Acute Graft-Versus-Host Disease (aGVHD) is a T cell mediated immunological disorder and the leading cause of non-relapse mortality in patients who receive allogeneic hematopoietic cell transplants. Based on recent observations that PRMT5 and arginine methylation is upregulated in activated memory T cells, we hypothesized that PRMT5 is involved in the pathogenesis of aGVHD. Here, we show that PRMT5 expression and enzymatic activity is upregulated in activated T cells in vitro and in T cells from mice developing aGVHD after allogeneic transplant. PRMT5 expression is also upregulated in T cells of patients who developed aGVHD after allogeneic hematopoietic cell transplant compared to those who did not develop aGVHD. PRMT5 inhibition using a selective small-molecule inhibitor (C220) significantly reduces mouse and human allogeneic T cell proliferation and inflammatory IFN-γ and IL-17 cytokine production. Administration of PRMT5 small-molecule inhibitors significantly improves survival, reducing disease incidence and clinical severity in mouse models of aGVHD without adversely affecting engraftment. Importantly, we show that PRMT5 inhibition retains the beneficial graft versus leukemia (GVL) effect by maintaining cytotoxic CD8 T cell responses. Mechanistically, we show that PRMT5 inhibition potently reduces STAT-1 phosphorylation as well as transcription of pro-inflammatory genes including Interferon Stimulated Genes (ISG) and IL-17. Additionally, PRMT5 inhibition deregulates cell-cycle in activated T cells and disrupts signaling by impacting ERK1/2 phosphorylation. Thus, we have identified PRMT5 as […]

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PRMT5 regulates T cell interferon response and is a target for acute graft-versus-host disease.

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COI Disclosure: The authors have declared that no conflict of interest exists.
Abstract

Acute Graft-Versus-Host Disease (aGVHD) is a T cell mediated immunological disorder and the leading cause of non-relapse mortality in patients who receive allogeneic hematopoietic cell transplants. Based on recent observations that PRMT5 and arginine methylation is upregulated in activated memory T cells, we hypothesized that PRMT5 is involved in the pathogenesis of aGVHD. Here, we show that PRMT5 expression and enzymatic activity is upregulated in activated T cells in vitro and in T cells from mice developing aGVHD after allogeneic transplant. PRMT5 expression is also upregulated in T cells of patients who developed aGVHD after allogeneic hematopoietic cell transplant compared to those who did not develop aGVHD. PRMT5 inhibition using a selective small-molecule inhibitor (C220) significantly reduces mouse and human allogeneic T cell proliferation and inflammatory IFN-γ and IL-17 cytokine production. Administration of PRMT5 small-molecule inhibitors significantly improves survival, reducing disease incidence and clinical severity in mouse models of aGVHD without adversely affecting engraftment. Importantly, we show that PRMT5 inhibition retains the beneficial graft versus leukemia (GVL) effect by maintaining cytotoxic CD8 T cell responses. Mechanistically, we show that PRMT5 inhibition potently reduces STAT-1 phosphorylation as well as transcription of pro-inflammatory genes including Interferon Stimulated Genes (ISG) and IL-17. Additionally, PRMT5 inhibition deregulates cell-cycle in activated T cells and disrupts signaling by impacting ERK1/2 phosphorylation. Thus, we have identified PRMT5 as a regulator of T cell responses and as a therapeutic target in aGVHD.
INTRODUCTION

T cell activation via the T cell receptor complex (TCR) involves a complex interaction of signaling networks and subsequent gene transcription pathways that dictate the phenotype of T cell response to antigenic insult. Acute graft versus host disease (aGVHD), a T cell mediated immunological disorder, is a frequent post-transplant complication associated with increased morbidity and mortality in patients who receive allogeneic hematopoietic stem cell transplants (1-3). The pathogenesis of aGVHD involves the recognition of host minor and major histocompatibility complex (MHC) antigens by immune-competent donor-derived T cells that mount an inflammatory reaction, initiating T cell allo-antigen recognition followed by expansion, migration, and finally end-organ damage due to a combination of pro-inflammatory cytokine secretion (IFN-γ, TNF-α, IL-17) and direct cytotoxic effects (1-3). Th1 cells producing inflammatory cytokines such as IL-2, IFN-γ, TNF-α are considered the crucial subset of T cells that induce aGVHD (4-6). Interestingly, studies have shown that absence of IFN-γ exacerbates aGVHD in part due to increased Th2/Th17 differentiation and Th2/Th17 mediated pathologies while absence of Th17 leads to aggravated Th1 mediated aGVHD (6-10). These studies demonstrate that the contribution of different T cell subsets (Th1, Th2 and Th17) on aGVHD pathogenesis is complex and likely interdependent involving multiple factors including preferential expression of chemokine receptors that regulate homing and thereby tissue-specific damage by donor T cells (6-10).

Post-translational modifications via arginine methylation have been shown to play an important role in several biological processes including gene transcription, cell-cycle progression and signal transduction (11-13). Arginine (R) methylation is catalyzed by a group of enzymes called protein arginine methyltransferases (PRMTs) that are classified into three main groups depending on the type of methyl groups added to the R residues. Type I PRMTs (PRMT1, PRMT3, PRMT4, and
PRMT6) catalyze asymmetric dimethylation, Type II PRMTs (PRMT5 and PRMT9) catalyze symmetric dimethylation and Type III enzymes (PRMT7) drive monomethylation of R residues (11, 12). All PRMT enzymes utilize S-adenosylmethionine (SAM) as their methyl donor, transferring a methyl group to terminal guanidine nitrogen atoms of arginine side-chains on histones and other protein substrates (14, 15). PRMT5 is the predominant type II enzyme responsible for the majority of symmetric dimethylation of R residues (11, 13, 16) and is unique among PRMT enzymes in requiring a co-factor, MEP50, that increases PRMT5’s methyltransferase capabilities by increasing protein substrate affinity (11, 14). Until recently, protein R-methylation was detected mostly on nuclear histones and therefore thought to regulate processes such as chromatin remodeling, transcription, cell cycle, and spliceosome assembly (15). However, new research shows that T cells are sensitive to protein R-methylation. T cell activation results in global upregulation of R-methyltransferase activity (17, 18) that controls signal transduction and T cell function. Disruption of PRMT5 activity by RNA interference resulted in inhibition of both IL-2- and NF-AT-driven promoter activities and IL-2 secretion (19). Using a conditional knock-out model, researchers have shown that PRMT5 plays a critical role in hematopoiesis by impairing proliferation of progenitor cells and cytokine signaling (20). Interestingly, loss of PRMT5 affected thymic cellularity indicating a role for PRMT5 in early thymocyte development, however no difference was seen in spleen weight or cellularity (20). T–cell specific deletion of PRMT5 results in defects in peripheral T cell maintenance as well as T cell signal transduction via splicing defects in γc and JAK3 mRNA (21). Our group has shown previously that PRMT5 blockade with a PRMT5 inhibitor suppressed memory T cell responses and reduced inflammation in Experimental Autoimmune Encephalomyelitis (EAE) mouse models (22). Given that aGVHD is predominantly a T-cell driven inflammatory disease, we sought to investigate the role of PRMT5 in promoting inflammation and wanted to determine the effect of PRMT5 inhibition on aGVHD disease pathogenesis.
In parallel to its role in immune cells, PRMT5 is overexpressed in many leukemias and lymphomas (23-26). Epigenetic changes driven by PRMT5 lead to repression of tumor suppressors such as ST7, PTPROT, microRNA-29b, consequently leading to aberrant proliferation and survival of cancer cells (24-28). This has led a surge of interest in developing specific and selective inhibitors of PRMT5 as a therapeutic strategy for its antitumor properties. Targeting PRMT5 has therapeutic benefit in preclinical models of lymphoma and AML (13, 23-27) and currently, PRMT5 inhibition is being evaluated in Phase I clinical trials for both solid tumors and advanced hematological malignancies (NCT03854227, NCT03573310, NCT02783300, NCT03886831). Acute leukemias and myelodisplastic syndromes (combined with myeloproliferative disorders) are the most common indications for allogeneic transplants accounting for 72% of allogeneic HCTs (29). Therefore, inhibition of PRMT5 in a post-transplant setting could be a novel approach to inhibit inflammatory T cell responses thereby preventing GVHD while simultaneously targeting residual leukemic cells.

C220, a potent, selective PRMT5 inhibitor, is a derivative of a published PRMT5 inhibitor (30) that directly occupies the SAM binding pocket (Supplemental Figure 1A). C220 demonstrates IC50 of 2.4 nM in biochemical assays using Histone H4-based peptide as substrate (Supplemental Figure 1B), and IC50>1 uM against 38 Methyltransferases including other members of PRMT family (Supplemental Figure 1C). Here, we show that PRMT5 expression and function is upregulated in activated T cells and in T cells of mice and humans with aGVHD. Inhibition of PRMT5 activity using a selective small-molecule inhibitor, C220, results in significant reduction of alloreactive T cell proliferation, impacts T cell signaling including STAT-1 and ERK1/2 phosphorylation and inflammatory IFN-γ and IL-17 cytokine production. PRMT5 inhibition in vivo delays aGVHD and improves survival in mouse models of aGVHD without compromising beneficial GVL effects. Together, our results demonstrate for the first time a role for PRMT5 in aGVHD pathogenesis and support future studies to investigate PRMT5 inhibitors as a therapeutic strategy for aGVHD.
RESULTS

PRMT5 expression and enzymatic activity is upregulated in activated T cells.

We first set out to investigate the expression and enzymatic function of PRMT5 in activated murine and human T cells. Prmt5 mRNA expression was significantly upregulated in murine T cells stimulated via TCR with CD3/CD28 beads (Figure 1A) as well as T cells stimulated by allogeneic BALB/c bone marrow derived dendritic cells (BMDCs) mimicking a mode of activation resembling GVHD (Figure 1B). Consistent with mRNA expression, PRMT5 protein expression was also upregulated in murine TCR-stimulated and allo-stimulated T cells (Figure 1C). The enzymatic activity of PRMT5 was also upregulated in activated T cells as evidenced by the concordant increased symmetric dimethylation of Arg-8(R8) on histone H3 (H3R8me2s), (Figure 1C). Similar to mouse cells, we observed increased PRMT5 mRNA expression (Figure 1D), PRMT5 protein and enzymatic activity (Figure 1E) in activated human T cells. Increased enzymatic function was further confirmed by in vitro PRMT5 methyltransferase activity assay, which demonstrated increased specific activity on an H4-Arg3 peptide in nuclear extracts derived from CD3/CD28 activated human T cells compared to control (Figure 1F). To investigate the status of PRMT5 expression/activity in T cells in an inflammatory disease setting, we used the B6 into B6D2F1 (F1) aGVHD mouse model. T cells were isolated from spleens of irradiated B6D2F1 recipient mice that received T cell depleted bone-marrows (TCD-BM, no disease) or mice that received TCD-BM + B6 allogeneic splenocytes (TCD-BM+allo spl, aGVHD mice) around day 25 post-transplant. PRMT5 mRNA (Figure 1G) as well as protein expression and enzymatic activity was clearly upregulated in T cells of mice that developed aGVHD compared to mice that do not develop disease (Figure 1H). Together, these data show that activation of T cells results in significant upregulation of PRMT5 gene and protein expression as well as function, determined by increased enzymatic activity. Lastly, to evaluate status of PRMT5 in aGVHD patients, we compared expression of PRMT5 in patients who received an allogeneic stem cell transplant but did not
develop aGVHD versus those who developed aGVHD (at time of diagnosis). We found that PRMT5 expression was significantly upregulated in T cells of patients who developed aGVHD as compared to non-aGVHD patients or healthy controls (Figure 1I).

PRMT5 inhibition significantly reduces mouse and human allogeneic T cell proliferation and inflammatory cytokine secretion.

Since PRMT5 expression was upregulated in activated T cells, we sought to determine if inhibition of PRMT5 would impact T cell proliferation and effector function (as measured by cytokine secretion). B6 T cells were stimulated with allogeneic BMDCs in the presence of increasing concentrations of PRMT5 small-molecule inhibitor (C220). Treatment with C220 significantly impaired both murine T cell proliferation and IFN-γ secretion (Figure 2A–C, Supplemental Figure 2). We observed selective reduction in symmetric dimethylation but not asymmetric R-methylation (Figure 2D, E), showing specificity and selectivity of our inhibitor to only PRMT5 and no other members of the PRMT family that mediate asymmetric R-methylation. Human PBMCs were stimulated in MLR experiments, and similar to the mouse T cells, we observed a dose-dependent decrease in human T cell proliferation, IFN-γ secretion (Figure 2F and G) and symmetric–R-dimethylation (Supplemental Figure 3) in response to PRMT5 inhibition.

PRMT5 inhibition significantly improves survival in mouse models of aGVHD

Since PRMT5 regulates T cell proliferation and cytokine secretion, we asked whether administration of PRMT5 inhibitor could improve overall survival and clinical disease severity in mice with aGVHD. Using the B6 into B6D2F1 model, lethally irradiated F1 mice were treated with C220 (2mg/kg) or vehicle (oral gavage, once weekly) starting at day +7 after infusion of allogeneic B6 splenocytes. PRMT5 inhibition with C220 significantly prolonged survival of allo-transplanted recipient mice (Figure 3A), and resulted in reduced aGVHD clinical scores (Figure 3B) and
histopathology scores (Figure 3C). The improved pathology score in the C220-treated mice was due to reduced periportal and perivascular inflammation in the liver as well as reduced inflammation and apoptotic bodies in the colon (Supplemental Figure 4). We observed significant downregulation of symmetric dimethylation of SmD protein, a pharmacodynamic marker for PRMT5 inhibition, in splenocytes of mice treated with C220 compared to vehicle (Figure 3D, E). We further tested therapeutic effect of PRMT5 inhibition using a mouse model of xenogeneic aGVHD. Briefly, NSG mice were conditioned with 50cGy and received T cell depleted PBMCs (10*10^6 cells, TCD-PBMC) or 15-20*10^6 human PBMCs. Mice that received T cell replete grafts were given C220 (2mg/kg, MWF, starting day 7 post-transplant) or vehicle and monitored for survival. Administration of C220 significantly prolonged survival of NSG mice (Figure 3F, G) compared to vehicle control.

**PRMT5 inhibition reduces donor Th1 and Th17 responses without adversely impacting engraftment.**

We used the B6 into F1 model to further characterize the impact of PRMT5 inhibition on donor cell engraftment and T cell functions in vivo. Using knock-out mouse models, it has previously been shown that while Prmt5 heterozygosity has no effect on hematopoiesis, homozygous loss of Prmt5 leads to BM aplasia and lethal pancytopenia (20). Donor stem cell engraftment is critical to the success of an allogeneic HCT; therefore, we wanted to evaluate the effect of PRMT5 inhibitor C220 on donor cell engraftment. There was no significant difference in total donor cell chimerism as evidenced by %CD45.1 BoyJ donor cells and absolute numbers between vehicle and C220 treated cohorts showing PRMT5 inhibition with C220 did not affect donor engraftment (Figure 4A, B). Concordantly, we did not observe any adverse effects on hematopoiesis in mice that received PRMT5 inhibitor C220 compared to vehicle (Supplemental Figure 5A), confirming that it is possible to pharmacologically inhibit PRMT5 without adversely affecting hematopoiesis. Mice treated with PRMT5 inhibitor had similar absolute numbers of neutrophils (Supplemental
Figure 5B), total WBC and platelets in circulation (Supplemental Figure 5C, D) compared to the vehicle treated counterparts. Interestingly, there were both lower percentage and absolute numbers of CD45.1+CD3+ T cells in mice receiving C220 (Figure 4C, D). Supporting our in-vitro data, we observed fewer Ki67+ proliferating donor T cells in the spleens of mice receiving C220 compared to vehicle (Figure 4E). We also observed significant reduction in CD4+ IFN-γ+ (Th1) as well as a reduction in in CD4+ IL-17+ donor Th17 cells in mice that received PRMT5 inhibitor compared to vehicle. Importantly, there was no reduction in the Foxp3+ Treg compartment in C220 treated mice compared to vehicle, confirming the Treg sparing effect (22) of PRMT5 inhibition (Figure 4F, G). Levels of serum IFN-γ was also lower in animals treated with C220 as compared to vehicle (Figure 4H). TNF-α and IL-17 serum levels were undetectable by ELISA, therefore comparisons could not be made. Altogether, our data suggests that pharmacological inhibition of PRMT5 reduces donor Th1 and Th17 but not Tregs in allo-transplanted recipient mice, improving survival while not adversely affecting engraftment.

**PRMT5 inhibition reduces donor T cell infiltration into GVHD target organs.**

The liver and gastrointestinal (GI) tract represent the major target organs involved in aGVHD pathogenesis, where donor T cell trafficking and cytotoxicity result in organ failure that is ultimately fatal to the recipients of an allogeneic hematopoietic stem cell transplants. Therefore, we wanted to evaluate the effects of PRMT5 inhibition on donor T cell infiltration and effector function in the liver and GI tract. Using the B6 into B6D2F1 model, we observed significantly lower percentages and absolute numbers of donor T cells (Figure 5A, B) in the liver of C220 treated mice compared to vehicle. There was no significant difference in the percentage and numbers of donor Foxp3+ Tregs between mice treated with vehicle and C220 (Figure 5C, D); however, the percentage and numbers of donor CD4 T cells secreting IFN-γ (Figure 5E-G) and IL-17 (Figure 5H-J) in the liver was significantly lower in mice receiving PRMT5 inhibitor C220 compared to vehicle. Similarly,
we observed reduced percentages and absolute numbers of donor T cells as well as reduced numbers donor CD45.1 CD4 T cells secreting IFN-γ and IL-17 in the small intestine lamina propria of mice in C220 treated mice compared to vehicle (Figure 5K).

PRMT5 inhibition maintains graft versus leukemia (GVL) response

The main goal of an allogeneic transplant is to induce a donor anti-tumor response to eliminate residual leukemia/lymphoma in the recipient. To investigate whether PRMT5 inhibition retained GVL, we used a luciferase-transduced murine mastocytoma P815 cell line in the B6 into F1 model. Briefly, lethally irradiated B6D2F1 mice were injected with 10,000-15,000 P815 cells along with B6 TCD-BM alone or along with allogeneic splenocytes. Recipients of allogeneic splenocytes were treated with vehicle or C220 (2mg/kg) or vehicle starting at day 7 post-transplant till the end of the study. PRMT5 inhibition retained the beneficial GVL effects as seen by an improvement in survival (Figure 6A), decreased luminescence (Figure 6B and C) compared to vehicle treatment. Cause of death in the recipients of allogeneic splenocytes was confirmed to be due to aGVHD by histological examination (Figure 6D) and clinical aGVHD scores (Supplemental Figure 6A). Presence/absence of tumor in was confirmed by flow cytometric evaluation of splenic GFP+ P815 cells. In mice that did not receive infusion of allogeneic splenocytes along with the tumor cells, there was high tumor burden in the spleen (>40% GFP+ P815 cells). In contrast, mice that received allogeneic splenocytes eradicated the tumor with no difference in percentage of GFP+ P815 cells between mice receiving vehicle or C220 showing retention of beneficial GVL effect (Supplemental Figure 6B, C). Murine CD8+ T cells were stimulated in vitro with allogeneic BMDCs in the presence of DMSO/C220 and degranulation in response to P815 tumor challenge was analyzed to evaluate the CTL capacity of CD8+ T cells. C220 treated CD8+ T cells showed comparable degranulation as measured by CD107a mobilization compared to control (Figure 6E-F). These results suggest that PRMT5 inhibition does not abrogate CD8 CTL capacity, an important factor that might contribute to retention of GVL effect observed in vivo.
PRMT5 inhibition deregulates cell-cycle and ERK signaling in activated T cells

PRMT5 has been shown to promote cell proliferation and play a critical role in G1-to-S cell cycle transition in neoplastic hepatocellular (31) and breast cancer cells (32). Additionally, PRMT5 inhibition in lymphoma leads to transcriptional repression of Cyclin D1 (25). Therefore, we hypothesized that deregulation of cell-cycle might be a mechanism by which PRMT5 modulates T cell proliferation. To investigate this, murine T cells were stimulated with CD3/CD28 beads in the presence or absence of C220. Inhibition of PRMT5 by C220 resulted in T cell cycle deregulation with a decrease in S phase T cells and a concomitant increase at G0/G1 phase (Figure 7A and B), demonstrating that PRMT5 regulates G1-to-S transition in activated T cells. CD3/CD28 stimulated human T cells treated with PRMT5 inhibitor, C220, also showed a similar decrease in Ki67+ IdU+ S phase T cells (Figure 7C). ERK1/2 phosphorylation is impacted by PRMT5 in BM cells (20), and ERK1/2 is important for G1 to S transitions (33, 34). Therefore, we wanted to investigate whether ERK1/2 phosphorylation was affected by PRMT5 in T cells. Here, we show that PRMT5 inhibition resulted in significant downregulation of ERK1/2 phosphorylation (Figure 7D-E) in T cells that could contribute to cell-cycle deregulation.

PRMT5 regulates interferon response in T cells

To further elucidate the molecular mechanisms affected in T cells by PRMT5 inhibition, we compared gene expression profiles of CD3/CD28 activated human T cells in the presence or absence of the PRMT5 inhibitor, C220. Interestingly, we observed a very strong reduction in expression of Interferon stimulated genes (ISG) in T cells subjected to PRMT5 inhibition compared to control (DMSO). Additionally, we also observed a strong reduction in IL-17 transcription in PRMT5 inhibited T cells compared to control (Figure 8A). The top genes affected (using IPA canonical pathway analysis) by PRMT5 inhibition are listed in Table 1. We also validated downregulation of ISG – IFIT1, IFIT3, IFI35, as well as inflammatory cytokine IL-17 in
four additional donors by real-time qPCR (Figure 8B). Our results show that PRMT5 inhibition resulted in both decreased IFN-γ secretion (in vitro and in vivo) as well as reduced ISG transcription. It is well known that signal transducer and activator of transcription-1 (STAT-1) mediates the biological activity of both Type I (IFN-α and IFN-β) and type II Interferon (IFN-γ) that ultimately results in the transcription of interferon-stimulated genes (ISGs) (35, 36). PRMT5 was originally identified as a Jak2 binding protein (37), and Jak2 is one of the kinases responsible for the phosphorylation of STAT-1 in response to IFN cytokine stimulation (38). Therefore, we hypothesized that PRMT5 may regulate STAT-1 phosphorylation in response to IFN stimulation. Confirming our hypothesis, we found that PRMT5 inhibition significantly suppressed STAT-1 phosphorylation (Figure 8C, D) without affecting total STAT-1 (Figure 8E). We also observed a similar reduction in STAT-1 phosphorylation in vivo in mice that received C220 compared to vehicle treated mice (Figure 8F, G). Therefore, decreased STAT-1 signaling and modulation IFN response observed due to PRMT5 inhibition could be a mechanism contributing to reduced pathogenicity of T cells.
DISCUSSION

Our results show that PRMT5 mRNA and protein expression as well as enzymatic activity is upregulated in activated T cells. Disruption of PRMT5 results in significant downregulation of T cell proliferation and function in response to allogeneic stimuli both in vitro and in vivo. We demonstrate that PRMT5 inhibition modulates multiple components of T cell signaling machinery including, ERK and STAT-1 phosphorylation culminating in significant downregulation of inflammatory ISGs and IL-17. PRMT5 inhibition by oral administration of a PRMT5 small-molecule inhibitor significantly improves survival and reduces clinical disease severity in mouse models of aGVHD. Importantly, we show that pharmacological targeting of PRMT5 with selective small-molecule inhibitors does not adversely impact donor stem cell engraftment and neutrophil recovery post-transplant. Neutrophil recovery after allo-HCT is an important parameter that dictates post-transplant outcomes, since prolonged periods of neutropenia leave the patient vulnerable to life-threatening infections(39). Additionally, we show that PRMT5 inhibition preserves Foxp3+ Tregs post-transplant. Tregs play a central role in the establishment and maintenance of tolerance after allo-HCT (40-42). Therefore, any therapies that can selectively maintain Treg presence and activity while eliminating Th1/Th17 inflammatory allo-responses will provide a superior outcome compared to existing therapies.

The main goal of an allogeneic transplant is to induce a donor anti-leukemia response to eliminate residual leukemia/lymphoma in the recipient (43). However, since aGVHD and GVL are two tightly linked processes, any therapeutic strategy targeting aGVHD must not abrogate the GVL response of the donor allograft. To that end, maintenance of CTL function is critical for the beneficial anti-neoplastic immune response of the donor allograft (40, 44). Using the P815 GVL model, we show that PRMT5 inhibition retains CD8+ CTL capacity that might explain why PRMT5 inhibition still preserves the beneficial GVL effect. Further studies are required to demonstrate whether CD4 and CD8 T cells have different responses to PRMT5 inhibition. Our studies show that PRMT5
inhibition is a viable post-transplant strategy that can alleviate aGVHD by reducing Th1/Th17 responses and maintaining Treg mediated tolerance while at the same time retaining GVL effect due to maintenance of donor CD8 CTL capacity.

One of the main steps in aGVHD induction and propagation is the proliferation of donor T cells triggered by DC antigen presentation, the appropriate co-stimulatory signals, and cytokine environment. This massive expansion begins with T cells entering into and rapidly progressing through the cell cycle (2, 3) and anti-proliferative regimens such as methotrexate, cyclophosphamide, or mycophenolate are routine prophylactic regimens for aGVHD in the clinic (45). Here, we show that inhibition of PRMT5 significantly reduces T cell proliferation with a concomitant decrease in S phase T cells. The relation between PRMT5 and ERK signaling is complex. PRMT5 has been shown to negatively regulate MAPK signaling in cancer cell lines (33), however, using the inducible PRMT5 KO mouse, researchers have shown that loss of PRMT5 decreased ERK1/2 phosphorylation (20). We extend these findings to T cells and show that inhibition of PRMT5 with a selective, small-molecule PRMT5 inhibitor results in decreased ERK1/2 phosphorylation. Targeting RAS-MEK–ERK pathway (46) using a MEK inhibitor such as trametinib has been shown to inhibit alloreactivity and suppress aGVHD by decreasing ERK1/2 phosphorylation and expansion of donor T cells and sparing Tregs (47). Therefore, down-regulation of ERK pathway could be a major mechanism by which inhibition of PRMT5 exerts its protective effects in aGVHD.

The role of cytokines such as the interferons (Type I and II) as well as IL-17 in aGVHD pathogenesis is intricate and depends on a number of factors including the cellular source (donor or recipient), conditioning regimen (myeloablative vs. reduced intensity) as well as timing of cytokine production (pre-transplant, early or late-post transplant) (48-51). While IFN-γ has been shown to promote aGVHD pathogenesis, it is also important for GVL (48, 52-54). Complete loss
of IFN-γ, paradoxically caused more severe GVHD, potentially due to the loss of anti-inflammatory
effects of donor-derived IFN-γ and IFNγR signaling (6, 8, 55, 56). Type I Interferons have been
shown to reduce (57, 58) or promote (59) GVHD while playing an important role in contributing to
donor derived GVL effects. Similarly, IL-17 producing Th17 cells have been shown to promote
aGVHD, however, absence of IL-17 has been shown to promote aGVHD by augmenting Th1
responses (6, 7, 10, 51, 60, 61). Despite the heterogeneous nature of the contribution of
interferons to aGVHD disease progression, there is consensus that inhibition of STAT-1; the
mediator of biological activity of interferons can ameliorate aGVHD (59, 62-64). Our results show
that inhibition of PRMT5 potently reduces IFN-γ and IL-17 production by activated alloreactive T
cells, accompanied by reduced STAT-1 phosphorylation, culminating in a reduction in
transcription of ISGs. The precise mechanisms by which PRMT5 modulates STAT-1
phosphorylation, i.e. whether by modulation of JAK kinase or SHP phosphatase activity is a
subject of ongoing investigation in our lab.

We demonstrate that PRMT5 inhibition causes significant downregulation inflammatory gene
transcription such as ISGs and IL-17. Previous studies have shown that PRMT5 mediated
histone-tail modifications, particularly symmetric dimethylation of histone arginine residues (H2A,
H4R3, H3R2, H3R8) is associated with transcriptional repression (15, 65, 66). PRMT5 is also
known to regulate transcription by conferring post-translational modifications on transcription
factors NF-κB (67), p53 (68, 69), and E2F-1 (70). In addition, PRMT5 symmetrically dimethylates
multiple members of the spliceosome machinery such as Sm proteins D1, D3, and B/B’ (21, 71)
that regulate post-transcriptional control of mRNA expression. Therefore, determining the exact
molecular mechanism underlying the reduction in gene expression – i.e. whether it is due to global
gene repression via histone modification, or more direct due to modulation of IFN-STAT signaling
or due to defects in splicing need to be further investigated. These investigations have the
potential to reveal cross-talk networks between epigenetic modulator such as PRMT5 and
signaling pathways influenced by PRMT5 inhibition (such as ERK/STAT-1) thereby opening the
door to synergistic interactions of future drug combinations.

Epigenetic modifiers are appealing targets for aGVHD not only because of the role epigenetics
has in immune regulation but also because many epigenetic modifying agents are also used as
treatment against hematopoietic neoplasms (72, 73). A variety of epigenetic modifiers are
currently being evaluated as potential aGVHD therapeutics in Phase I and Phase II clinical trials,
primarily as combinatorial therapies with other well-established aGVHD treatments (74, 75).
DNMT inhibitors such as 5-azacitidine and 5-aza-2'-deoxycytidine (decitabine) have shown great
promise in ameliorating GVHD in both preclinical and clinical studies by increased regulatory T
cell production and impairing effector T cell proliferation (76-81). HDAC inhibitors suberoylanilide
hydroxamic acid 22(SAHA; vorinostat), ITF2357 (givinostat), and LBH589 (panobinostat) have
also been very successful in modulating aGVHD because of their multimodal roles in reducing
inflammatory cytokine secretion, boosting regulatory T cell production, and suppressing
alloreactive APC activation (64, 82-85).

We and others have shown that targeting PRMT5 has therapeutic benefit in preclinical models of
lymphoma and AML (13, 23-27). Here, we propose inhibition of PRMT5 in a post-transplant
setting as a “two-for one approach” that can inhibit inflammatory T cell responses and maintain
tolerance thereby preventing GVHD, retain GVL effects while simultaneously targeting residual
leukemic cells. Our studies have provided mechanistic insight into the role of PRMT5 in T cell
function as well as regulating aGVHD pathogenesis, and reveal PRMT5 as a therapeutic target
for aGVHD.
METHODS

Mice

C57BL/6 (B6, H2b), B6.SJL-Ptprca Pepc/BoyJ (CD45.1 B6), B6D2F1 (F1, H2bd) and BALB/c (H2d) mice were purchased from Jackson ImmunoResearch Laboratories (Bar Harbor, ME). Female NSG mice were acquired from the NSG mouse colony maintained by the Target Validation Shared Resource (TVSR) at the Ohio State University; breeders (Strain #005557) for the colony were received from the Jackson laboratory. All mice were bred and maintained in an OSU animal care facility. NSG mice were housed in a specific pathogen-free facility in micro-isolator cages. For transplant experiments, recipient mice were between 12 and 16 weeks of age, for all other experiments, mice were between 8-10 weeks of age.

aGVHD murine models

Only age- and sex-matched mice were used for transplant experiments. Briefly, B6D2F1 mice were irradiated with 1200 cGy administered in 2 fractions (to minimize toxicity) one day before transplant. T cell depleted bone marrow (TCD-BM) cells (10x10^6) plus 15x10^6 total splenocytes from CD45.1 B6 donors were administered via tail vein injection on the day of transplant. T cell depletion from BM cells was carried out by CD90 magnetic bead separation (Miltenyi Biotec). Recipients of allogeneic splenocytes were treated with vehicle or PRMT5 inhibitor C220 2mg/kg, administered by oral gavage once a week starting day +7 post-transplant until the end of the study.

Mouse model of xenogeneic GVHD

A xenogeneic GVHD model was used based on previous studies(42). One day before transplant, mice were irradiated with 50 cGy. On the day of transplant, mice were injected with T cell depleted PBMCs (10*10^6 cells, TCD-PBMC) or 15-20*10^6 human PBMCs. Mice receiving T cell replete
grafts were treated with C220 (2mg/kg) or vehicle administered by oral gavage, three times weekly starting day +7 post-transplant until the end of the study.

Clinical and histologic assessment of aGVHD

Recipient mice were weighed 2-4 times a week and monitored daily for clinical signs of aGVHD and survival. GVHD scores were performed using a system modified from Cooke et al (86). Briefly, this scoring system incorporates 5 clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity. Individual mice were ear tagged and graded (in a scale from 0 to 8) twice a week. Mice who reached an aGVHD score of more than or equal to 7 were very sick and were euthanized and their tissues harvested (86, 87). GVHD was also assessed by detailed histopathology analysis of H&E stained liver and gut tissues using a previously reported scoring system with a range of 0 (absence of signs of GVHD) to 4 (maximal GVHD damage) (88). A separate cohort of mice were euthanized around day 25 (± 3 days) post-transplant and used for histopathological assessment of target tissues.

PRMT5 inhibitor C220

PRMT5 inhibitor C220 was synthesized by WuXi Apptech, Shanghai, China. It was administered in a vehicle consisting of 0.5% methylcellulose, 0.1% Tween-80 and 99.4% sterile water. Mice were given C220 (2mg/kg) by oral gavage once a week starting day +7 post-transplant till the end of the study. For the NSG experiment alone, mice were dosed three times weekly. Mice in the control group were treated with the same volume of vehicle as the C220 group.

Cells and cell culture

Mouse B6 or CD45.1 B6 T cells were isolated from splenocytes using Pan-T Cell Isolation Kit or Naïve CD8 T cell isolation kit (Miltenyi Biotec) per manufacturer’s protocol. All cells were cultured in RPMI 1640, 20% FBS, and 1%Pen-Strep unless otherwise specified. Healthy donor-derived
PBMC were isolated by Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare). T cells were isolated from PBMCs using Pan-T Cell Isolation Kit (Miltenyi Biotec).

Western blots

T cells were lysed in RIPA buffer, and Western blot performed according to standard protocols. For time-course experiments, T cells were isolated from mouse spleen or human T cells were isolated from healthy donor leukopacs and stimulated using CD3/CD28 Dynabeads according to manufacturer’s protocol (Invitrogen). Cells were lysed in RIPA buffer and immunoblotted using primary Abs against PRMT5 (Abcam), H3R8me2s, and H3R8me2a (EpiGentek).

In vitro T cell proliferation:

CD45.1 B6 T cells and human T cells were labeled with Cell Trace Violet (CTV) and incubated with allogeneic BALB/c bone marrow derived dendritic cells (BMDCs) or T cell depleted PBMCs (for human MLR). Cell division was measured by CTV dilution after 4-5 days using LSRII and FACS Diva software (Becton Dickinson).

Cytokine ELISA

Mouse and human T cells were stimulated as described above. Supernatant cytokines were analyzed by ELISA according to manufacturer’s protocol (BioLegend). Results are shown as mean + SD of biological duplicates of three donors. Serum was collected by cardiac puncture of mice that were euthanized on day 21 from transplant experiments for cytokine and flow cytometry experiments; and cytokines measured by ELISA (BioLegend).

In vitro PRMT5 methyltransferase assay

Methylation activity in nuclear extracts derived from indicated human T cells was measured using Epigenase PRMT5 Methyltransferase (Type II-Specific) Activity/Inhibition Assay Kit (EpiGentek,
Farmingdale, NY) according to the manufacturer’s protocol. Standard curves were generated using Methylated H4-Arg3 standard provided by the manufacturer, with a linear detection range between 0.1 – 2 ng of methylated product. Reactions contained 20 µg of the indicated nuclear extracts (prepared with Nuclear Extraction kit # OP-0002 from EpiGentek), and were incubated for 120 min at 37°C. Absorbance (450 nm with a reference wavelength of 655 nm) was measured with a Tecan Infinite M1000Pro microplate reader (Thermo Fisher Scientific). Each experiment included at least three independent replicate measurements per sample, and P-values for each sample comparison were calculated using a two-tailed unpaired t-test.

mRNA-seq

Human T cells were isolated from healthy donor PBMCs (n=4 donors) by negative selection. T cells were stimulated with CD3/CD28 Dynabeads for 48hrs and treated with either vehicle (DMSO) or C220 (100nM). RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and treated with DNase (Qiagen, Hilden, Germany). RNA quality was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the RNA integrity number values were greater than 7 for all samples. Sequencing libraries were generated with polyA+ RNA using the TruSeq RNA sample prep kit (Illumina, San Diego, CA). Libraries underwent paired end 50bp sequencing using the Illumina HiSeq2500 sequencer to a depth of 17 – 20 million passed filter clusters per sample. Demultiplexed and quality-filtered reads were mapped to the human genome GRCh38 using Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT2) (89). Raw read counts for each gene were quantified using featureCounts software, with GENCODE v.27 transcript reference (GENCODE annotation) (90). RNAs with less than twenty read counts on average across all samples were excluded from further analysis. Differential gene expression analysis between PRMT5 and DMSO treated cell was performed using R package edgeR (91). The read counts were normalized using TMM method (92). Differentially expressed genes were
selected based on adjusted p-value and log2 fold change (log2FC). Biological pathways and interactome analysis were performed using Ingenuity Pathway Analysis (IPA) software. The data supporting the results of this article are available in the GEO repository (accession ID: GSE145527).

Real-time PCR

RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was prepared using the SuperScript III cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA) and detected by commercially available Taqman probes for specific genes (Applied Biosystems) on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was normalized to housekeeping gene β-actin.

Flow cytometry analysis

Around day 25, cohorts of mice were euthanized and splenocytes, whole liver, and small intestine were harvested for flow cytometric analysis. Hepatic tissue and lamina propria were digested into a single cell suspension using a commercial mouse Liver Dissociation Kit (Miltenyi Biotec) and mouse Lamina Propria Tissue Dissociation Kit (Miltenyi Biotec). To select only the donor T cells, a specific gating strategy was used (Supplemental Figure 7). A complete list of antibodies used is listed in Supplemental Table 1. For cytokine evaluation, splenocytes were incubated for 5 hours with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors, Thermo Fisher Scientific) for T cell stimulation and protein transport inhibition. Cells were then stained with surface antibodies, permeabilized, fixed, stained with intracellular antibodies and analyzed within 24 hours. Analysis was performed with a FACS LSR II cytometer; FACSDiva software (Becton Dickinson) data analysis was performed using FlowJo (Tree Star).
IdU incorporation and mass cytometry analysis

Following healthy donor T-cell stimulation as described above cells were incubated with IdU (Sigma-Aldrich) at a final concentration of 10 uM for 15 minutes at 37°C. Following IdU incubation cells were fixed using SmartTube Proteomic Stabilizer (SmartTube Inc.) at a 1:1.4 ratio for 10 minutes at room temperature before being stored in a -80°C freezer before staining. Mass cytometry staining and measurement was performed as previously described (93). Briefly surface antibody staining was performed in 100 uL of CSM (1×PBS with 0.5% bovine serum albumin and 0.02% sodium azide) for 50 min at room temperature with continuous shaking. Cells were washed twice with CSM and surface antibodies were fixed using 1.5% paraformaldehyde solution (Electron Microscopy Services). Cells were pelleted following surface antibody fixation and permeabilized while vortexing with ice-cold methanol. Cells were washed with PBS and CSM twice before intracellular staining. Intracellular staining was performed in 100uL of CSM for 50 minutes at room temperature with continuous shaking. Cells were washed twice with CSM and then incubated for overnight at 4°C in PBS with 500 nM iridium intercalator pentamethylcyclopentadienyl-Ir(III)-dipyridophenazine (Fluidigm, Toronto Canada) and 1.6% paraformaldehyde. Excess intercalator was removed with two CSM washes and a single wash in PBS. Cells were resuspended in DI water at approximately 1 million cells/mL. Cell events were acquired on the Helios Mass Cytometer (Fluidigm, Toronto Canada) and FCS file analysis was performed using the Cytobank data analysis platform. A singlet gate was drawn in Cytobank using cell length by DNA (Ir intercalator) to remove doublets and debris from downstream analysis (Supplemental Figure 8). Cell cycle phases were gated according to previous methods (94, 95).

For cell cycle analysis, S-phase cells were gated on an IdU and Ki67 double positive population.

GVL experiments

Firefly luciferase transduced P815 mastocytoma (96, 97) cells (10,000) were injected
intravenously into F1 recipients on day 0 along with TCD-BM (10x10^6 cells) and B6 donor splenocytes (20 x 10^6 cells). Treatment groups included vehicle and PRMT5 inhibitor C220 2mg/kg, administered by oral gavage once a week starting day +7 post-transplant. TCD-BM and P815 cells (leukemia alone) served as the control group. P815-induced leukemic death was defined by the occurrence of either macroscopic tumor nodules in liver and/or spleen or hind-leg paralysis. GVHD death was defined by the absence of leukemia and the presence of clinical and histo-pathological signs of GVHD.

In vivo imaging

Xenogen IVIS imaging system (Caliper Life Sciences) was used for live animal imaging. Mice were anesthetized using 1.5% isofluorane (Piramal Healthcare). XenoLight RediJect D-Luciferin Ultra Bioluminescent Substrate (150 mg/kg body weight; 30 mg/mL in PBS; Perkin Elmer) was injected intraperitoneally and IVIS imaging was performed 10 minutes after substrate injection. Whole body bioluminescent signal intensity was determined weekly using IVIS Living Image software v4.3.1 (Caliper Life Sciences), and pseudocolor images overlaid on conventional photographs are shown. Data were analyzed and presented as photon counts per area.

Degranulation assay

CD8 degranulation assay was measured by CD107a mobilization and experiments were set up based on previously published protocols (40, 98). P815 cells were incubated at 37°C overnight. On the next day, the culture supernatant was removed from the wells, and effector cells were added to the wells. Effector T cells: Naive CD8 T cells isolated from BoyJ CD45.1+ splenocytes by negative selection (StemCell Technologies) were used as effector T cells: (a) CD8+ T cells without any treatment (CD8-resting), (b) CD8+ T cells stimulated with allogeneic BMDCs in the presence of DMSO or (c) C220 (100nM) for 5 days. CD8+ T cells were then mixed with P815 cells at effector/target (E/T) ratio (5:1), centrifuged at 1000 RPM for 3 minutes, and co-cultured in
the presence of anti-CD107a at 37°C overnight. On the next day, the cells were stained with surface markers CD45.1 and CD8, and analyzed by flow cytometry.

Statistical analysis
Survival data were analyzed using Kaplan-Meier and log-rank test methods. Differences between continuous variables at a single time point were analyzed using two-tailed t-tests. Data represent mean ± SD. One-way analysis of variance (ANOVA) with Holm-Sidak post-hoc test was used for comparisons >2 groups unless indicated otherwise. Differences between two groups over time analyzed with Mann-Whitney U. All analyses were performed using GraphPad Prism 7.0. *, p<0.05; **, p<0.01; ***, p<0.001. A P-value less than 0.05 was considered significant.

Study Approval
All animal studies were conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee at The Ohio State University. Peripheral blood samples from healthy donors and allogeneic transplant patients were obtained following written informed consent in accordance with the Declaration of Helsinki. Healthy PBMCs were obtained from Versiti and transplant patient samples were selected from a biorepository study, both approved by the IRB at The Ohio State University.
AUTHOR CONTRIBUTIONS

K.J.S, N.C.Z, Y.G., N.E.S., L.N.C. and A.I. performed in-vivo murine aGVHD experiments, ex-vivo FACS analyses, serum/supernatant ELISAs, western blots, real-time PCR analyses, in-vitro experiments with mouse cells and human PBMCs, signaling experiments, analyzed the data and interpreted the results. C.K., Y.G., N.E.S., R.D. performed mass cytometry experiments. G.B. was involved in supervision and discussion of results of the mass cytometry experiments. M.G.A and J.E.J. performed the in vitro methylation experiments. M.P. provided the bioinformatics data. M.W. and Y.W.Z. performed C220 biochemical assay, H.L. synthesized C220. H.K.C discussed experimental design, provided data and edited the manuscript. R.A.B. provided data discussion and edited the manuscript. R.G. and K.V. edited the manuscript. P.R. designed the study, supervised research, interpreted the data and wrote the manuscript along with NCZ.

ACKNOWLEDGEMENTS

This work was supported by a New Investigator Award from the Leukemia Research Foundation, Pelotonia New Investigator Award, K12 Paul Calabresi Award and Division of Hematology start-up funds (P.R.). Research reported in this publication was supported by the Ohio State University Comprehensive Cancer Center and the National Institutes of Health under grant number P30 CA016058. We thank the Target Validation Shared Resource (TVSR) at the Ohio State University Comprehensive Cancer Center for providing the NSG mice used in the preclinical studies described herein. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.
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Figure 1. PRMT5 expression and activity is upregulated in activated mouse and human T cells in vitro and in vivo. Murine B6 T cells were stimulated with either CD3/CD28 Dynabeads or with allogeneic BALB/c DCs for 4 days. (A, B) PRMT5 mRNA expression in T cells quantified by real-time PCR (n=5). Expression relative to unstimulated (US) T cells, β-actin used as a normalizer. Results are represented as mean ± S.D. of 2-3 independent experiments. Each symbol represents an individual donor. (C) Prmt5 protein and Histone H3R8 symmetric dimethyl protein (H3R8me2s) analyzed by western blot. One representative western blot of three independent experiments is shown. (D) T cells isolated from healthy human donor (HD) PBMCs were stimulated with CD3/CD28 Dynabeads and PRMT5 mRNA expression (n=3 donors) quantified by real-time PCR (E) immunoblotted for PRMT5 protein expression and function (n=2 donors). (F) In vitro PRMT5 methyltransferase activity in nuclear extracts derived from US vs. CD3/CD28 stimulated human T cells. Results show specific activity measured in ng dimethylated H4-Arg3 produced per 20 μg nuclear extract. Each symbol represents an individual replicate measurement, with mean ± S.D. One representative assay of 3 independent experiments is shown. (G) Splenic T cells isolated from lethally irradiated B6D2F1 mice that received TCD-BM or TCD-BM+ B6 allogeneic splenocytes around 25 days post-transplant. Messenger RNA expression (n=9) and (H) and protein expression. 2-3 spleens were pooled from recipient mice to make one pooled sample and three pooled samples were analyzed for PRMT5 protein expression by western blot. (I) Healthy human PBMCs were collected from healthy donors (HD PBMCs, n=7) and from patients after allogeneic BMT with or without GVHD at the time of collection (Supplemental Table 2). Patient samples were matched for day of transplant at which GVHD occurred. PBMCs were stained and analyzed for PRMT5 expression on T cells using mass cytometry; the gating strategy is shown in Supplemental Figure 8. Each symbol represents an individual donor. Data represent mean ± S.D. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.
Figure 2. Inhibition of PRMT5 blunts mouse and human T cell alloreactive response. Cell trace violet (CTV) labeled B6 T cells were stimulated with allogeneic BALB/c BMDCs for 4 days in the presence of increasing concentrations of PRMT5 inhibitor C220 (A) Histogram plots of one representative donor. (B) Percent alloreactive T cell proliferation normalized to no drug. (C) IFN-γ cytokine in supernatant analyzed by ELISA. B6 T cells were stimulated with BALB/c BMDCs for 4 days, T cells re-isolated and immunoblotted for (D) symmetrically dimethylated arginine residues (SDMA) and (E) H3R8me2s and H3R8me2a. β-actin was used as loading control. One representative western blot of three independent experiments is shown. (F) Cell trace violet (CTV) labeled CD3+ human T cells were stimulated with allogeneic T cell depleted PBMCs for 4 days in the presence of increasing concentrations of C220. Percent alloreactive T cell proliferation normalized to no drug. (G) IFN-γ cytokine in supernatant analyzed by ELISA. Mean ± SD of biological replicates of 3 mouse/human donors shown. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 compared to allo-stimulated T cells in absence of C220.
Figure 3: Inhibition of PRMT5 improves survival in mouse models of aGVHD. Lethally irradiated B6D2F1 recipients received CD45.1+ B6 T cell depleted bone marrow (TCD-BM, 10 x 10^6 cells) or TCD-BM + CD45.1+ B6 splenocytes (15 x 10^6). Recipients of allogeneic splenocytes were treated with C220 (2mg/kg) or vehicle by oral gavage once weekly starting day 7 post-transplant. (A) Kaplan-Meier survival curve. Log-rank test was used to compare survival. Data pooled from 3 independent transplant experiments, with n=6-8 in TCD-BM+ splenocytes group in every transplant experiment, TCD-BM group was included for 2 transplants, with n=4 each transplant. Total n=8 for TCD-BM group, n= 18-24 for TCD-BM +splenocytes groups. (B) Clinical GVHD scores. Mann-Whitney U test was used for comparison between groups. B6 into F1 transplant was performed as described above, and separate cohort of mice were euthanized at day 22 post-transplant (after receiving 3 doses of vehicle/C220 on days 7, 14 and 21) and used for (C-E). (C) Histopathological assessment of target tissues (liver and GI), n=5 per group. (D) Splenocytes harvested for immunoblotting for pharmacodynamics marker SmD3. (E) Densitometry values normalized to β-actin. Data represent mean ± S.D. Each symbol represents an individual donor, n=4-5. (F) NSG mice were irradiated with 50 cGy on day -1. On day 0, irradiated NSG mice received either T cell depleted- human PBMCs (TCD-PBMC, 10^6, n=3) or human PBMCs (15*10^6). Mice that received T cell replete grafts were treated with either C220 (2mg/kg, n=12) three times weekly or vehicle (n=10). Survival curve. Log-rank test was used to compare survival. (G) Clinical GVHD scores of xenogeneic GVHD model. Data shown is combined from 2 independent xeno-aGVHD experiments. *, p<0.05; ****, p<0.0001.
**Figure 4.** PRMT5 inhibition reduces donor Th1 and Th17 responses. B6 into F1 transplant was performed as described in methods. Mice were sacrificed around day 25 post-transplant (n=5–7 per group) and spleen harvested.  

(A) Percentage and (B) Absolute numbers of CD45.1+ donor cells. (C) Percentage and (D) Absolute numbers of CD45.1+ CD3+ donor T cells. (E) Percent CD45.1+ Ki67+ donor CD4+ and CD8+ T cells. (F) Representative histograms showing intracellular staining for IFN-γ, IL-17 and CD25/Foxp3 on donor CD45.1+CD4+ T cells. (G) Percent IFN-γ+, IL-17+ and CD25/Foxp3+ donor CD45.1+CD4+ T cells. (H) Serum IFN-γ analyzed by ELISA. Data shown is combined from two independent transplant experiments. Each symbol represents an individual mouse. *, p<0.05; **, p<0.01; ***, p<0.001.
Figure 5. PRMT5 inhibition reduces donor T cell infiltration into liver and GI tract.

B6 into F1 transplant was performed as described in methods. Mice were sacrificed around day 25 post-transplant, (n=5-7 per group) and liver (A-J) and small intestine of GI tract (K) harvested. Single cell suspensions were made as described in methods. (A) Percentage and (B) Absolute numbers of CD45.1+ CD3+ donor T cells in the liver. (C) Percentage and (D) Absolute numbers of Foxp3+ donor CD45.1+CD4+ T cells. (E) Representative histograms showing intracellular staining, (F) percentage and (G) absolute numbers of donor IFN-γ + CD45.1+CD4+ T cells in the liver. (H) Representative histograms showing intracellular staining, (I) percentage and (J) absolute numbers of donor IL-17+ CD45.1+CD4+ T cells in the liver. (K) Percentage and absolute numbers of CD45.1+ CD3+ donor T cells absolute numbers of donor Foxp3+ Tregs, donor IFN-γ +, IL-17+ CD45.1+CD4+ donor T cells in the lamina propria of the small intestine. One representative experiment of two independent transplant experiments is shown. Each symbol represents an individual mouse. *, p<0.05; **, p<0.01; ***, p<0.001.
Firefly luciferase-transduced P815 cells (10,000 cells) were injected i.v. into lethally irradiated F1 recipients on day 0 along with TCD-BM and B6 donor splenocytes. Treatment groups included PRMT5 inhibitor C220 or vehicle control. TCD-BM and P815 cells (leukemia alone) served as the control group. (A) Kaplan-Meier survival curve. Log-rank test was used to compare survival. Data pooled from 3 independent transplant experiments. N=22 for TCD-BM + P815 alone group, n=17-19 for TCD-BM + P815 + B6 splenocytes groups. (B-C) Whole-body bioluminescent signal intensity of recipient mice (n=5 per cohort). Mice were imaged on indicated days. Average radiance expressed as mean ± SD. One representative experiment of two is shown. (D) H&E stained liver sections at original magnification 200 and 400 of two representative recipients showing leukemic infiltration in the liver in the TCD-BM + P815 only group. There were no leukemic cells in either of the groups that received B6 allogeneic splenocytes. There was lymphocytic infiltration in mice receiving B6 allogeneic splenocytes (triangles). (E) Murine CD45.1 BoyJ CD8+ T cells were stimulated with allogeneic BALB/c BMDCs ± C220 (100nM) for 5 days. CTL capacity against P815 tumor cells was assessed by flow cytometric evaluation of intracellular CD107a expression. Contour plots of one representative donor. (F) Data expressed as mean ± SD of biological duplicates of three independent experiments. *, p<0.05.
Figure 7. Inhibition PRMT5 deregulates cell-cycle and ERK signaling in mouse and human T cells. (A) Murine CD3+T cells stimulated with CD3/CD28 Dynabeads ± C220 for 48hrs. Cell cycle analysis based on propidium iodide (PI) incorporation. Representative PI histogram of one experiment. (B) Percentage T cells in G0/G1, S and G2/M phases. Mean ± SD of biological triplicates of two independent experiments. Significance was determined using two-tailed t-test with Holm-Sidak correction for multiple comparisons. (C) Healthy donor T cells were stimulated with CD3/CD28 beads for 48hrs ± C220 (100nM). Cells were labeled with IdU for 15 mins at the end before collection for analysis by mass cytometry. Percent Ki67+ Idu+ T cells of 4 individual donors. (D) PBMCS were pre-treated with DMSO or C220 (100nM) for 2hrs and stimulated with PMA/ionomycin for 5 mins. Phosphorylation of ERK1/2 on CD3+ T cells analyzed by flow cytometry. Histogram of two representative donors. Mean Fluorescence Intensity (MFI) of (E) phospho-ERK1/2 and (F) total ERK1/2 in 7 donors. **, p<0.01; ***, p<0.001
Figure 8. PRMT5 regulates interferon response in T cells

Human T cells were isolated from healthy PBMCs (n=4) by negative selection. T cells were stimulated CD3/CD28 Dynabeads for 48hrs and treated with vehicle (DMSO) or C220, RNA isolated and RNA-seq performed. (A) Volcano plot showing the top dysregulated genes. (B) Real-time qPCR validation in four additional human donor T cells performed for indicated genes. Fold change compared to DMSO, gene expression normalized to β-actin. (C) PBMCs were stimulated with CD3/CD28 ± C220 for 48hrs and then rested for 4hrs followed by 15min pulse with IFN-α (100ng/ml). STAT-1 phosphorylation on CD3+ T cells was analyzed by flow cytometry. Histogram of 3 representative donors. (D) Mean Fluorescence Intensity (MFI) of p-STAT and (E) MFI Total STAT-1, n=6 donors. (F) B6 into F1 transplant was performed, and recipients treated with C220 or vehicle as described in methods. Splenocytes were isolated around day 25 post-transplant and pulsed with IFN-α (100ng/ml) for 40 minutes. MFI of p-STAT-1 and (G) total STAT-1 on CD45.1+ CD3+ donor T cells was evaluated by intracellular flow cytometry in both resting and IFN-α pulsed cells. *, p<0.05; **, p<0.01; ***, p<0.001.
Table 1. List of top 20 genes with significantly different expression in PRMT5 inhibitor treated T cells compared to control (DMSO).

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