Role of the HDAC6/STAT3 pathway in regulating PD-L1 expression in osteosarcoma cell lines

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Abstract
Histone deacetylases (HDACs), initially described as histone modifiers, have more recently been verified to target various other proteins unrelated to the chromatin environment. On this basis, findings of the current study demonstrates that the pharmacological or genetic abrogation of HDAC6 in osteosarcoma cell lines down-regulates the expression of program death receptor ligand-1 (PD-L1), an important co-stimulatory molecule expressed in cancer cells, which activates the inhibitory regulatory pathway PD-1 in T cells. As shown by our results, the mechanism by which HDAC6 regulated PD-L1 expression was mediated by the transcription factor STAT3. In addition, we observed that selective HDAC6 inhibitors could inhibit tumor progression in vivo. Crucially, these results provide an essential pre-clinical rationale and justification for the necessity of further research on HDAC6 inhibitors as potential immuno-modulatory agents in osteosarcoma.

Keywords Osteosarcoma · HDAC6 · PD-L1 · STAT3 · Immunoregulation

Abbreviations

- APC: Antigen-presenting cell
- CTLA-4: Cytotoxic T-lymphocyte-related protein 4
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- HBSS: Hank's buffered salt solution
- HDAC: Histone deacetylase
- PD-L1: Program death receptor ligand-1
- PVDF: Polyvinylidene fluoride
- SDS–PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction
Osteosarcoma remains the most common pediatric bone cancer, the morbidity of which ranks the 8th place among childhood malignancies globally [1, 2]. Osteosarcoma develops from osteoblasts, typically during the periods of rapid bone growth, with a median age of onset at 14 years [2]. However, new findings provide optimism for patients with metastatic osteosarcoma. This is due to the improved clinical outcomes observed in patients receiving targeted therapies aiming to impede negative immuno-modulatory pathways such as program death receptor-1 (PD-1), program death receptor ligand-1 (PD-L1), and cytotoxic T-lymphocyte-related protein 4 (CTLA-4) [1]. This work has promoted a new role in development of rational combinatorial approaches proposed to improve the efficacy of immune-modulatory drugs and antibodies targeting immunological checkpoints. The role of epigenetic modifiers in regulating immune-modulatory pathways has been recently addressed. Among these, histone deacetylases (HDACs) are fascinating targets, which can be attributed to the validity of a wide range of inhibitors targeting the enzymatic activities. In addition, the influences of HDACi have been perfectly recorded under the control of the cell cycle and apoptosis. However, their roles in regulating the immune-related pathways have not been observed completely [1, 4]. Hence, the generation of selective HDACi...
and mechanistic insight into their role in the immune response against cancer cells is highly desirable goals, and has the potential to augment anti-tumor immunity.

HDACs, originally described as histone modifiers, are currently verified to modify various other proteins, which are involved in different cellular processes unassociated with chromatin environment. This includes deacetylation of multiple non-histone targets, such as the members of immune and oncogenic virus-related pathways [3, 5]. In this regard, the role of unspecific inhibition of HDACs through pan-HDACi in cancer as well as processes of immune regulation is analyzed by a considerable number of researches. Under such context, it is reported that the pharmacological and genetic inhibition of a single HDAC, HDAC6, resulted in decreased proliferation of cancer cells both in vitro and in vivo [6, 7]. In addition, HDAC6 was also demonstrated to modulate the expression of co-stimulatory molecules, especially the tumor-associated antigens and MHC class I [8]. Moreover, it seems that HDAC6 is a crucial regulator in the STAT3 pathways [9], which can be commonly activated in osteosarcoma and other malignancies [10]. Here, we report that HDAC6 plays a role in regulating the co-inhibitory molecule PD-L1. This protein is one of the natural ligands for the PD-1 receptor present on T cells, which suppresses T-cell activation, proliferation, and induces T-cell anergy and apoptosis [11]. PD-L1 is found in cancer cells by many important studies [4, 12], and its over-expression is usually related to the poor prognosis of respective malignancies, including osteosarcoma [13], ovarian [14], gastric [15], and breast cancer [16].

It is reported in this study that HDAC6 could regulate the immunity-associated pathways in osteosarcoma, so as to moderate the negative pathways that influence the reaction of T cell against cancer. Furthermore, it works as a regulator, which makes it possible to use its selective inhibition as a potential immuno-modulatory option in ongoing therapies.

Materials and methods

Mice

Male C57BL/6 mice were purchased from the Experimental Animal Center of Xinjiang Medical University (Urumqi, China). All animal experiments were approved by the Animal Research Committee of Xinjiang Medical University, and all animals were maintained under specific pathogen-free conditions. For tumor studies in vivo, K7M2-WT-Luc cells (2 × 10⁵) suspended in Hank’s buffered salt solution (HBSS) were subcutaneously injected into the shaved flank of mice.

Cells

The K7M2-WT-Luc mouse osteosarcoma cell line was purchased from the American-Type Culture Collection (ATCC; Rockville, MD) and cultured as described previously [17]. Human osteosarcoma cell lines were obtained from the Cell Bank of Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured as described above [18].

Reagents and plasmids

The recombinant cytokines human IL-6 (570804) and mouse IL-6 (575704) were purchased from Biolegend (San Diego, CA). The selective HDAC6 inhibitors Tubastatin A and Nexturatstat were purchased from Sigma-Aldrich (San Diego, CA). Lipofectamine 2000 was obtained from Invitrogen (Grand Island, NY), and the plasmid GFP-STAT3c was a gift presented from Dr. Linzhao Cheng (Addgene plasmid # 24983) [19].

Cell transfection

Cells were transfected using Lipofectamine 2000 (Invitrogen) in strict accordance with the manufacturer’s protocol. Briefly, cells were transfected with plasmids for 24 h, followed by 24 h of stimulation with the respective cytokine, and cells expressing each protein variant were treated for further analysis. In addition, shRNA plasmids for human HDAC6 and human STAT3, as well as the non-target shRNA were obtained from Genechem (Shanghai, China). The sequences of HDAC6 shRNA were: #16, 5′-GUC ACU UCG AAG CGA AAU ATT-3′, 3′-TTT AGA ACU GGU CUC CCA AUA-5′; #20, 5′-GAG GGU CUC AUU CUA AUA-5′; #21, 5′-GGU GUC ACC UGA GGG UUA UTT-3′, 3′-TTT AGA GCU GCA CCA AUA-5′. The sequence of Nonsense control was: 5′-UUC UCC GAA CGU GUC ACC UTT-3′, 3′-TTT AGA GCU GCA CCA AUA-5′. The sequence of STAT3 ShRNA was: #16, 5′-GCA AAC UAG GUG CAG AAU CTT-3′, 3′-TTT GGU UGC UGC UGC UGC ACA UCC AUA-5′. Transduction was performed according to the manufacturer’s instructions as previously described [20]. In brief, osteosarcoma cells were grown in antibiotic-free medium, followed by transduction with shRNA particles. 72 h later, puromycin was added into the culture medium, and cells were cultured until 50% confluence and analyzed using...
western blotting. Moreover, monoclonal populations were generated by serial dilutions of polyclonal populations.

**Western blotting analysis**

Cells were washed with ice-cold PBS and suspended in radioimmunoprecipitation assay (RIPA) lysis buffer (Biyuntian Biotech, Shanghai, China) containing 1% phenylmethylsulfonyl. The suspension was then centrifuged at 14,000 rpm for 10 min at 4 °C, and the protein content of supernatant was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Aliquots (20 µg protein per lane) were separated through 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were then incubated with primary antibodies and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, UK) at room temperature for 1 h, followed by labeling with horseradish peroxidase-conjugated goat anti-rabbit IgG for 40 min at room temperature. Subsequently, signals were detected with enhanced chemiluminescence combined with reagents (Amersham Pharmacia, Piscataway, NJ). GAPDH was used as the internal control. Specifically, the following primary antibodies were used: anti-acetyl-Tubulin (ab24610, Abcam), anti-HDAC6 (ab1440, Abcam), anti-PD-L1 (ab205921, Abcam), anti-α-Tubulin (2144, Cell Signaling Technology, CST, Danvers, MA), anti-STAT3 (12640, CST), anti-p-STAT3 Tyr705 (9131, CST), anti-p-STAT3 Ser727 (9134, CST), anti-acetyl-STAT3 Lys685 (2523, CST), anti-GAPDH (Ab2302, Millipore), and anti-Flag (F1804, Sigma-Aldrich).

**Flow cytometry**

The attached cells were digested with 0.2% trypsin supplemented with 0.25% ethylenediaminetetraacetic acid. Afterwards, the suspending cells (1 × 10^6) were fixed with 4% formaldehyde in PBS for 10 min, followed by staining with the PD-L1 antibody or with the corresponding isotype control (Rabbit [DA1E] monoclonal antibody IgG, CST) for 1 h at room temperature. Cells were then washed twice with PBS by centrifugation, followed by staining with the anti-rabbit IgG (H + L), F(ab’)^2 Fragment (Alexa Fluor® 647 Conjugate, Life Technologies, Baton Rouge, LA) for 30 min. Later, cells were washed and analyzed by flow cytometry on a FACScan (BD Biosciences, Piscataway, NJ) instrument. Data analysis and graphical output were performed using the FlowJo 7.6.1 software (Tree Star, Inc. Ashland, OR).

**Quantitative real-time RT-PCR (qRT-PCR)**

To quantify the *PD-L1* mRNA level, total RNA was isolated from osteosarcoma cells using Trizol reagent (Invitrogen) according to the manufacturer’s instruction protocol. Briefly, 1 mg total RNA was reversely transcribed into cDNA using Bestar qPCR RT Kit (DBI Bioscience, Shanghai, China). The qRT-PCR reaction was carried out in a total volume of 20 µl, which contained 10 µl DBI Bestar™ SybrGreen qPCR Master Mix (DBI Bioscience), cDNA derived from 0.2 µg input RNA, 5 µM each primer, and 7 µl double-distilled H2O. In addition, PCR was conducted on the Stratagene Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA). Fluorescent quantity PCR was conducted under the following conditions: pre-denaturation at 95 °C for 2 min, followed by 40 cycles at 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s. Primers used were shown as follows: *PD-L1* forward 5′-GAA CTA CCT CTG GCA CAT CCT-3′, and *PD-L1* reverse 5′-CAC ATC CAT CAT TCT CCC TTT-3′. Each reaction was replicated three times. Finally, the fold changes in cDNA relative to the *gapdh* endogenous control were calculated using the 2− ΔΔCt method [21].

**Studies in vivo**

Mice were given subcutaneous injection with K7M2-WT-Luc cells at the density of 2 × 10^5/mouse in the shaved rear flank. Subsequently, Tubastatin A (25 mg/kg) or Nexiturastat (25 mg/kg) was injected daily intraperitoneally once the tumor was barely palpable. Tumor growth was monitored every 5 days starting from the injection of drugs (Day 0) by measuring the longest diameter (length) and the orthogonal diameter (width) using calipers. Results were presented as the mean tumor size (volume in mm^3) and standard deviation for every treatment group at various time points until the experiment endpoint.

**Statistical analysis**

Continuous variables were compared using a two-tailed Student’s *t* test, and categorical variables were compared using the Chi-square test or the Fisher’s exact test. Differences were considered statistically significant with a two-sided *P* value of < 0.05. Statistical analyses were performed using the SPSS version 17.0 software (SPSS, Inc., Chicago, IL).
Results

HDAC6-mediated IL-6-induced PD-L1 expression in osteosarcoma cells

HDAC6 can regulate critical functions in mammalian cells, such as cell proliferation, survival, differentiation, and immune response [22]. Actually, HDAC6 has been suggested to regulate the expression of some essential immunity-related pathways, including those involved in the regulation of pro- and anti-inflammatory pathways and anti-tumor responses [8, 23, 24]. Meanwhile, HDAC6 is reported to be an effective regulator of the co-stimulatory molecule PD-L1 in melanoma cells [25]. To verify these previous findings in osteosarcoma cells, PD-L1 expression was evaluated in HDAC6 knock-down (sh-HDAC6) MG63, U2OS, and SaOS-2 osteosarcoma cells. Among three predicted HDAC6 shRNAs, sh-HDAC6-#21 and #20 had the most obvious inhibitory effect, which was used in the followed experiment (Supplemental Fig. S1). As expected, IL-6 treatment resulted in the enhanced expression of PD-L1 in sh-NC control cells. However, its expression was impaired in sh-HDAC6 cells after IL-6 stimulation (Fig. 1a). In addition, the PD-L1 mRNA level was analyzed using qRT-PCR, and the results of which ensured that HDAC6 could mediate PD-L1 expression at transcriptional level (Fig. 1b). Meanwhile, the changed expression of PD-L1 protein in sh-HDAC6-transfected osteosarcoma cells was similar when the expression of surface PD-L1 was analyzed by flow cytometry (Fig. 1c). Considering the potential off-target activity of shRNAs [26], the regulatory effect of HDAC6 on PD-L1 expression was further confirmed by another HDAC6 shRNA #20 transfection in osteosarcoma cells (Supplemental Fig. S2).

HDAC6 selective inhibitors recapitulated the impact of sh-HDAC6 on PD-L1 production

Next, we evaluated the enzymatic activity of HDAC6 is responsible for the regulation of IL-6 induced PD-L1 production. To test this hypothesis, we used the selective HDAC6 inhibitor Tubastatin A and Nexturastat A in osteosarcoma cells. First, we evaluated the expression level of acetylated tubulin, which has been demonstrated as a quantifiable target to evaluate HDAC6 activity [27]. As expected, acetylated tubulin was greatly up-regulated in different osteosarcoma cell lines (Fig. 2a), confirming the pharmacological inhibition of HDAC6 under these conditions. In the meantime, the following evaluation of PD-L1 production verified that the enzymatic inhibition of HDAC6 mirrored the outcomes previously discovered in the sh-HDAC6-transfected cells (Fig. 2a). At the mRNA level, a down-regulated PD-L1 mRNA level was observed after Tubastatin A treatment analyzed by qRT-PCR (Fig. 2b). Nexturastat A is another HDAC6 selective inhibitor [17]. Results of western blotting

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** HDAC6 modulated PD-L1 expression in osteosarcoma cells. a Results of Western blotting on sh-NC- and sh-HDAC6-transfected MG63, U2OS, and SaOS-2 osteosarcoma cells under IL-6 stimulation (30 ng/mL). b Total RNA was isolated from Sh-NC- and Sh-HDAC6-transfected osteosarcoma cells treated with IL-6 or control. PD-L1 expression was analyzed by qRT-PCR. These results were expressed as a percent over control cells, and data were normalized by gapdh expression. The experiment was performed for three times. Error bars represented the standard deviation from triplicates, \( *P < 0.05 \). c PD-L1 expression was measured using flow cytometry in Sh-NC- and Sh-HDAC6-transfected osteosarcoma cell lines with or without IL-6 stimulation.
and qRT-PCR revealed that HDAC6 inhibition induced by Nexturastat A treatment caused the results similar with previously found with Tubastatin A treatment (Fig. 2c, d).

**STAT3 was involved in HDAC6-regulated PD-L1 expression in osteosarcoma cells**

The previous studies reported that the transcription factor STAT3 is involved in PD-L1 expression in mesenchymal stromal cells (MSCs) [18], lung adenocarcinoma cells [19], nasopharyngeal carcinoma cells [20], and melanoma cells [25]. Furthermore, the dysregulation of STAT3 has been considered to play a vital role in the pathogenesis of osteosarcoma [28]. Therefore, we questioned whether HDAC6 regulates the activation of STAT3 in osteosarcoma cells. As shown in Fig. 3a, HDAC6 knock-down impaired STAT3-Tyr705 phosphorylation after IL-6 stimulation, which showed the function of HDAC6 in regulating STAT3 activity in osteosarcoma cells (Fig. 3c). Specifically, acetylation of STAT3 at Lys685 is another post-translational modification, which is considered to be crucial in regulating STAT3 [29, 30]. In our results, the acetylation status of STAT3 was not changed when comparing sh-HDAC6 against NC control (Fig. 3c).

**PD-L1 production was rescued upon constitutive STAT3 activation in sh-HDAC6-transfected osteosarcoma cells**

Next, we examined whether the impact of HDAC6 on PD-L1 production was a consequence of its regulatory role over STAT3 activation or mediated by other cellular mechanism. A constitutively active variant of STAT3 (STAT3c) was overexpressed to active its target genes in the absence of cytokine stimulation [19]. As expected, in the absence of either STAT3 or HDAC6, we observed a decreased expression of PD-L1 regulated by IL-6/STAT3 (Fig. 3b).

The necessary role of HDAC6 in regulating the STAT3 pathway has been previously reported in antigen-presenting cells (APC) [9] and melanoma cells [25]. Although the exact regulatory mechanism is not completely comprehended, it is shown that the genetic abrogation and pharmacological inhibition of HDAC6 would diminish STAT3 phosphorylation and inhibit the expression of STAT3-targeted genes [9, 25]. Therefore, we questioned whether HDAC6 regulates the activation of STAT3 in osteosarcoma cells. As shown in Fig. 3a, HDAC6 knock-down impaired STAT3-Tyr705 phosphorylation after IL-6 stimulation, which showed the function of HDAC6 in regulating STAT3 activity in osteosarcoma cells (Fig. 3c). Specifically, acetylation of STAT3 at Lys685 is another post-translational modification, which is considered to be crucial in regulating STAT3 [29, 30]. In our results, the acetylation status of STAT3 was not changed when comparing sh-HDAC6 against NC control (Fig. 3c).
the qRT-PCR results also demonstrated that PD-L1 mRNA expression was rescued after STAT3c over-expression in sh-HDAC6-transfected cells (Fig. 4b).

**HDAC6 inhibition impaired tumor growth and PD-L1 production in vivo**

Considering the potent impact of HDAC6 disruption on PD-L1 production in vitro, we wondered whether such observation would be translated in vivo. Tubastatin A (25 mg/kg) and Nexturastat A (25 mg/kg) could inhibit enzymatic activity of HDAC6 in vivo, as shown by Tubulin acetylation (Fig. 5a, b). The results suggested a crucial reduction in K7M2-WT-Luc osteosarcoma tumor growth in mice treated with HDAC6 inhibitor Tubastatin A (Fig. 5c) or Nexturastat A (Fig. 5d). Besides, as we hypothesized, the pharmacological inhibition of HDAC6 impaired STAT3 phosphorylation and PD-L1 production in the tumors isolated at the end point (Fig. 5e, f), which closely resembled the abrogation resulted from the genetic or pharmacological inhibition of HDAC6 in vivo.

**Discussion**

Osteosarcoma remains one of the leading causes of cancer-related deaths worldwide in spite of the development of targeted therapies [31]. Moreover, the morbidity and mortality of osteosarcoma are still related to the development of metastasis [31]. Numerous studies have been carried out in the last few years to uncover the molecular markers during the development of osteosarcoma [31, 32]. Recent clinical trials using PD-1 blocking antibodies have demonstrated promising results in several malignancies [33]. In osteosarcoma, combined therapy targeting PD-L1 and CTLA-4 showed control of osteosarcoma growth in a mouse model [13, 34]. In this study, our data indicate that a novel mechanism of PD-L1 regulation is mainly mediated by the influence of HDAC6 upon the activation of the transcriptional factor STAT3. Furthermore, it is also found that selective HDAC6 inhibitors impaired tumor growth and reduced PD-L1 production in vivo. These findings have provided novel insight into the function and the interplay between HDAC6 and PD-L1 in osteosarcoma, which have remarkably enriched our understanding towards the role of HDAC6 in osteosarcoma development.

In recent time, there is a special interest to identify new potential therapeutic options and/or adjuvants targeting multiple cellular processes, thereby improving immunotherapeutic options in osteosarcoma treatment [35]. Some HDACs have captured special attention due to their modulatory roles in oncogenesis and the immune response [36]. Among them, the genetic and/or pharmacological inhibition of HDAC6 has been reported to inhibit tumor growth in vitro and in vivo and control the expression of...
tumor-associated antigens [8]. In addition, HDAC6 is an essential regulator in the activation of the pro-oncogenic STAT3 pathway, and the previous studies demonstrated that selective inhibitors for this deacetylase effectively down-regulate the expression of STAT3 target genes, such as IL-10 [9]. These findings, along with the important known oncogenesis, role of STAT3 in osteosarcoma [37] encouraged us to further study the potential involvement of HDAC6 in the regulation of STAT3 target genes in osteosarcoma. The results showed that HDAC6 modulates STAT3 Tyr-705 phosphorylation in several osteosarcoma cell lines and does not exert an influence on acetylation status of STAT3 (Fig. 3c). The previous studies reported similar results [25], which suggested that HDAC6 might control STAT3 phosphorylation by indirect pathway. A potential regulatory mechanism is the enhanced interaction of phosphatase PP2A with STAT3 in the HDAC6 knock-down cells [25], which could facilitate the dephosphorylation of STAT3. The exact mechanisms need further investigation in the future.

Some other studies reported that STAT3 is a tolerogenic pathway influencing both professional APCs and tumor cells to inhibit T-cell function and evade immune recognition [38]. In addition to STAT3, several other cellular pathways have been reported to be involved in the regulation of PD-L1, including those activated by IL-6, IL-10, IL-4, GM-CSF, interferons, and TNFα [39, 40]. Since most of the previous observations have been made in immune cells, we evaluated whether PD-L1 was also up-regulated by cytokines in osteosarcoma cell. Our results showed that IL-6 induced PD-L1 expression in several osteosarcoma cell lines, and HDAC6 knock-down can outstandingly inhibit PD-L1 expression both in vitro and in vivo. Similar results were reported in human melanoma cells [25]. The question whether this regulatory mechanism exists in other solid tumors is worthy of further exploration.

In summary, the present study demonstrates that the pharmacological or genetic inhibition of HDAC6 in osteosarcoma cell lines would down-regulate the production of PD-L1.
of PD-L1, which is an important co-stimulatory molecule expressed in cancer cells. Thus, it can activate the inhibitory regulatory pathway PD-1. Importantly, our data suggest that the mechanism by which HDAC6 regulates PD-L1 is mainly mediated by STAT3. Furthermore, we observe that selective HDAC6 inhibitors would impair tumor growth and reduce PD-L1 expression in vivo. In conclusion, our results have provided a rational framework for the use of selective HDAC6 inhibitors as anti-tumor agents in osteosarcoma.

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Compliance with ethical standards

Conflict of interest  The authors declare that they have no conflict of interest.

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