

LETTER TO THE EDITOR

Immune regulatory effects of panobinostat in patients with Hodgkin lymphoma through modulation of serum cytokine levels and T-cell PD1 expression

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The Hodgkin Reed–Sternberg (HRS) cells of classical Hodgkin lymphoma (cHL) are surrounded by a number of reactive and inflammatory cells.¹ The ability of HRS cells to evade immune surveillance is linked to a network of cytokines and chemokines that are produced by HRS and the surrounding cells,^{1–3} in addition to the occasional aberrant expression of programmed death ligand-1 (PDL-1) by HRS⁴ that inhibits the activation of PD1-expressing T lymphocytes. Histone deacetylase (HDAC) inhibitors can alter the levels of cytokines and chemokines *in vitro*, favoring a TH1-type immune response.⁵ HDAC inhibitors also enhance antitumor immunity by HDAC11-mediated upregulation of OX40L.⁵

Panobinostat is a potent oral pan-HDAC inhibitor, which induces cell death, autophagy and increase the expression of natural killer cell receptors in cHL cell lines.⁶ It triggers an enhanced lymphocyte-dependent lyses of cHL cells, suppresses the IFN-gamma release and increases TNF-alpha secretion.⁶ A recent phase II study of panobinostat in 129 patients with relapsed or refractory cHL showed overall response rate of 27% including complete response in 4%.⁷ Tumor size reduction was observed in 74% of patients, and the median progression-free survival duration was 6.1 months.⁷ In a subset of patients in this study, several serum cytokine and chemokine levels were analyzed, and the decrease of TARC levels were found associated with clinical responses.⁸ The present study shows the *in vivo* effect of the pan-HDAC inhibitor panobinostat on an expanded panel of 52 serum cytokines and chemokines from the phase II study. In addition, a pilot study of peripheral blood lymphocytes for PD1 expression before and after panobinostat treatment was conducted.

The phase II clinical trial of single-agent panobinostat (clinicaltrials.gov NCT00742027)⁷ with exploratory correlative study was approved by the institutional review board at each center. All patients provided written informed consent. To be eligible for this clinical trial, patients were required to have age ≥ 18 years, histopathologically confirmed cHL that had relapsed or were refractory to autologous stem cell transplant, and had ≤ 5 prior systemic treatment regimens. The treatment consisted of oral panobinostat (40 mg) three times per week every week in 3-week cycles. Response assessment was based on Cheson Criteria,⁹ performed at the end of every two cycles.

Serial serum samples were collected from 65 consenting patients with relapsed or refractory cHL treated in this study before (day 0) and during (days 8 and 21) therapy. Serum was prepared at each study site, and the samples were frozen, de-identified and shipped to UT MD Anderson Cancer Center. We analyzed serum levels of 52 cytokines (Figure 1a) and the levels were analyzed for their association with the eventual response. Serum cytokines and chemokine were measured using the Human Cytokine/Chemokine Magnetic Beads Panel kits (Millipore, Billerica, MA, USA, Cat. Nos HCYTMAG-60K-PX29 and

MPXCHCYP2MK23) on Luminex-100 ELISA System (Luminex Corporation, Austin, TX, USA). Cytokine levels were plotted relative to the median value of healthy controls in box-and-whisker plots. Levels of IL2 and IL4 were plotted for their measured values because those cytokines were not detectable in healthy controls. Comparison of the levels in two groups or different time points were conducted by analysis of variance test. For a pilot analysis of peripheral blood lymphocytes for PD1 expression using a flow cytometry, blood samples were collected with EDTA and mononuclear cells were extracted and analyzed within 24 h. The expression levels were marked by the relative value (delta) to the immunoglobulin isotype expression in peripheral blood mononuclear cells with CD4 or CD8 expression.

Serial serum samples (pretreatment, days 8 and 21) were available from 65 patients. In this group, median age was 32 years (range 18–70), 55% were male, complete response rate was 2%, overall response rate was 31%. The waterfall chart of the changes in the sum of the product of the diameters was similar to that of the entire population in the clinical trial.⁷ Thus, our population is generally considered representing the entire population treated with panobinostat in this study.

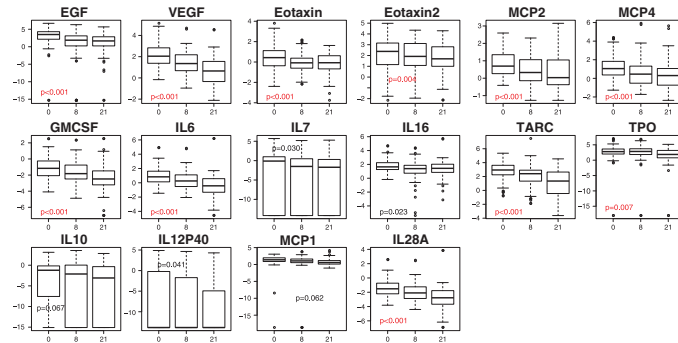
Changes in the level of each cytokine are summarized in Figure 1a. Baseline values of 21 cytokines (EGF, Eotaxin2, IL6, IL16, MCP1, MCP2, MCP4, TARC, TPO, VEGF, 6Ckine, BCA1, cutaneous T-cell-attracting chemokine (CTACK), Eotaxin3, I309, MIP1 delta, IL20, SCT, SDF1alpha-beta, IL2 and IL4) were higher than the maximum value of normal controls in majority (>75%) of patients. We have identified 14 cytokines whose serum levels significantly decreased after treatment with panobinostat (Figure 1a), and 11 of them were still considered significant after multiple testing adjustment with a method controlling false-discovery rate (Figure 1a, EGF, VEGF, Eotaxin, GM-CSF, IL6, IL12p40, Eotaxin2, MCP2, MCP4, TARC, TPO and IL28A). IL8 levels were found to increase after treatment ($P=0.008$) but the actual changes of the levels were small.

We compared the levels of cytokines in responders (patients achieving complete or partial response⁹) and nonresponders (patients without response). Cytokines whose levels were significantly different between responders and nonresponders on at least one of the days (day 0, 8 or 21) are shown in Figure 1b. Most notably, IL3 levels were consistently lower in responders than in nonresponders throughout the course of treatment. In addition, posttreatment levels of IL1-alpha (day 8), IL2 (day 8) and IL21 (day 21) were lower in responders than in nonresponders. Posttreatment levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (days 8 and 21) and CTACK (day 8) were higher in responders than in nonresponders. However, none of the changes from baseline was significantly related to response after multiple testing adjustment with a method controlling false-discovery rate.

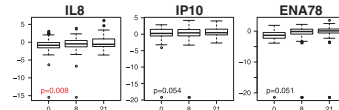
We next analyzed the absolute (day 8 – day 0) or relative ((day 8 – day 0)/day 0) changes in the cytokine levels for their association with response (data not shown). Responders showed greater absolute decrease in the IL20 levels from day 0 to 8

a

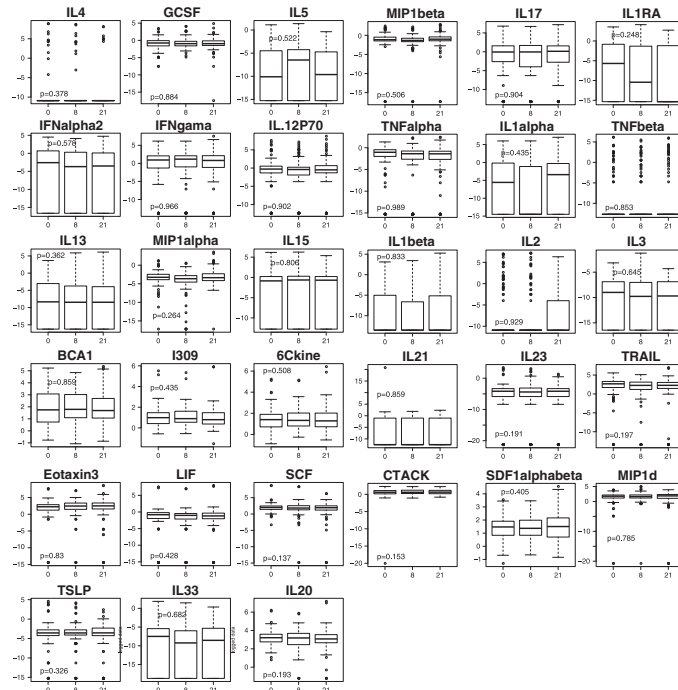
Cytokines whose levels decreased after panobinostat



Cytokines whose levels increased after panobinostat



Cytokines whose levels did not change significantly



b

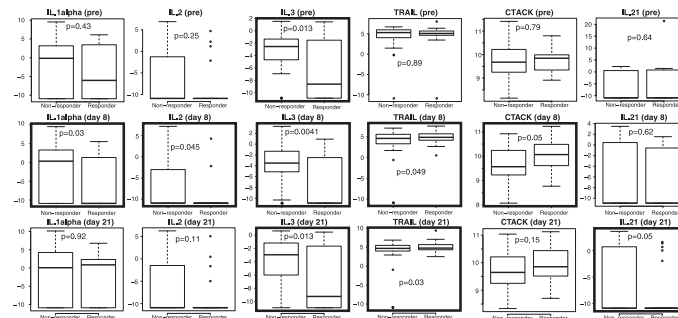


Figure 1. (a) Changes in cytokine levels. **(b)** Cytokine levels that are different between responders and nonresponders. CTACK, Cutaneous T-cell-attracting chemokine; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

($P=0.049$), greater relative increase in CTACK levels from day 0 to 8 ($P=0.01$), greater absolute increase in CTACK levels from day 0 to 21 ($P=0.046$). However, the significance was not confirmed after multiple testing adjustments.

Finally, we performed a pilot analysis of peripheral blood mononuclear cells from two patients for their expression levels of CD4, CD8 and PD1 by flow cytometry (Figure 2). PD1 expression levels decreased after treatment with panobinostat in both

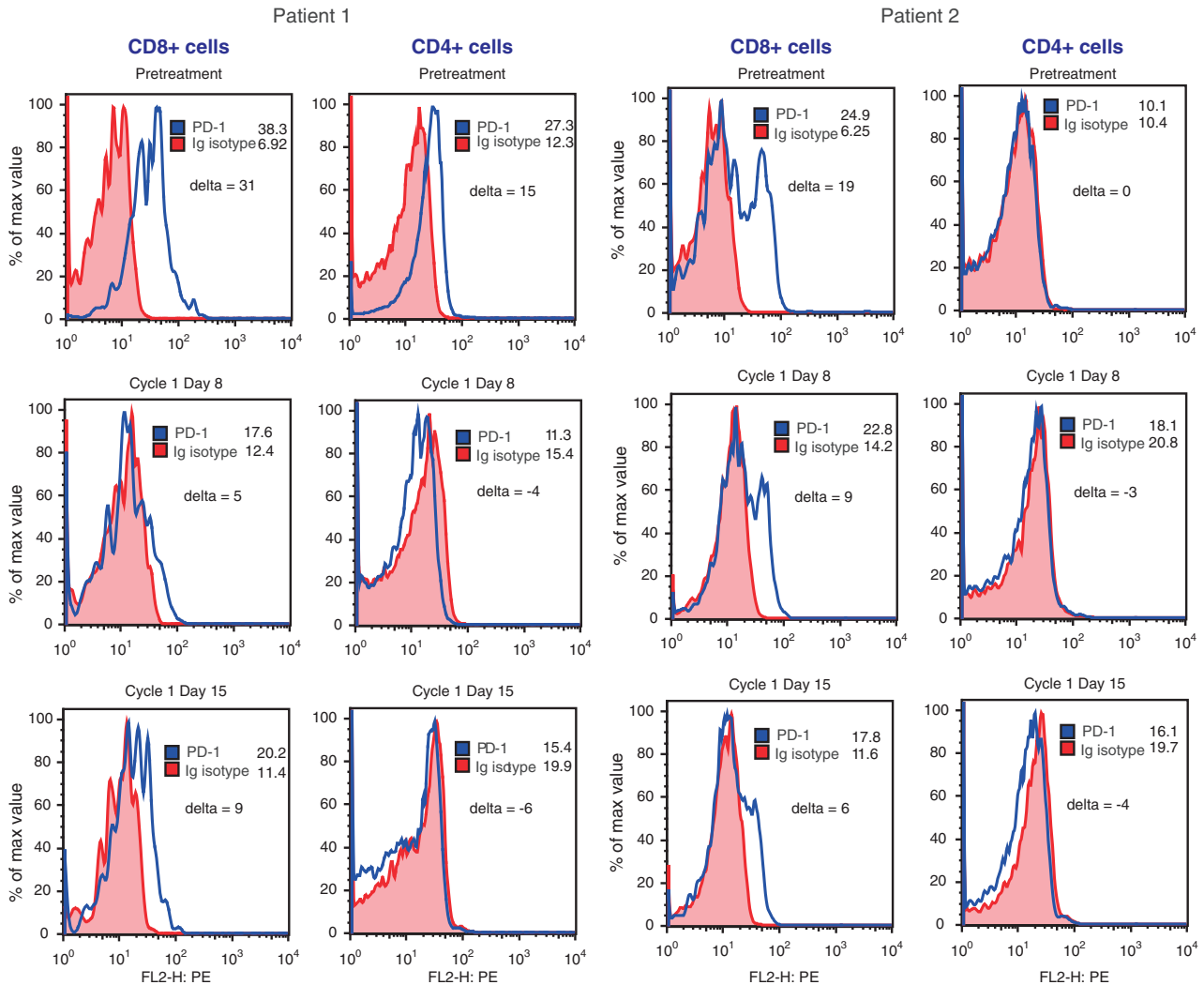


Figure 2. PD1 expression in peripheral T cells.

CD4- and CD8-positive cells, suggesting the suppression of PD1 expression by panobinostat treatment. This information is limited to two patients but warrants further investigation in both *in vitro* and *in vivo* studies.

Biomarkers are of value as prognostic indicators or surrogates of clinical response, and help stratifying patients for different treatment modalities. In fact, a recent study suggested the prognostic significance of pretreatment serum cytokine levels in patients with newly diagnosed cHL.¹⁰ In our study, levels of cytokines were often found elevated at initiation of the study compared with normal values. Moreover, after the treatment with panobinostat, numbers of cytokine levels decreased ($n=14$ for $P < 0.05$ and $n=16$ for $P < 0.1$), suggesting that panobinostat does affect the tumor survival factors *in vivo*. Although the clinical response rate was only 28%, waterfall plots demonstrated tumor size reduction in $> 70\%$ of patients,⁷ consistent with the cytokine level changes seen in majority of patients in this study. In the recent report, TARC levels were related to a clinical response after panobinostat treatment.⁷ In this study we used a smaller data set due to the limited availability of the specimen. As a result, we did not observe the same significant difference in TARC levels between responders and nonresponders. Instead, the decrease in IL20 levels was associated with response warranting further investigation of this cytokine's levels in association with clinical

response. In addition, we observed an increase in the levels of CTACK and TRAIL after treatment in responders but not in nonresponders. As TRAIL is considered a possible biologic antitumor agent, these two cytokines should be analyzed in larger-scale studies.

PD1 is a negative regulator of immune response in T cells. In the peripheral blood samples of patients, we observed a suppression of PD1 expression after treatment with panobinostat. Antibodies against PD1 have been investigated and have attracted a large interest as new treatment modalities for lung cancer,¹¹ renal cell carcinoma,¹¹ melanoma¹² and non-Hodgkin lymphoma.^{13,14} Our data suggest that panobinostat may exert antitumor activity by affecting PD1 expression in normal lymphocytes, stimulating the immune reaction against lymphoma.

In summary, we showed that levels of selected cytokines were generally higher at baseline than in normal controls, and decreased levels were seen in one-third of cytokines analyzed after treatment with panobinostat. Posttreatment levels of IL1, IL2, IL3, IL20, IL21, TRAIL and CTACK may serve as potential biomarkers. Further studies are needed to evaluate and validate the role of these serum cytokine levels and T-cell PD1 expression as biomarkers.

CONFLICT OF INTEREST

YO and AY received research grant from Novartis. BvT received honoraria from Novartis and Takeda, and travel grants from Takeda. ASH is an employee of Novartis. The remaining authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

YO collected and analyzed the data and wrote the paper. DB conducted laboratory work. JZ, YY and SZ conducted statistical analyses. ASu, DB-Y, PLZ, HMP, SJH, MK, PJ, ASH and BvT contributed to the clinical trial and reviewed the paper. AY designed and supervised the study, contributed to the clinical trial and edited the paper.

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