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Supplemental Data

Short title: HDAC inhibition regulates immunity after human HCT

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Key Points

- HDAC inhibition reduced pro-inflammatory cytokines and increased regulatory T cell number and function after allo-HCT
- HDAC inhibition enhanced STAT-3 acetylation and induced IDO after allo-HCT
Abstract

We examined immunological responses in patients receiving histone deacetylase (HDAC) inhibition (vorinostat) for graft-versus-host disease (GVHD) prophylaxis following allogeneic hematopoietic cell transplant (allo-HCT). Vorinostat treatment increased histone acetylation in peripheral blood mononuclear cells (PBMC) from treated patients, confirming target HDAC inhibition. HDAC inhibition reduced pro-inflammatory cytokine levels in plasma and from PBMC, decreased ex vivo responses of PBMC to pro-inflammatory TLR-4 stimuli, but did not alter the number or response of conventional T cells (Tconv) to non-specific stimuli. However, the numbers of regulatory T cells (Tregs) were increased, which revealed greater demethylation of the Foxp3 T regulatory-specific demethylation region. Vorinostat-treated patients showed increased expression of CD45RA and CD31 on Tregs, and these Tregs demonstrated greater suppression on a per-cell basis. Consistent with preclinical findings, HDAC inhibition also increased STAT-3 acetylation and induced indoleamine-2,3-dioxygenase (IDO). Our data demonstrate that HDAC inhibition reduces inflammatory responses of PBMC but enhances Tregs after allo-HCT.
Introduction
Acute GVHD remains a major contributor of non-relapse mortality (NRM) after allo-HCT.\textsuperscript{1} Its pathogenesis involves a complex network of interactions between alloreactive T cells, antigen presenting cells (APCs), pro-inflammatory cytokines, and effector cells, leading to target organ injury in the host.\textsuperscript{2} In experimental models of allo-HCT, an HDAC inhibitor (vorinostat) reduced pro-inflammatory cytokines\textsuperscript{3} through the induction of IDO in a STAT-3-dependent manner\textsuperscript{4,5} and increased Tregs\textsuperscript{6} to attenuate GVHD. On the basis of these experimental observations, we recently performed a clinical trial of HDAC inhibition after allo-HCT that demonstrated significantly decreased acute GVHD without increase in relapse.\textsuperscript{7} However, whether HDAC inhibition had similar immunological impact on inflammatory cell and Treg responses in humans is not known.\textsuperscript{8} In the present study, we explored the role of HDAC inhibition on inflammation, Tconv, and Tregs in patients from the clinical trial\textsuperscript{7} of vorinostat post-allogeneic transplant.

Methods
Study Cohort and Sample Collection
Laboratory studies were conducted in 50 patients who underwent a clinical trial (clinicaltrials.gov #NCT00726375).\textsuperscript{7} Oral vorinostat was administered 10 days before the stem cell infusion and continued until day 100. Study patients were compared with patients who were similarly transplanted and given standard-of-care treatment but did not receive vorinostat (control cohort). Samples were collected under informed consents and IRB-approved research protocols.\textsuperscript{7} Research was conducted in accordance with the Declaration of Helsinki. No differences in clinical characteristics were observed between study participants and control cohort (Supplementary Table 1).

Immunoblotting
Histones and STAT-3 in PBMC were assessed by Western blot, as previously described, using antibodies listed in the Supplement.

**Cytokine Analyses**

Cytokine production was determined in plasma and PBMC supernatant samples by ELISA and in CD11c+ PBMC by intracellular staining, as previously described.

**Phenotype of T lymphocytes**

Routine absolute lymphocyte counts (ALC) were performed in the Clinical Hematology Laboratory. To enumerate CD4+, CD8+ and Treg cells, PBMC were stained using fluorochrome-conjugated anti-human mAbs detailed in the Supplement. To detect recent thymic emigrants (RTE), PBMC were identified using anti-human CD4, CD25, CD31 and CD45RA.

**RNA Isolation and RT-PCR**

Total cellular RNA was isolated from PBMC, reverse-transcribed, and used for real-time PCR analysis of Foxp3 and IDO using previously described methods and primer pairs.

**Treg-specific-demethylation region (TSDR) Demethylation Assay**

CD4+CD25+ Tregs and CD4+CD25− Tconv were sorted from PBMC using the Regulatory T Cell Kit (Miltenyi Biscience). Genomic DNA was isolated and TSDR analysis was performed, as previously described. PCR conditions and specific primers pairs are listed in the Supplementary Methods.

**Treg Suppression assay**

Tregs and Tconv were isolated, as described above. Tregs were mixed at ratios of 1:4 and 1:8 with Tconv and incubated with the presence of anti-CD3/anti-CD28 coated beads (Life
Technologies, Grand Island, NY) for 72 h. \(^3\)H-thymidine (1 μCi/well, NEN Life Sciences Products) was incorporated by proliferating cells for the last 8h of incubation and was measured using a TopCount NXT Microplate Scintillation Counter (Perkin Elmer, Waltham, MA).

**Statistical Analysis**

Samples from the study and control cohorts were compared using the Mann-Whitney non-parametric test. Comparison of paired samples from the same patient was made using the Wilcoxin matched-pairs signed rank non-parametric test. These statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc. La Jolla, CA). A two-sided p-value of <0.05 indicated statistical significance.

**Results and Discussion**

Administration of vorinostat increased H3 and H4 acetylation, confirming HDAC inhibition,\(^8,11\) and these increases had prolonged effects without significant decline 100 days post-transplant (Figure 1A and Supplementary Figure 1). Because HDAC inhibition mitigated inflammation and reduced GVHD in pre-clinical models,\(^1-4,8\) we explored the impact of vorinostat on inflammatory cell responses. Similar to pre-clinical observations, vorinostat treated patients experienced significant reductions in the plasma levels of pro-inflammatory cytokines such as, IL-1β, TNF-α, IL-6 and IL-8 fourteen days post-transplant (Figure1B). IFN-γ, IL-17, and IL-2 cytokines were also evaluated at this time point. While IL-17 was significantly reduced, IFN-γ and IL-17 were not different between study and control cohorts (Supplementary Figure 2). We measured plasma cytokines thirty days post-transplant, and found significant reductions in TNFR1 and IL-8 levels (Supplementary Figure 3). While plasma IL-6 levels were reduced in vorinostat treated patients, statistical significance was not reached. IL-1β was also unchanged at day 30 between the two cohorts (Supplementary Figure 3). Furthermore, we measured plasma biomarkers of GVHD fourteen and thirty days post-transplant,\(^12,13\) such as Reg3-α, ST2, IL-2Ro, HGF, and
Elafin (Supplementary Table 2). We did not find a clear correlation with clinical outcomes, which we speculate may have been due to the conditioning regimen (reduced intensity) and small number of patients who developed GVHD in our study.\(^7\)

HDAC inhibition also reduced the release of pro-inflammatory cytokines by PBMC ex vivo in response to LPS (Figure 1C) and by CD11c\(^+\) after PMA/ionomycin stimulation of bulk PBMC (Figure 1D). These observations of reduced pro-inflammatory cytokines in the presence of HDAC inhibition are also consistent with other studies.\(^{14,15}\) Importantly, we did not observe significant differences in percentages of CD19\(^+\), CD14\(^+\) or CD11c\(^+\) cells in the PBMC of study patients compared to controls (Supplementary Table 3), nor did the percentage of CD11c\(^+\) PBMC differ between groups following PMA/ionomycin stimulation (data not shown). In contrast to pre-clinical findings,\(^3\) expression of MHC class II or costimulatory (CD86) molecules by CD11c\(^+\) PBMC were not changed in patients treated with vorinostat on either day 30 (Supplementary Table 3) or 100 days post-transplant (data not shown).

Patients with GVHD have reduced frequencies of Tregs.\(^{16-18}\) In murine models, Tregs attenuate GVHD,\(^{19}\) and HDAC inhibition increased Treg numbers and enhanced their suppressive function, but did not directly alter non-Treg, i.e., Tconv responses.\(^{3,6,14,20}\) HDAC inhibition can also enhance suppression by human Tregs\(^{21}\) and promote the conversion of human T cells into Tregs in vitro.\(^{22}\) We therefore explored the impact of HDAC inhibition with vorinostat on both Tregs and Tconv after clinical allogeneic BMT. HDAC inhibition did not significantly alter total lymphocyte or, CD4\(^+\) or CD8\(^+\) T cell counts, nor did it alter the proliferative response of Tconv to non-specific stimuli (Supplementary Table 4 and Supplementary Figure 4). However, patients treated with vorinostat exhibited increased numbers of Tregs (CD4\(^+\)CD25\(^+\)CD127\(^-\)) in the peripheral circulation (Figure 2A and 2B and Supplementary Figure 5). Consistent with this increase, Foxp3 mRNA expression was increased in PBMC 30 days post-transplant,\(^7\) which remained significantly higher 100 days post-transplant (Figure 2C). Foxp3 expression was
verified on a per-cell basis by flow cytometry, where >80% of CD4^+CD25^+CD127^- cells were Foxp3^+ (data not shown).

Foxp3 is a critical regulator of Treg development and function^{23} and is subject to epigenetic regulation through methylation of CpG islands within the Treg-specific demethylation region (TSDR).^{24} Thymus-derived Tregs with stable expression of Foxp3 show TSDR demethylation, whereas recently activated conventional human T cells and Treg induced in the periphery with transient Foxp3 expression show methylated TSDR.^{25} HDAC inhibition with vorinostat increased demethylation of the Foxp3 TSDR in CD4^+CD25^+ T cells 100 days post-transplant (Figure 2D). Consistent with increased TSDR demethylation, HDAC inhibition also increased the numbers of CD45RA^-CD31^- Treg RTE (Figure 2E and Supplementary Figure 6), whereas Tconv RTEs (CD4^-CD25^-CD45RA^-CD31^-) were not changed (p=0.42; data not shown). Preliminary analysis also demonstrated a trend toward increased TRECs in CD4^+CD25^+ Treg (Supplementary Figure 7). Thus, although we cannot rule out the possibility that vorinostat also induced Tregs in the periphery, the data suggest that at least a portion of the increased Tregs observed in study patients were natural Tregs derived from the thymus. We next determined the functionality of these Tregs. Tregs isolated from vorinostat-treated patients suppressed effector T cell proliferation more effectively on a per-cell basis (Figure 2F). However, antigen-specific functional studies (e.g., recovery of CMV- or EBV-specific T cells) were not performed.

HDAC inhibition in mice increased acetylation of the non-histone protein STAT-3,^{5} which is critical for the induction of IDO in APCs,^4 reduction in inflammation, and the regulation of acute GVHD. Because vorinostat reduced acute GVHD and mitigated inflammatory cytokines, we next analyzed STAT-3 acetylation and IDO induction in these patients. Concordantly, levels of acetylated STAT-3 protein were significantly increased with HDAC inhibition 30 days post-transplant and levels remained high at day 100 (Figure 2G). Phosphorylated STAT-3 levels
were unchanged (Figure 2G), consistent with a previous study.\textsuperscript{26} IDO is an intracellular enzyme that degrades tryptophan, suppresses APC function, and induces T cell anergy.\textsuperscript{27} IDO mRNA was also induced with HDAC inhibition on day 30 post-transplant (p<0.0001),\textsuperscript{7} and remained high at day 100 (Figure 2H).

Our data in murine models of HCT have shown that vorinostat modulates the production of inflammatory cytokines and induces IDO expression by host APCs to modulate GVHD after allo-HCT.\textsuperscript{3-5} Additional murine data by others indicate that HDAC inhibitors promote the generation and function of regulatory T cells.\textsuperscript{6} Ex vivo studies using human cells have confirmed the abilities of HDAC inhibitors to modulate both APCs and Tregs.\textsuperscript{20,22} Thus, collective findings suggest that HDAC inhibition can have potent and distinct direct regulatory effects on various immune cell populations. While the mouse continues to be an important in vivo model for human immunology, we understand that potential limitations of extrapolating data from mice to humans exist.\textsuperscript{28} It is likely significant that human recipients of allo-HCT receive treatment with vorinostat concomitant with immunosuppressive therapy (tacrolimus and mycophenolate mofetil). It is possible that our inability to find quantitative reproducible differences in HLA and costimulatory molecule expression in human PBMC samples is reflective of direct effects of immunosuppression on APCs. Additional ex vivo analysis will be required to investigate this possibility. Nonetheless, the findings herein demonstrate that HDAC inhibition with vorinostat after allo-HCT in humans regulated inflammation and enhanced Tregs. They further suggest that regulation of inflammation and increased Tregs might have been crucial for the ability to reduce clinical GVHD in the first-in-human clinical trial,\textsuperscript{7} and collectively, suggest that HDAC inhibition has immune-regulatory effects in humans. Future studies are needed to further delineate which immune cells are impacted by HDAC inhibition after allo-HCT.
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Author Contributions

S.W.C. and P.R. designed the clinical trial and the laboratory experiments, reviewed clinical and laboratory data, and wrote the manuscript. E.G. reviewed laboratory data, designed and analyzed laboratory experiments, and wrote the manuscript. G.H. performed and analyzed laboratory experiments and reviewed and helped write the manuscript. Y.S. reviewed laboratory data and prepared figures for publication and reviewed the manuscript. C.D. analyzed data, and reviewed and helped write the manuscript. The remaining authors (K.O.W. and Y.S.) contributed to the experiments and reviewed and helped write the manuscript. All authors vouch for the accuracy and completeness of the data and for the analyses.
Disclosure of Conflicts of Interest

All authors declared no conflicts of interest.
References


**Figure Legends**

**Figure 1.** HDAC inhibition modulated histone acetylation and pro-inflammatory cytokine production after human allo-HCT. Triangles and black bars denote patients in the study who received vorinostat. Circles and open bars denote control patients that did not receive vorinostat. Median values ± IQR are plotted. Each data point in a dot plot represents a single patient. All plots include data from at least two independent experiments. (A) Levels of acetylated (Ac-) H3 and H4 30 and 100 days after allo-HCT. Levels in each patient normalized to β-actin. (B) Levels of IL1-β, TNF-α, IL-6 and IL-8 in the plasma of study patients and controls 14 days after allo-HCT. Control, n=18; study, n=31. (C) TNF-α and IL-6 production by PBMC after ex vivo stimulation with LPS (500ng/mL) for 16-24h, 30 days after allo-HCT. Control, n=12; study, n=14. (D) Intracellular staining of IL1-β, TNF-α and IL-6 in CD11c+ PBMC of study and control patients 30 and 100 days after allo-HCT, following ex vivo stimulation with PMA and ionomycin. Day 30: control, n=6; study, n=10. Day 100: control, n=6; study n=6.

**Figure 2.** Patients treated with vorinostat have increased Treg with greater suppressive function and increased acetylation of STAT-3 and IDO expression after allo-HCT. Triangles and black bars denote patients who received vorinostat. Circles and open bars denote control patients. All plots include, or are representative of, data from at least two independent experiments. Mean values ± SEM are plotted in panels B and F. Median values ± IQR are plotted in all other panels. (A, B) CD25+CD127- cells within the CD4+ population of PBMC 30, 100 and 180 days after allo-HCT. Day 30: control, n=22; study, n=36. Day 100: control, n=11; study, n=16. Day 180: control, n=6; study, n=8. *control vs study, p=0.01; **p=0.007; ***p=0.06. (C) Foxp3 expression in PBMC 100 and 180 days after allo-HCT. Data are expressed relative to GAPDH copy number. Day 100: control, n=6; study, n=6. Day 180: study, n=9. (D) Methylation of the Foxp3 TSDR in
purified CD4+CD25- conventional (Tconv, open icons) and CD4+CD25+ regulatory (Treg; black icons) T cells 100 days after allo-HCT. Control, n=9; Study, n=11. (E) CD4+CD25+CD45RA+CD31+ RTE 100 days after allo-HCT. Control, n=5; Study, n=6. (F) Suppression of effector Tconv proliferation to anti-CD3 and anti-CD28 stimulation by autologous Tregs 100 days after allo-HCT. Control, n=8; Study, n=10. *p=0.04; **p=0.07. (G) Levels of Ac- and phosphorylated (P)-STAT-3 30 and 100 days after allo-HCT. Ac- and P-STAT3 levels normalized to total STAT-3. Day 30, control, n=3-6; study, n=5; Day 100: study, n=6. (H) IDO mRNA expression 100 days after allo-HCT. Values expressed relative to GAPDH mRNA copy number. Control, n=6; Study, n=6.
Figure 1

A. Ac-H3 and Ac-H4 normalized levels.

B. IL-1β, TNF-α, IL-6, and IL-8 levels in Control vs. Study.

C. TNF-α and IL-6 levels in Control vs. Study.

D. Intracellular IL-1β, TNF-α, and IL-6 in Control vs. Study.

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Figure 2