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Expression of PD-1 and PD-Ls in Kaposi's sarcoma and regulation by oncogenic herpesvirus lytic reactivation

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV) causes several cancers such as Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL). PD-1/PD-Ls immune checkpoint molecules play important roles in cancer cell immune escape. The expression of PD-1/PD-Ls and their regulation by oncogenic viruses, in particular KSHV, remain largely undefined. Here we demonstrate strong PD-1/PD-L1/PD-L2 expression in KS tissues from a cohort of HIV + patients. We found that induction of KSHV lytic reactivation significantly upregulates PD-L1 expression on infected tumor cells, potentially through several major cellular signaling pathways and IL-1β, which may represent a novel mechanism for virus-associated tumor cell immune escape.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of several human malignancies including Kaposi's sarcoma (KS), Primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (Cesarman et al., 2019). The high levels of immune cell infiltration and a pro-inflammatory cytokine milieu present in early KS lesions strongly suggest that the immune system may respond during the progression of KS disease (Mesri et al., 2010). Although the morbidity of KSHV-associated diseases is relatively low in patients with fully competent immune systems, in immunosuppressed patients, particularly HIV-infected individuals, KSHV infections are capable of causing severe, and sometimes fatal, diseases (Mesri et al., 2010). Moreover, it is likely that virus-regulated immune evasion is vital for the development of these malignancies.

Several recent studies have associated KSHV infection with immune checkpoint molecules, such as programmed death-1 (PD-1) and programmed death ligand 1 (PD-L1), suggesting novel perspectives for research related to oncogenic virus-associated immune responses (Beldi-Ferchiou et al., 2016; Chen et al., 2013; Host et al., 2017). PD-1 in particular plays an essential role in regulating T cell and B cell immunity due to its function as an inhibitory receptor expressed by activated lymphocytes (Jin et al., 2011). PD-L1, is expressed on the cell surface where it acts as an immune-regulating factor. PD-L1 interaction with PD-1 is important in peripheral immune tolerance. Programmed death ligand 2 (PD-L2), a second ligand for PD-1, dramatically inhibits T cell receptor (TCR)-mediated proliferation and cytokine production by $CD4^+$ T cells when interacting with PD-1 (Latchman et al., 2001). Interestingly, many viruses including HIV, HBV, HSV-1, Hantavirus and EBV regulate PD-1/PD-Ls expression during viral infection and utilize PD-1/PD-Ls interactions to induce T cell exhaustion to generate an immune-tolerant environment for viral replication and disease development (Geng et al., 2006; Larbcharoensub et al., 2018; Said et al., 2010). In patients with KS, PD-1 expression on NK cells was increased, which inhibited degranulation and production of interferon (IFN)-γ (Beldi-Ferchiou et al., 2016), implying that PD-1 overexpression on NK cells may be a strategy KSHV uses to escape NK cell-mediated immune surveillance. Another study reported that PD-L1 expression was increased in KSHV-infected monocytes undergoing lytic viral replication and this correlated an inflammatory milieu (Host et al., 2017), suggesting that viral lytic replication may induce PD-1 or PD-L1 activation in KSHV-associated diseases.

KSHV infections alternate between latency and lytic phases during their infection cycles within host cells. Following primary infection,

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KSHV establishes latency, characterized by limited viral gene expression, with only a small proportion of infected cells entering the lytic phase and expressing the majority of viral genes and producing progeny virions. Other studies showed that entering the viral lytic phase may be required for the development of KSHV-associated diseases (Gantt and Casper, 2011). In the current study, we show strong PD-1/PD-Ls expression in KS tissues collected from a cohort of HIV + patients. Further, mechanistic studies indicate that induction of lytic reactivation upregulates PD-L1 expression from KSHV-infected tumor cells, potentially through several major cellular signaling pathways (e.g., MAPK, NF-κB and AKT) together with IL-1β, which may represent a previously unknown mechanism for oncogenic virus-mediated immunoregulation.

2. Result and discussion

2.1. Detection of PD-1/PD-Ls expression in AIDS-KS tumor tissues

PD-1/PD-Ls molecules are highly expressed in a variety of human cancers. In contrast, there are only limited reports regarding their expression in KSHV-related tumor tissues, especially AIDS-KS. One study reported robust PD-L1 expression in a subset of aggressive B-cell lymphomas and virus-associated malignancies, including KSHV + PEL (Chen et al., 2013). Here we evaluated the expression of PD-1/PD-Ls in AIDS-KS tumor tissues of 10 patients from LSUHSC-NO HIV + Outpatient Clinic. Patient demographic information including age, gender, and ethnicity are listed in Table 1. Immunohistochemical analysis of patient samples indicated that 60% (6/10) of AIDS-KS tissues were strongly positive (+ + +/+ + + +) for PD-1/PD-L1, and 70% (7/10) of AIDS-KS tissues were strongly positive for PD-L2 (Table 1). PD-1, PD-L1 and PD-L2 expression were highly correlative with each other in these patients (all p-value < 0.01), with the expression of these immune checkpoint molecules observed in tumor cells and infiltrated immune cells from different AIDS-KS tumor tissues (Fig. 1A–B).

2.2. Activation of PD-L1 expression by induction of KSHV lytic reactivation

To assess whether lytic reactivation and/or replication induces the expression of PD-L1 in virus infected tumor cells, BCBL-1, a KSHV + PEL cell line, were treated with sodium butyrate (NaB) and/or 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) to induce lytic reactivation. As expected, the expression of PF (Processivity Factor, ORF59), a representative viral lytic protein, was increased following the treatment with either NaB or TPA, and was increased to a greater extent by the combination of both chemicals (Fig. 1C). Interestingly, these treatments similarly increased the expression of PD-L1 suggesting that viral lytic reactivation can upregulate PD-L1 expression in KSHV + tumor cells. To provide additional support for this conclusion, we used KSHV + iSLK.219 cells in which the lytic stage is inducible with doxycycline (Dox). Similar to results using BCBL-1 cells, induction of the lytic phase in iSLK.219 cells with Dox and various concentrations of NaB increased the expression of PD-L1 (Fig. 1C).

During the lytic phase, the expression of most viral genes is induced in an orderly fashion based on the time of expression. To identify the stage at which PD-L1 expression is activated during viral lytic replication, iSLK.219 cells were treated with inhibitors targeting different stages of viral lytic replication. Our data indicated that cidofovir (CDV), a KSHV polymerase inhibitor targeting viral DNA synthesis, had a minimal inhibitory effect on PD-L1 expression. In contrast, LY2228820, a p38-MAPK inhibitor targeting the early stages of KSHV lytic reactivation, resulted in a significant inhibition of PD-L1 expression suggesting that early viral lytic events may promote PD-L1 expression. As expected, LY2228820 but not CDV also inhibited the expression of PF protein (Fig. 1D).

The KSHV-encoded replication and transcription activator (RTA) protein is the key molecular switch for KSHV lytic reactivation from latency and is required for lytic initiation and subsequent viral DNA replication (Lukac et al., 1998). To identify whether RTA protein directly regulates PD-L1 expression, HEK293T cells were transiently transfected with or without two different RTA recombinant vectors (pCR3.1-RTA and pFLAG-RTA), and the ectopic expression of RTA was confirmed by western blotting. However, increased RTA expression had minimal effects on PD-L1 expression (Fig. S1), indicating that viral RTA protein alone is insufficient for activation of PD-L1 expression and that additional viral components are required.

2.3. Several major cellular signaling pathways and IL-1β are involved in KSHV lytic reactivation induced PD-L1 expression

To further explore the underlying mechanisms for KSHV lytic reactivation induced PD-L1 expression, we tested several major cellular signaling pathways including MAPK, NF-κB and AKT, all of which have been reported to regulate PD-L1 expression in cancer cells (Chen et al., 2016). We found that blockade of MAPK, NF-κB and AKT signaling by their respective inhibitors, U0126, Bay 11–7082 and A6730, all effectively reduced PD-L1 expression from lytically-induced iSLK.219 and BCBL-1 cells in a dose-dependent manner (Figs. 2A and S2). One recent study reported that inflammasome/IL-1β pathways modulate PD-1/PD-L1 checkpoint molecules (Guo et al., 2017). Interestingly, our recent RNA-Seq analyses (data not shown) comparing iSLK.219 cells with or without lytic induction identified IL-1β is one of top significantly upregulated genes (as well as PD-L1) in lytic cells (Fig. 2B). We further demonstrated that blockade of these 3 signaling pathways especially MAPK and AKT significantly reduced IL-1β expression from lytically-induced iSLK.219 cells (Fig. 2C). Moreover, directly silencing IL-1β by RNAi reduced PD-L1 expression from lytically-induced iSLK.219 cells (Fig. 2D). Taken together, our findings reveal a novel mechanism for oncogenic virus-mediated immunoregulation to promote tumor development, although the underlying mechanisms for these signaling

Table 1
KS tumor tissues collected from cohort HIV + patients.

No.	AGE	GENDER	ETHNICITY	DIAGNOSIS	LESION LOCATION	IMMUNOHISTOCHEMISTRY		
						PD-1	PD-L1	PD-L2
#01	31 y/o	Male	African-American	Kaposi's Sarcoma	Right arm	–	+	+ +
#02	49 y/o	Male	African-American	Kaposi's Sarcoma	Upper abdomen	+	+ +	+ +
#03	54 y/o	Male	Caucasian	Kaposi's Sarcoma	Upper leg	+ + +	+ + + +	+ + + +
#04	46 y/o	Male	Caucasian	Kaposi's Sarcoma	Right inner thigh	+ + +	+ + + +	+ + + +
#05	50 y/o	Male	African-American	Kaposi's Sarcoma	Right thigh	+ + + +	+ + +	+ + + +
#06	34 y/o	Male	African-American	Kaposi's Sarcoma	Not documented	+ + +	+ + + +	+ + + +
#07	26 y/o	Male	African-American	Kaposi's Sarcoma	Not documented	+	+ +	+ + +
#08	30 y/o	Male	Caucasian	Kaposi's Sarcoma	Right upper arm	+ + +	+ + + +	+ + + +
#09	53 y/o	Male	Caucasian	Kaposi's Sarcoma	Left forearm	+	+	+ +
#10	29 y/o	Male	Caucasian	Kaposi's Sarcoma	Right anterior chest	+ + +	+ + + +	+ + + +

−/+: weak; + +: intermediate; + + +/+ + + +: strong.

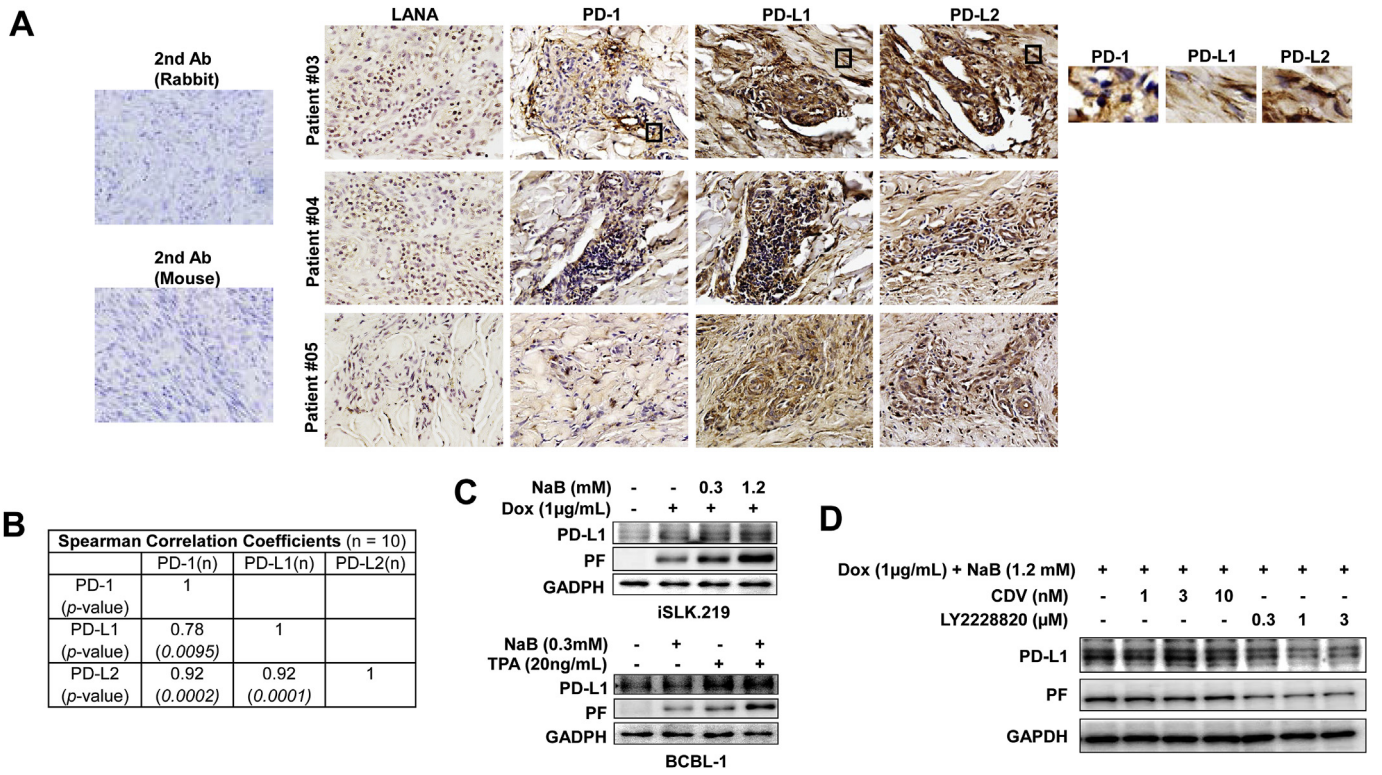

Fig. 1. PD-1/PD-Ls are highly expressed within AIDS-KS tumor tissues and upregulated from lytically-induced cells. (**A-B**) Expression of LANA, PD-1, PD-L1 and PD-L2 in representative formalin-fixed paraffin-embedded KS tissues from 3 HIV + patients were determined by immunohistochemical staining as described in the Methods. The higher magnification for PD-1, PD-L1 and PD-L2 detection from representative AIDS-KS patient and 2nd Ab negative controls (rabbit and mouse Ab, respectively) were also shown. The pairwise correlations among PD-1, PD-L1 and PD-L2 expression in total 10 AIDS-KS tumor tissues were tested using the Spearman Correlation analysis. (**C**) KSHV + PEL cell line, BCBL-1, or human iSLK.219 cells carrying a recombinant and inducible rKSHV.219 virus were treated with different chemical inducers for 48 h. (**D**) iSLK.219 cells were exposed to CDV or LY2228820 for 48 h prior to induction. Protein expression was analyzed by western blotting. Representative blots from one of three independent experiments are shown. TPA: 12-*O*-tetradecanoyl-phorbol-13-acetate; NaB: sodium butyrate; Dox: doxycycline.

pathways regulation of immune checkpoint molecules in KSHV-infected cells still require further investigation.

3. Materials and methods

Cell culture and reagents. The KSHV + PEL cell line BCBL-1 was kindly provided by Dr. Dean Kedes (University of Virginia) and cultured as described previously (Dai et al., 2018). Human iSLK.219 cells and HEK293T were cultured in DMEM medium with 10% FBS. The doxycycline (Dox), sodium butyrate (NaB), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and other signaling pathway inhibitors were purchased from Sigma. The cidofovir (CDV) and LY2228820 were purchased from Selleck.

Plasmids and siRNA transfection. HEK293T cells were transfected with RTA recombinant vectors, pCR3.1-RTA (A gift from Dr. Yan Yuan, University of Pennsylvania) or pFLAG-RTA, using Lipofectamine™ 3000 reagent (Invitrogen). Transfection efficiency was normalized through co-transfection of a lacZ reporter construct and determination of β-galactosidase activity using a commercial β-galactosidase enzyme assay system (Promega). For RNAi assays, ON-TARGET plus SMART pool siRNA for *IL-1β* (Dharmacon), or the negative control siRNA, were delivered by using the DharmaFECT transfection reagent.

Immunoblotting. Total cell lysates (30 μg) were resolved by 10% SDS–PAGE, transferred to nitrocellulose membranes, and incubated with 100–200 μg/mL of antibodies for PD-1, PD-L1, PD-L2 (Abcam), KSHV-PF (Advanced Biotechnologies), KSHV-RTA (ABBIOTEC), *p*-ERK/t-ERK, p-p65/t-p65, *p*-AKT/t-AKT, IL-1β (Cell Signaling). For loading controls, lysates were also incubated with antibodies detecting GAPDH (Cell Signaling). Immunoreactive bands were developed using an enhanced chemiluminescence reaction (PerkinElmer).

KS tumor tissues from HIV + patients and immunohistochemistry. KS tissues from HIV-infected patients were provided by the Louisiana State University Health Sciences Center (LSUHSC) HIV Clinical/ Tumor Biorepository (IRB approved No. 8079). Formalin-fixed, paraffin-embedded tissues were microtome-sectioned to a thickness of 4 μm, placed on electromagnetically charged slides (Fisher Scientific), and stained with hematoxylin & eosin (H&E) for routine histologic analysis. Immunohistochemistry was performed as described previously (Dai et al., 2018). Images were collected using an Olympus BX61 microscope equipped with a high resolution DP72 camera and CellSense image capture software.

Statistical analysis. The PD-1, PD-L1 and PD-L2 expressions were measured as an ordinal variable with the three strength levels: weak (−/+), intermediate (++) and strong (+++/++++). The pairwise correlations among PD-1, PD-L1 and PD-L2 expressions in AIDS-KS tumor tissues were tested using the Spearman Correlations. Significance for differences of gene transcription from iSLK.219 cells with or without lytic induction was determined using the two-tailed Student's t-test (Excel, 2016), and p values < 0.01 were considered highly significant.

Conflicts of interest

All the authors have declared no conflict of interest.

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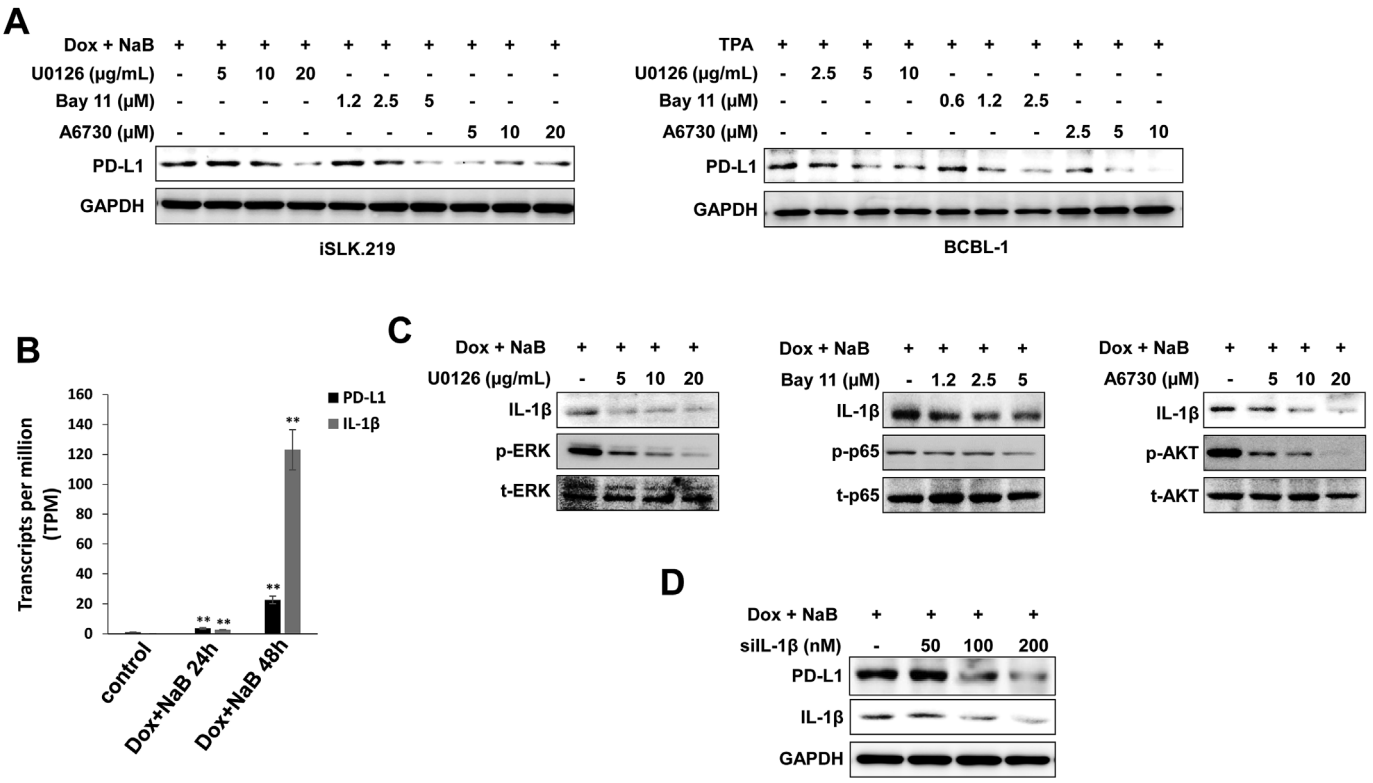

Fig. 2. Cellular signaling pathways and IL-1β involved in KSHV lytic reactivation induced PD-L1 expression. (**A/C**) iSLK.219 and/or BCBL-1 cells were treated with indicated concentrations of MAPK, NF-κB and AKT signaling inhibitors, U0126, Bay 11–7082 and A6730, respectively, for 12 h prior to chemicals induction. (**B**) Illumina RNA-Seq analyses of IL-1β and PD-L1 mRNA levels between iSLK.219 cells with or without lytic induction. Error bars represent S.D. for 3 independent experiments. ** = $p < 0.01$. The completed RNA-Seq data has been submitted to NCBI Sequence Read Archive (SRA, accessions SAMN11941049, SAMN11941050, SAMN11941051). (**D**) iSLK.219 cells were transfected with non-target control siRNA or IL-1β-siRNA for 48 h prior to chemicals induction. Protein expression was analyzed by western blotting. Representative blots from one of three independent experiments are shown.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.07.024.

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