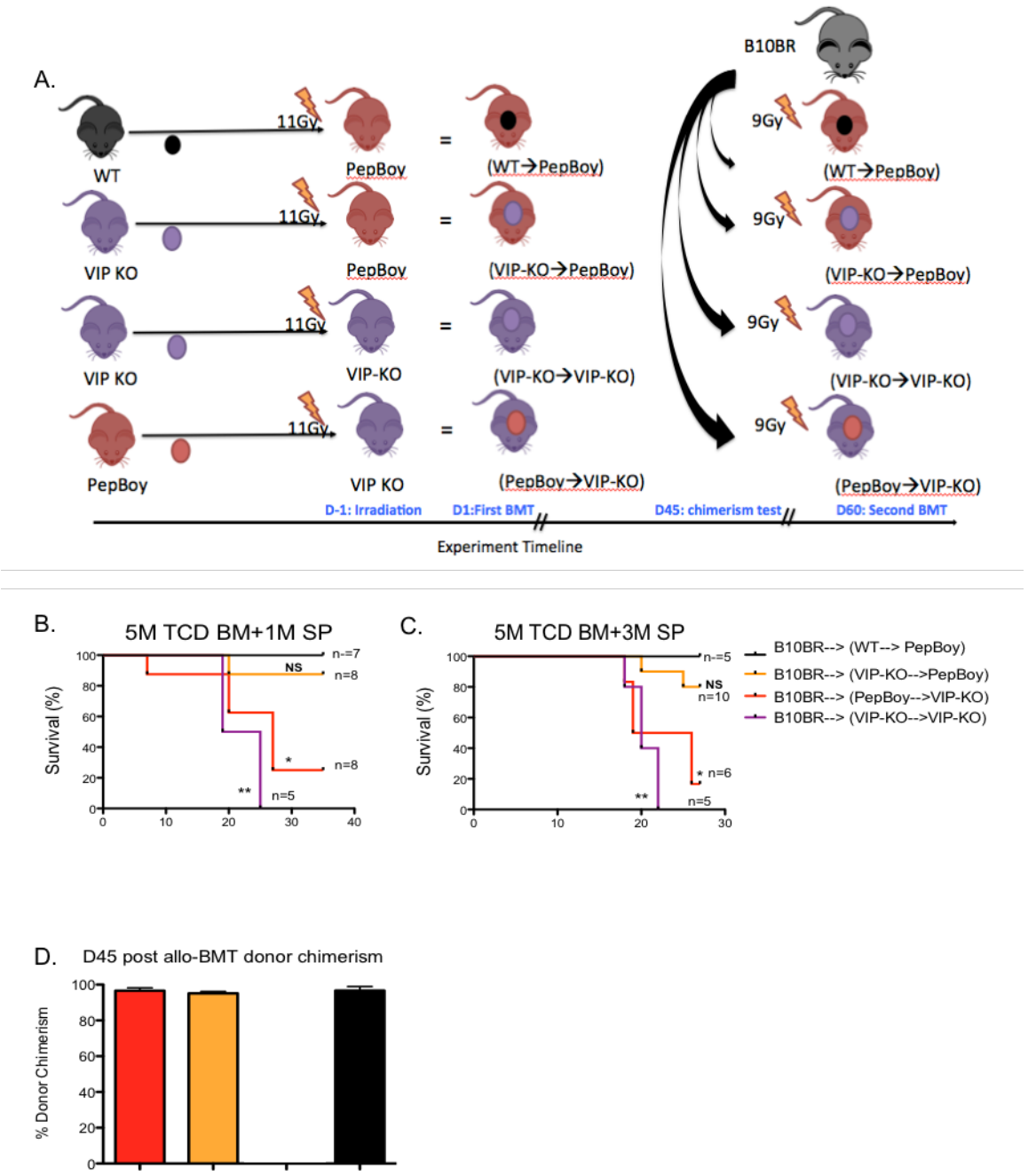


Figure 8. VIP expression in on-hematopoietic tissues in radiation chimeric recipients was suffient to prevent GvHD lethality after second allogeneic BMT. Lethally irradiated (11Gy in 2 equal splits 3-4 hours apart on D-1) C57/BL6 WT, VIP-KO or PepBoy mice were infused with 5M TCD corresponding

BM for syngeneic transplant to create control mice with VIP production in both hematopoietic or non-hematopoietic cells, or lacked VIP production in both hematopoietic or non-hematopoietic cells and to create radiation chimeras either lacked VIP production in hematopoietic or non-hematopoietic compartments. On D45 post syngeneic transplant, submandibular bleeding approach was used to collect blood testing for donor chimerism for WT→PepBpy (CD45.2→CD45.1), VIP-KO→PepBoy (CD45.2→CD45.1) and PepBoy→ VIP-KO (CD45.1→CD45.2). On D59 post syngeneic transplant, control and radiation chimera recipients were lethally irradiated (9Gy equally split in 2 dosage) and were infused with 5M TCD BM plus 1 or 3 $x10^6$ splenocytes from B10. BR mice. Survival rate was recorded daily. (A) Schematic illustration of the timeline of experiment design. (B) Kaplan-Meier survival plot of radiation chimera infused with 5M TCD BM plus 1 $x10^6$ B10.BR splenocytes (C) Kaplan-Meier survival plot of radiation chimera infused with 5M TCD BM plus 3 $x10^6$ B10.BR splenocytes. (D) Donor chimerism on D45 post the 1st syngeneic transplant in four radiation chimeras. Data represent mean ± SD, and p-values were calculated by 2 way- ANOVA.*p<0.05, **p<0.01,***p<0.001, ****p<0.0001

Detection of VIP production in hematopoietic and non-hematopoietic cells up to D15 post allo-BMT

To characterize the source of VIP produced by cells in transplant recipients, we used a transgenic mouse in which the GFP was placed down-stream of the VIP promoter (Li *et al*, 2016). The original strain of VIP-GFP mice on a FVB/N background was back-crossed to C57BL/6 mice for more than 10 generations, using a PCR assay for GFP to track the presence of the transgene. We found the presence of the VIP-GFP transgene with VIP (Vip-31009 F1 5-GCTAGACCCTCTGAAATGTTGCCAA-3) and GFP (GS eGFP R3 5-GGTCGGGGTAGCGGCTGAA-3) primers. Among non-transplanted VIP-GFP mice, we found that VIP-GFP^+ cells were super-imposable with $MAP2^+$ in lung tissue, indicating neuronal origin of VIP in the lung (Figure 10B).

Next we tested whether VIP production in host tissues persists after allogeneic transplantation. We transplanted C57/BL6 recipients expressing a GFP transgene under the control of the VIP promoter (n=10) with 5×10^6 TCD BM plus 3×10^6 splenocytes from WT B10.BR donors, and visualized VIP gene promoter activity as GFP expression in cells in recipient lymphoid tissues and GvHD target organs co-stained with DAPI and anti-CD45. Additional lung, liver and intestine were co-stained with DAPI and MAP2 for detection of neurons. Small number of $CD45^+$ VIP-GFP^+ cells were detected from in host spleen but not in liver, lung or intestines on day15 post-transplant, suggesting that a small number of residual host leukocytes, particularly dendritic cells, continued to express VIP for at least 15 days post-transplant (Figure 9B). Additionally, a high frequency of VIP-GFP^+ anuclear cell processes were seen in host lungs, indicating that relatively high level of VIP production continues in host-type nerve cells after allogeneic transplantation (Figure 10).

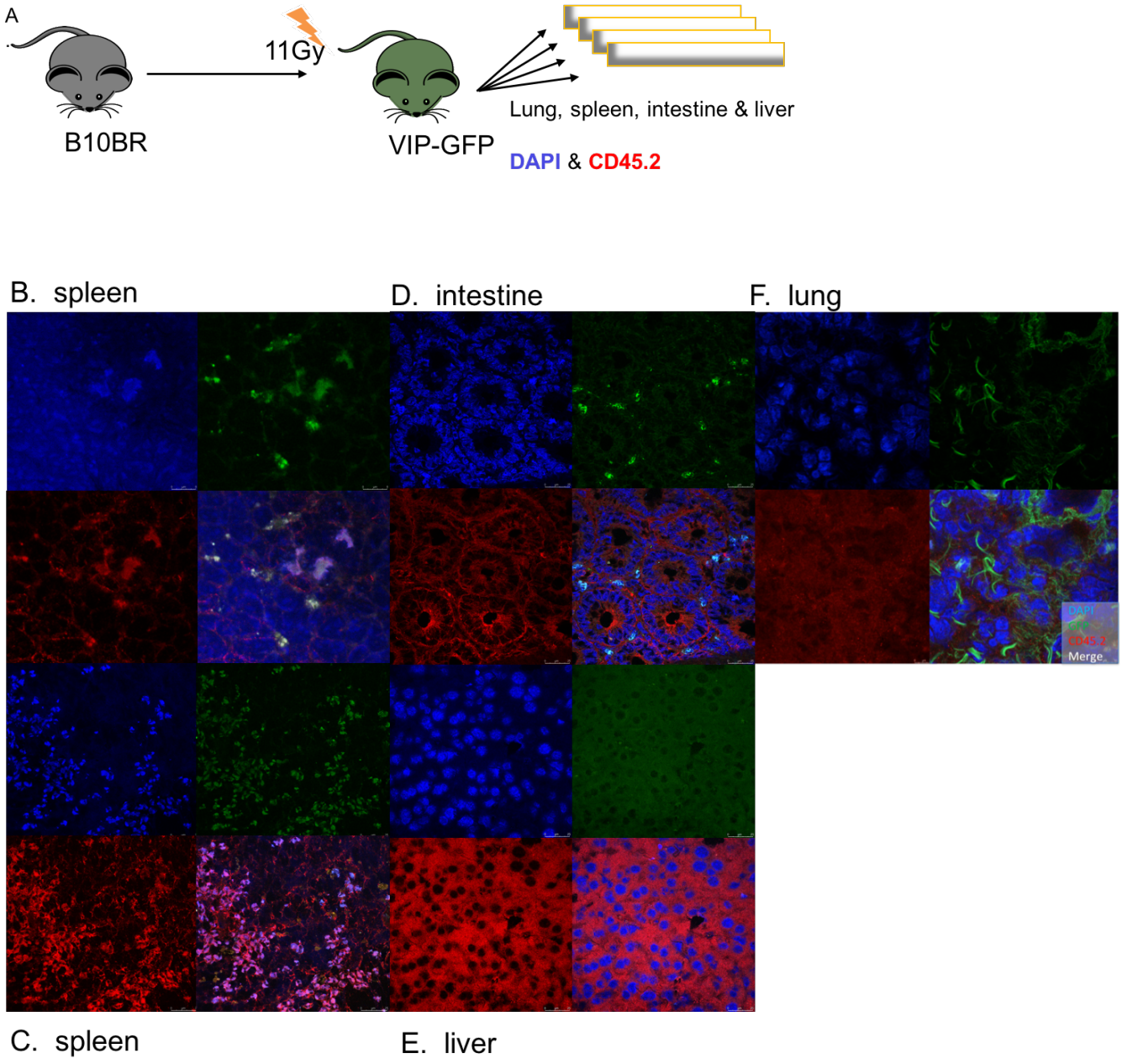


Figure 9. VIP-GFP^{+} producing host leukocytes can be detected at least for 15 days post allo-BMT. Lethally irradiated VIP-GFP B6 recipients were infused with 5M TCD BM plus 3x10^{6} B10.BR splenocytes (n=5) and were sacrificed on D15 post allo-BMT. Intestine, lung, liver, thymus and spleen specimens were collected in OCT embedded cryomolds and frozen sections of the collected tissues were processed and sectioned by the Pathology Core Laboratory at Winship Cancer Institute of Emory University. (A) Schematic diagram of obtaining specimen for frozen section immunofluroscent staining.

(B-F) confocal microscopic images of spleen, instestine, liver and lung immunofluroscent stained for DAPI and Alexa Flouro 568 anti-CD45.2 from VIP-GFP mice infused with 5M TCD BM plus 3 x 10^6 splenocytes.

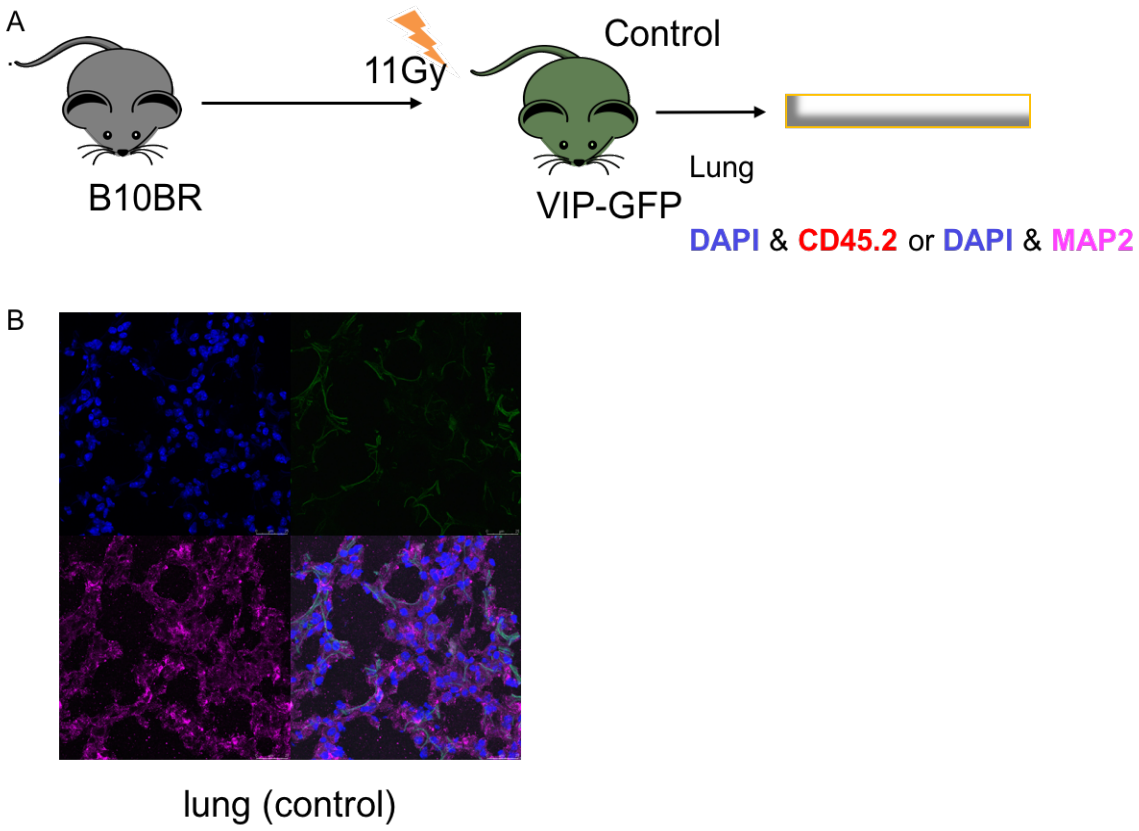


Figure 10. VIP-GFP$^+$ producing neural cells can be detected at least for 15 days post allo-BMT. Control VIP-GFP B6 recipients (n=3) and were sacrificed. Lung specimens were collected in OCT embedded cryomolds and frozen sections of the collected tissues were processed and sectioned by the Pathology Core Laboratory at Winship Cancer Institute of Emory University. (A) Schematic diagram of obtaining specimen for frozen section immunofluroscent staining. (B) confocal microscopic image of lung from VIP-GFP control mice stained DAPI and Alexa Fluro 647 anti-MAP2.

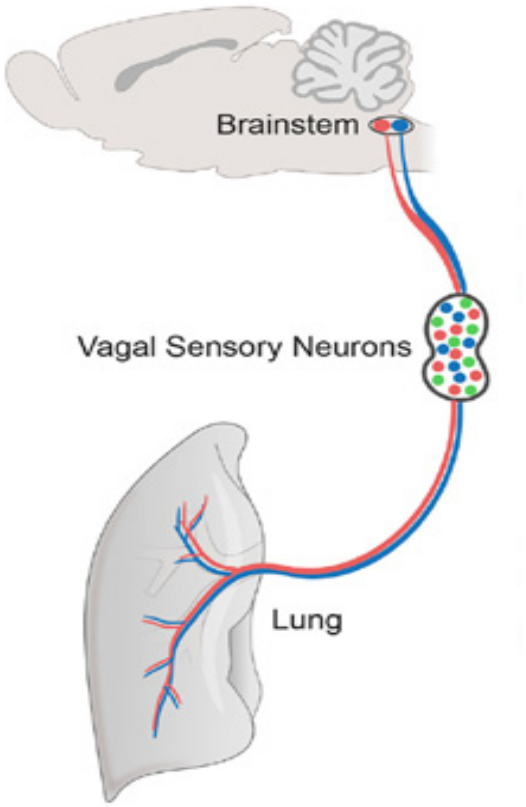


Figure 11. The vagal nerve serves as a linkage from brain-to lung. Vagal sensory neurons provide ascending sensory information from lung back to the brainstem.

Discussion

The pleiotropic effects of VIP are mediated through its receptors widely distributed in the central nervous system and peripheral tissues (immune, pulmonary, GI and cardiovascular systems) (Dickson et al, 2009). VIP expression in immune cells represents a significant co-inhibitory pathway, as VIP signaling dampens systemic inflammatory responses (Ganea, 2014) and the *in vivo* immunomodulatory role of endogenous VIP produced by hematopoietic compartment has been confirmed by the higher frequencies of antiviral CD8 T cells detected in WT mice transplanted with bone marrow cells from VIP-KO mice (Li JM, 2011). The augmentation in antiviral response was attributed to the absence of hematopoietic VIP production as normal levels of neuronal VIP were produced in the bone marrow chimeric mice. In allo-BMT setting, GvHD is mediated by early activation of alloreactive donor T cells, which secondarily upregulate co-inhibitory pathway molecules, including VIP expression on T cells and dendritic cells. The role of VIP expression by recipient cells in allo-BMT setting has not been studied. Here, we studied whether absence in VIP expression by recipient cells would enhance donor T cell activation and increase GvHD in allo-BMT recipients.

The longstanding challenges in clinical setting following allo-HSCT is the detrimental consequences of allo-reactive T cell induced GvHD (Ferrara et al, 2009), but yet T cell depletion of the graft exposes recipients to the high risk of poor engraftment and leukemia relapse (Marmont et al, 1999). Consistent with these findings, both WT and VIP-KO recipients received allo-BMT with high dose of splenocytes (3 x 10^6) had higher GvHD-induced mortality compared the corresponding WT and VIP-KO recipients received grafts with low-dose splenocytes (1 x 10^6) or no splenocytes. Among the WT and VIP-KO recipients transplanted with same bone marrow plus splenocyte dosages (0, 1 x 10^6 or 3 x 10^6), VIP-KO recipients had reduced survival compared to WT recipients suggesting that the absence in endogenous VIP expression by recipient cells enhanced GvHD progression. Reduced survival in WT and VIP-KO group receiving BM only (73.3% vs 33.3% correspondingly) suggested higher levels of early graft rejection in VIP KO mice, possibly due to host versus graft effect of residual host T cells or NK cells in the absence of endogenous VIP. A potential caveat in interpreting the survival differences

among WT and VIP-KO groups as a consequence of immune modulation is the possibility that the absence of endogenous VIP could result in enhanced sensitivity to irradiation. We confirmed that VIP-KO mice had comparable radiation sensitivity as WT mice by showing equivalent survival pattern among WT and VIP-KO groups received 11Gy irradiation split into two equal dosages in the absence of allo-BMT (Figure 3B).

Cytokine storm is a signature step in GvHD pathogenesis. We measured Th1, Th2 and Th17 cytokine production in a time course among WT and KO recipients transplanted with 5M TCD BM plus 1 x 10^6 or 3 x 10^6 splenocytes. VIP signaling has been shown to inhibit pro-inflammatory Th1 polarization and favor anti-inflammatory Th2 differentiation in both *in vivo* and *in vitro* studies (Ganea *et al*. 2003). Consistent with these reports, pro-inflammatory Th1 cytokines (IFN-γ and TNF-α) were consistently produced in significantly higher frequencies on CD4+ and CD8+ T cells in VIP-KO recipients received 5M TCD BM plus 1 x 10^6 or 3 x 10^6 splenocytes compared to WT recipients at the peak of post-transplant inflammation following allo-BMT. Significantly elevated CD4+ and CD8+ TNF-α Th1 cytokine production in VIP-KO recipients received 3 x 10^6 splenocytes are consistent with higher levels of inflammatory cytokines leading to increased death incidences in VIP-KO groups compared to the WT group. In addition, WT recipients transplanted with 3 x 10^6 splenocytes had significantly increased frequencies of CD8+ IL4 and CD8+ IL10 ($p<0.01$) compared to VIP-KO recipients on D19 and D5 correspondingly post allo-BMT, confirming greater anti-inflammatory Th2 polarization in the presence of endogenous VIP in transplant recipients.

T cells phenotypically characterized as Th17 cells plays a key role in autoimmunity promoting inflammatory response in Crohn's disease (Ferraccioli & Zizzo, 2011). Previous studies have controversial conclusions on whether VIP signally negatively regulates Th17 differentiation and Th17 cytokine production. In experimental murine models of type I diabetes delayed disease onset and reduced level of IL-17, RORγt and IL-22 productions were observed after VIP administration, which

indicates VIP exhibits inhibitory effect on Th17 polarization (Deng *et al*. 2010). However other the *in vivo* and *in vitro* experiments showed increased numbers of IL-17$^+$ T cells were correlated with increased exposure to TGFβ and VIP treatment for Langerhans cells, or for cultured human Th17 cells (Yadav *et al*. 2008; Jimeno *et al*. 2014). In our allo-BMT experimental setting, we found significantly increased CD4+ IL17 and CD8+ IL17 correlated with significantly elevated RORγt level in VIP-KO recipients on D25 post allo-BMT compared to WT recipients received 3 x10^6 splenocytes, which may contribute to the pro-inflammatory cytokine storm stage and GvHD maintenance.

Given the immunosuppressive nature of VIP and its production in immune cells, central and peripheral nervous system, it is critical to be able to separate out which source of VIP production plays a more critical role in GvHD control. Thus, we generated radiation chimeric mice, in which either their hematopoietic or non-hematopoietic system lacked VIP production and used mice went through syngeneic transplant (VIP-KO→ VIP-KO or WT B6 CD45.2→PepBoy CD45.1) as controls to assess allo-BMT outcome. Analysis of the levels of donor chimerism after syngeneic transplant confirmed no significant differences among four chimeric mice groups. Following a second allogeneic transplant that included a donor T cells, significant differences in survival rates were seen across the radiation chimera groups clearly demonstrating the indispensable contribution of VIP production in non-hematopoietic compartment in the transplant recipients in limiting GvHD, as well as a potential minor contribution by the host hematopoietic cells in the WT→VIP KO radiation chimeric mice.

To further characterize VIP production in the non-hematopoietic compartment of recipients post allo-BMT, we used a transgenic mouse in which the GFP was placed down-stream of the VIP promoter (Li JM, 2016). Non-transplanted VIP-GFP mice were used as controls to examine VIP production prior to allo-BMT. We observed that VIP-GFP$^+$ cells were superimposable with MAP2$^+$ staining in lung tissue, suggesting that the source of VIP production was from a neuronal origin. This finding is consistent with physiological phenomenon that the vagal nerve, as the tenth cranial nerve, is known for its extensive

innervation of the neck, chest, and abdomen region (Berthoud and Neuhuber, 2000) (Figure 10). The efferent vagal nerve functions not only respiration modulation, but also has basic physiological functions of the cardiovascular, immune, and digestive systems. Eighty percent vagal neurons collect input from thoracic tissues, including lung and inputs from abdomen region and relay them back to the brain. A high percentage of VIP-GFP$^+$ anuclear cell processes were also detected in host lungs on day15 post transplant, indicating that VIP production continues in host-type nerve cells after allogeneic transplantation. These data suggest the interesting hypothesis that VIP production by the extensive network of nerves cells in the lung may have a key role in regulating post-transplant immune responses. In addition, small portion of CD45$^+$ VIP-GFP$^+$ cells in host liver and spleen, but not in lung or intestines, were observed on day15 post allo-BMT, suggesting that a minority of residual host leukocytes in immune and GvHD selected targeted organ continue to express VIP at this time-point. Overall, these data suggest that regulation of donor Tcell allo-reactivity and immune polarization by host neurons surrounding lung alveoli represents a new and promising avenue for pharmacological intervention of allo-reactive T cells and control of GVHD.

Reference

Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. 2010. Auton Neurosci. 85, 1-17.
Deng S, Xi Y, Wang H et al. 2010. Regulatory effect of vasoactive intestinal peptide on the balance of Treg and Th17 in collagen-induced arthritis. Cell Immunol 265, 105–110.
Jimeno R., Leceta, J et al..2014. Vasoactive Intestinal Peptide Maintains the Nonpathogenic Profile of Human Th17-Polarized Cells. J Mol Neurosci54, 512–525.
Yadav M, Huang MC et al. 2011. VPAC1 (vasoactive intestinal peptide (VIP) receptor type 1) G protein-coupled receptor mediation of VIP enhancement of murine experimental colitis. Cell Immunol 267, 124–132.
Ferraccioli G, Zizzo, G. 2011. The potential role of Th17 in mediating the transition from acute to chronic autoimmune inflammation: rheumatoid arthritis as a model. Discov Med 11, 413–424.
Li JM, Waller EK et al. 2011. Absence of vasoactive intestinal peptide expression in hematopoietic cells enhances Th1 polarization and antiviral immunity in mice. J Immunol 187, 1057–1065.
Dickson, L. & Finlayson, K. 2009. VPAC and PAC receptors: from ligands to function. Pharmacol Ther 121, 294–316.
Pachnio A, Dietrich S, Klapper W, Humpe A, Schwake M, Sedlacek R, Gramatzki M, Beck C. Proliferation-based T-cell selection for immunotherapy and graft-versus-host-disease prophylaxis in the context of bone marrow transplantation. Bone Marrow Transplant. 2006 Jul;38(2):157-67. PubMed PMID: 16820783.
Sarantopoulos S, Ritz J. Aberrant B-cell homeostasis in chronic GVHD. Blood. 2015 Mar 12;125(11):1703-7.
Review. PubMed PMID: 25645355; PubMed Central PMCID: PMC4357579.
Yi T, et al. Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. Blood. 2008;112:2101–2110.
Steinman L. 2007. A brief history of TH17, the first major revision in the TH1/TH2 hypothesis of T cell–mediated tissue damage. Nat. Med. 13: 139–145.
Fulton L. M., et al. 2012. Attenuation of acute graft-versus-host disease in the absence of the transcription factor RORγt. J. Immunol. 189: 1765–1772.
Hossain MS et al. PD-1 and CTLA-4 up regulation on donor T cells is insufficient to prevent GvHD in allo-HSCT recipients. PLoS One. 2017 Sep 27;12(9):e0184254. doi:
Bashey, Soiffer RJ, Ball ED. CTLA4 blockade with ipilimumab to treat relapse of malignancy after allogeneic hematopoietic cell transplantation. Blood. 2009 Feb 12;113(7):1581-8. doi: 10.1182/blood-2008-07-168468. Epub 2008 Oct 30.
Serody J. S., G. R. Hill. 2012. The IL-17 differentiation pathway and its role in transplant outcome. Biol. Blood Marrow Transplant. 18(1, Suppl.)S56–S61.
Tawara I, et al. Combined Th2 cytokine deficiency in donor T cells aggravates experimental acute graft-vs-host disease. Exp Hematol. 2008;36:988–996.
Cooke KR, et al. Tumor necrosis factor- alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. J Clin Invest. 1998;102:1882–1891
Nestel FP, Price KS, Seemayer TA, Lapp WS. Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease. J Exp Med. 1992;175:405–413
Bellinger et al. The significance of vasoactive intestinal peptide (VIP) in immunomodulation. Adv Neuroimmunol 6, 5-17.
Brok HP, Vossen JM, Heidt PJ. IFN-gamma-mediated prevention of graft-versus-host disease: pharmacodynamic studies and influence on proliferative capacity of chimeric spleen cells. Bone Marrow Transplant. 1998;22:1005–1010.

Lu Y, Waller EK. Dichotomous role of interferon-gamma in allogeneic bone marrow transplant. Biol Blood Marrow Transplant. 2009;15:1347–1353.
Reddy P. Pathophysiology of acute graft-versus-host disease. Hematol Oncol. 2003;21:149–161.
Koyama M, et al. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. Nat Med 2011;18(1):135-142.
Toubai T, et al. Induction of acute GVHD by sex-mismatched H-Y antigens in the absence of functional radiosensitive host hematopoietic-derived antigen-presenting cells. Blood 2012;119(16):3844-3853.
Matte CC, et al. Donor APCs are required for maximal GVHD but not for GVL. Nat Med2004;10(9):987-992.
Barnes D.Wet al. “Secondary disease” of radiation chimeras: A syndrome due to lymphoid aplasia. Ann. N. Y. Acad. Sci. 1962;99:374–385. doi: 10.1111/j.1749-6632.1962.tb45321.
Billingham R.E. The biology of graft-versus-host reactions. Harvey Lect. 1977–1967;62:21–78.
Ferrara J.L., Levine J.E., Reddy P., Holler E. Graft-versus-host disease. Lancet. 2009;273:1550–1561. doi: 10.1016/S0140-6736(09)60237-3
Villa NYet al. Therapeutics for Graft-versus-Host Disease: From Conventional Therapies to Novel Virotherapeutic Strategies. Lamfers MLM, Chiocca EA, eds. Viruses. 2016;8(3):85. doi:10.3390/v8030085.
Andrea S. Henden and Geoffrey R. Hill. J Immunol May 15, 2015, 194 (10) 4604-4612; DOI: https://doi.org/10.4049/jimmunol.1500117
Wang X, et al. Mechanisms of antigen presentation to T cells in murine graft-versus-host disease: cross-presentation and the appearance of cross-presentation. Blood. 2011 Dec 8;118(24):6426-37. doi: 10.1182/blood-2011-06-358747. Epub 2011 Sep 30. PubMed PMID: 21963602; PubMed Central PMCID: PMC3236124.
Marmont AM, et al. T-cell depletion of HLA-identical transplants in leukemia.Blood 1991;78(8):2120-2130.
↲Horowitz MM, et al. Graft-versus-leukemia reactions after bone marrow transplantation.Blood 1990;75(3):555-562.
Motoko Koyama and Geoffrey R. Hill. Alloantigen presentation and graft-versus-host disease: fuel for the fire. Blood 2016 127:2963-2970; doi: https://doi.org/10.1182/blood-2016-02-697250
Saha A,et al. Programmed death ligand-1 expression on donor T cells drives graft-versus-host disease lethality. J Clin Invest. 2016 Jul 1;126(7):2642-60. doi: 10.1172/JCI85796. Epub 2016 Jun 13. PubMed PMID: 27294527; PubMed Central PMCID: PMC4922691.
McDonald-Hyman C, Turka LA, Blazar BR. Advances and Challenges in Immunotherapy for Solid Organ and Bone Marrow Transplantation. Science translational medicine. 2015;7(280):280rv2.
Freeman GJ, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med. 2000;192(7):1027–1034. doi: 10.1084/jem.192.7.1027.
Latchman Y, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol. 2001;2(3):261–268. doi: 10.1038/85330.
Yamazaki T, et al. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol. 2002;169(10):5538–5545. doi: 10.4049/jimmunol.169.10.5538.
Keir ME, et al. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008;26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331.
Blazar BR, et al. Advances in graft-versus-host disease biology and therapy. Nature reviews Immunology. 2012;12(6):443-458. doi:10.1038/nri3212.
Blazar BR, et al. Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. J Immunol. 2003;171:1272–1277.
D. Ganea et al. The neuropeptide vasoactive intestinal peptide: direct effects on immune cells and involvement in inflammatory and autoimmune diseases. Acta Physiol 2015, 213, 442–452
Ryan CE, Sahaf B, Logan AC, O'Brien S, Byrd JC, Hillmen P, Brown JR, Dyer MJ,

Mato AR, Keating MJ, Jaglowski S, Clow F, Rezvani AR, Styles L, Coutre SE, Miklos DB. Ibrutinib efficacy and tolerability in patients with relapsed chronic lymphocytic leukemia following allogeneic HCT. Blood. 2016 Dec 22;128(25):2899-2908. doi: 10.1182/blood-2016-06-715284. Epub 2016 Nov 1. PubMed
Kitko CL, Braun T, Couriel DR, Choi SW, Connelly J, Hoffmann S, Goldstein S, Magenau J, Pawarode A, Reddy P, Schuler C, Yanik GA, Ferrara JL, Levine JE. Combination Therapy for Graft-versus-Host Disease Prophylaxis with Etanercept and Extracorporeal Photopheresis: Results of a Phase II Clinical Trial. Biol Blood Marrow Transplant. 2016 May;22(5):862-8. doi: 10.1016/j.bbmt.2015.11.002.
Bellinger, D.L., Lorton, D., Brouxhon, S., Felten, S. & Fel-ten, D.L. 1996. The significance of vasoactive intestinal polypeptide (VIP) in immunomodulation. Adv Neuroim-munol 6,5–27