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Journal of Cancer Immunology Commentary

Exosomes, PD-L1 and aGvHD: Perspectives for WJMSC-mediated Therapy

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Commentary

Tumor-derived small extracellular vesicles or exosomes which carry the checkpoint PD-L1 are directly involved in immune evasion and uncontrolled tumor growth. We have recently reported that PD-L1 is also enriched on Wharton's Jelly Mesenchymal Stromal Cell (WJMSC)-associated exosomes [1]. This biological property is likely associated with the physiologic role of WJMSCs atthe maternal-fetal interface. These cells have been shown to protect the developing fetus from attack by the maternal immune system due to the foreign paternal antigens which the fetus express. In this manuscript, we review the important roles of exosome-enriched PD-L1 (exo-PD-L1) during WJMSC-mediated therapy for acute graft-versus-host disease (aGvHD). We focus on exo-PD-L1's immunomodulation on T cell receptor (TCR) signaling in CD4+ T cells. In addition, we extend our discussions to other exosome-associated immune regulators, highlighting the potential to develop a cell-free, WJMSC-derived exosome therapy to treat complex immune diseases.

Exo-PD-L1 Underlies WJMSC-mediated aGvHD Therapy

Allogeneic hematopoietic stem cell transplantation (HSCT) provides a reliable treatment for various hematological malignancies [2]. Although the donor's immune cells help eliminate the tumor cells (an effect of graft-versus leukemia, GVL), they also cause the serious complication ofacute graft-versus-host disease (aGvHD) [3,4]. aGvHD occurs due to donor T cells recognizing and attacking the recipient's non-malignant tissues, impacting the clinic application of HSCT for patients [2,5].

Immunomodulatory mesenchymal stem cells (MSCs) have been widely used to treat aGvHD andother immune diseases [6,7]. We recently reported the first investigator-initiated clinical trial investigating the safety and efficacy of WJMSC in steroid-refractory GvHD [8]. To date, there wereat least 25 registered clinical trials for the exploration of the treatment of MSCs for aGvHD patients and 122 for other immunological diseases (http://clinicaltrials.gov/; accessed February 2021). One of the important molecular mechanisms lies in the immune regulatory checkpoint PD-L1. It is well known that checkpoint PD-L1 signal suppresses the T cell activation mediated by T cell receptors (TCRs) [9,10]. By way of *in vivo* studies, Kim et al. showed that transplantation of tonsil-derived-MSCs into imiquimod-induced psoriatic skin inflammation in mice significantly abrogated disease symptoms, mainly by blunting the Th17 response in a PD-L1-dependent manner [11]. Furthermore, Nie et al. demonstrated that human MSCs alleviated the pulmonary fibrosis by the PD-L1/PD1 signaling pathways [12]. In the bleomycin-induced pulmonary fibrosis model, abnormal PD1 expression in circulating T cells and lung tissues of patients with pulmonary fibrosis was observed [12].

Biologically, MSC-associated PD-L1 may interact with its receptor PD1 on the surface of T cells, leading to the exhaustion of T cells pathologically involved in the development of aGvHD. In aGvHD patients, Erkers et al. reported that decidual stromal cells have cell-cell

contact in order to suppress lymphocytes and blocking PD-L1 impaired their antiproliferative ability [13]. In liver transplant recipients diagnosed with aGvHD, higher PD1 expression was observed on donor CD4 and CD8 T cells. Blocking PD-L1 on the host-derived cells significantly enhanced alloreactivity by CD8 T cells *in vitro* [14]. This finding suggests that augmenting PD1/PD-L1 pathway may be a therapeutic strategy to control graft-versus-host-reactive T cells. In a mouse model of aGVHD, Tang et al. reported that systemic overexpression of PD-L1 inhibits the donor T cells activation, effector memory status, as well as Th1 and Th17 cells' responses *in vivo* [15]. Furthermore, they demonstrated that PD-L1 Ig treatment led to decreased T cells proliferation, promoted apoptosis, and reduced pro-inflammatory cytokine expression by effector T cells *in vitro*, in the setting of anti-CD3/CD28 stimulation and allogeneic dendritic cell stimulation [15]. These studies suggest that MSCs provide the regulatory checkpoints necessary to target pathological CD4+ T cells during stem cell transplantation treatments.

Extracellular vesicles (EVs) include a heterogeneous group of lipid bilayer membranous structures released from their host cells including mesenchymal stem cells (MSCs). Small EVs (sEVs, also referred to as exosomes) usually have an average size between 30-150 nm [16]. It has been suggested that exosomes are effective at delivering many types of bioactive molecules, including mRNAs, miRNAs, lncRNAs, lipids, and proteins to a variety of target cells and that theycan actively regulate the target cells even in organs distant from the cell of origin [17].

Meanwhile, more and more studies also suggest that tumor exosomal PD-L1 contributes to immunosuppression and is a valuable target for therapy and diagnosis. For instance, Cheng et al.reported that metastatic melanoma-derived exosomal PD-L1 is a rational predictor for patients and suppressed the function of CD8 T cells related to tumor growth [18]. In non-small cell lung cancer, Kim et al. reported that exosomal PD-L1 promotes tumor growth through immune escape [19]. Very similar observations were obtained in the studies of head and neck cancer [20] and gastric cancer [21]. Poggio et al. suggested that suppression of tumor exosomal PD-L1 may induce complex anti-tumor immunity and memory [22]. Therefore, both WJMSC-derived and tumor-associated exosomes which carry PD-L1 demonstrate the specific capability to target T cells through intervening with signaling pathways related to TCRs' functions.

Like tumor-derived exosomes, recent studies have suggested that that MSC-derived exosomes can affect CD4+ and CD8+ T cells in the aGvHD patients. Lai et al. reported that MSC exosomes effectively prolonged the survival of chronical GVHD mice and diminished the clinical and pathological scores [23]. They observed that activated CD4 T cells and their infiltration into the lung were reduced in these animals. Wang et al. reported that WJMSC-EVs alleviated the *in vivo* manifestations of aGVHD and the associated histologic changes and significantly reduced the mortality of the recipient mice [21]. They also found that recipients treated with hUC-MSC-EVs had significantly lower frequencies and absolute numbers of CD3+CD8+ T cells. Similar findings were obtained in aGvHD mouse model. Fujii et al. reported that the systemic infusion of human bone marrow MSC EVs prolonged the survival of mice with aGVHD and reduced the pathologic damage in multiple GVHD-targeted organs [24]. They reported that both CD4+ and CD8+ T cells were suppressed in EV-treated GVHD mice. As shown in Figure 1,we have recently reported that WJMSC sEVs suppress activated CD4+ T cells that were stimulated with anti-CD3/CD28 beads [1]. This observation suggests that WJMSC sEVs intervened on the CD4+ T cell activation and we have shown that multiple inhibitory checkpoints exist on WJMSC sEVs.

We identified PD-L1 enriched on this WJMSC sEVs as one of the key regulators for suppressing T cells [1]. We observed that exo-PD-L1 from WJMSCs demonstrated some unique features. First, exo-PD-L1 is a membraned-bound protein even though exosomes themselves are very diffusible; second, PD-L1 is highly enriched on the sEVs and preserves its bioactive glycosylation as previously reported [25]; and third, exo-PD-L1 is inducible through licensing WJMSCs by proinflammatory cytokines, such as interferon-gamma (IFNγ). These features support that exosomes may provide a functional form of PD-L1 that can be isolated to create a cell-free therapy. Exosomal PD-L1 is also likely superior to a recombinant PD-L1 for its ability to persist *in vivo*, partially due to co-expression of CD47 on sEVs [26,27], and home to various inflammatory sites,although the mechanism behind this ability is poorly characterized [26]. Consistent with the above findings from tumor exosomes, we found that WJMSC-derived sEVs also rely on PD-L1 to enhance the exhaustion of activated CD4+ T cells. Both pharmaceutic blocking of PD-L1 protein and genetic disrupting of the PD-L1 gene can dramatically impair WJMSC sEV-mediated inhibition of CD4+ T cells. More importantly, WJMSC exo-PD-L1 seems more efficient to block the activation of CD4+ T cells than both soluble PD-L1 or cell surface PD-L1. These observations support the concept that therapeutic WJMSCs provide other exosome-derived checkpoint signals which synergize with the exo-PD-L1 to regulate the immune activities in aGvHD patients.

Kordelas et al. reported that MSC EVs reduced the pro-inflammatory cytokine response of patient's PBMCs and the clinical GvHD symptoms during the course of MSC EVs therapy [28]. In support, we observed a rapid increase of peripheral plasma exo-PD-L1 in aGvHD patients shortly after infusing with therapeutic WJMSCs [1]. These

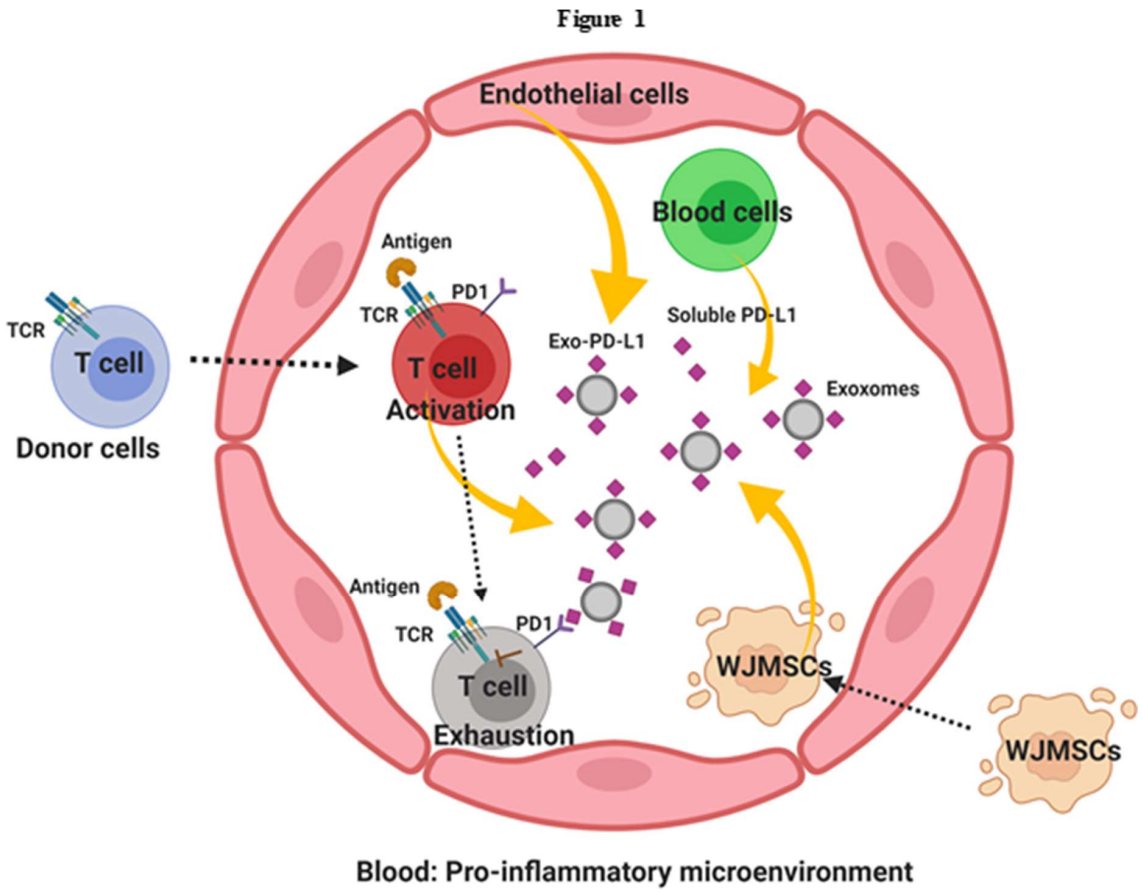

Figure 1: WJMSCs contribute to the circulatory Exo-PD-L1 pool in aGvHD patients. Infused WJMSCs are induced by cytokine IFN-γ, and enhance the release of Exo-PD-L1 in the blood. Exo-PD-L1 interacts with its PD1 receptor on the surface of T cells, subsequently inhibiting the TCR-mediated T cell activation. TCR: T cell Receptor; Exo: Exosomes; Exo-PD-L1: Exosomal PD-L1; IFN-γ, Interferon gamma; WJMSCs: Wharton's Jelly Mesenchymal Stromal Cells.

findings suggest that increased levels of exo- PD-L1 are correlated with the therapeutic infusion of WJMSCs. In aGvHD patients, we hypothesize that licensing WJMSCs is a necessary step to induce high levels of exo-PD-L1, further contributing to the "pool" of exo-PD-L1 in patient blood (Figure 1). However, we also noticed that the distribution of exo-PD-L1 in patients' circulation system is completely dependent on the subsequent cell uptake from other blood cells. Thus, our group's study as well as other groups' studies provide strong evidence to support that exo-PD-L1 is essential for WJMSC- mediated therapy of aGvHD in acute AML patients after HSCT.

Perspectives: Other Exo-regulators underlay WJMSC-mediated Therapy

Beside checkpoints (PD-L1, PD-L2 and CD276), exosomal markers (CD9, CD63, CD81, and HSP70), stem cell markers (CD73, CD105 and CD90) and other antigens (CD24, CD44, CD151and CD248) are enriched on WJMSC exosomes [1,29]. Their potential roles remain to be further determined. For instance, antigen CD73, recognized as a stem cell maker owns the enzyme activity of 5'-nucleotidase. Clayton et al. reported that exosomes from diverse cancer celltypes exhibited potent ATP- and 5' AMP-phosphohydrolytic activity [30]. They found that these exosomes can perform hydrolytic steps sequentially to form adenosine from ATP, triggering a cAMP response in adenosine A (2A) receptor-positive but not A (2A) receptor-negative cells [30]. Ludwig et al. reported that tumor exosomes carried enzymatically active CD39/CD73 andadenosine [31]. They found that tumor exosomes promoted A2BR-mediated polarization of macrophages toward an M2-like phenotype and enhanced their secretion of angiogenic factors [31]. Theodoraki et al. reported the highest level of CD39/CD73 ectoenzymes of adenosine

production was found in CD3 (-) exosomes in patients with advanced-stage (III/IV) head and necksquamous cell carcinoma. Also, the production of 5'-AMP and purines was significantly higher inTreg co-incubated with CD3 (-) than CD3 (+) exosomes [32].

WJSMC exosomes were defined as positive for CD9, CD63, and CD73 [33]. Cytokine TNF-α stimulation not only increased the amount of exosome secreted from gingiva-derived MSCs but also enhanced the exosomal expression of CD73 [34]. Bone marrow-derived MSC recipients had increased serum CD73 expressing exosomes that promoted adenosine accumulation *ex vivo* [18].Using exosomes that were isolated from HPV-16 E6/E7 transformed human bone marrow stromalcells, Hettich et al. found that both CD73 and constitutional lipids on these exosomes areidentified as key bioactive components promoting the exosome-driven acceleration of processesrequired for wound repair [35]. Using chondrocyte cultures, Zhang et al. reported the rapid cellular proliferation and infiltration during exosome-mediated cartilage repair is due to exosomal CD73-mediated adenosine activation of AKT and ERK signaling [36]. Chew et al. found that MSC exosomes could increase bone and periodontal ligament cell migration and proliferation through CD73-mediated adenosine receptor activation of pro-survival AKT and ERK signaling [37]. Accordingly, WJMSC exo-CD73 may inhibit T cells through adenosine signal pathways. However, the detailed mechanism needs to be further characterized by either molecular or pharmaceutical methods.

In summary, exosome-associated proteins derived from therapeutic WJMSCs can provide important regulatory signals to impact adaptive immune activities. Further, identifying and characterizing these exosomal immune regulators will help us gain deeper insights into the therapeutic value of WJMSCs and WJMSC-derived exosomes in the treatment of complex human immune diseases. Finding the balance between inhibiting alloreactive T-cells mediating aGvHD without impairing the GvL effect and tumor elimination is a complex issue when it comes to leveraging immunosuppressive treatments after HSCT. However, we believe it is possible to find this balance through the proper timing and dosing of the WJMSC-associated sEVs. Critically, these small nano-size particles may be very helpful in developing novel cell-free therapies for many patient populations.

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