

A checkpoint on innate myeloid cells in pulmonary arterial hypertension

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To the Editor

Immature myeloid cells that are in a pathologic state of activation have been identified in many inflammatory disorders, including pulmonary arterial hypertension (PAH).¹ Myeloid-derived suppressor cells (MDSC) are a subtype of immature myeloid cells that were initially described in relation to the immune escape and proliferation of certain cancers. It is known that peripheral blood MDSC are elevated in pediatric patients with pulmonary hypertension (PH) secondary to congenital heart disease compared to healthy controls, especially in female patients.² In addition, in several murine models of disease, polymorphonuclear MDSC (PMN-MDSC), a subpopulation of MDSC, promote PH, with a reduction in PMN-MDSC trafficking to the lung attenuating pulmonary vascular remodeling.³ Despite these findings, a profile of MDSC, including potentially important differences in PMN-MDSC and monocytic MDSC (Mo-MDSC) sub-groups, is unknown in adult patients with either familial or idiopathic PAH.

The interaction between programmed cell death protein 1 (PD-1; also known as CD279) and programmed death-ligand 1 (PD-L1; also known as CD274) are key immunoregulatory components of innate myeloid cells; PD-L1 is highly expressed on MDSC. This pathway is a therapeutic target in oncology and may be similarly relevant in PAH, but it is unclear whether PD-L1 is differentially expressed on sub-populations of myeloid cells, such as Mo-MDSC and PMN-MDSC, in patients with PAH compared to healthy individuals. We therefore hypothesized that PD-L1 surface expression is elevated on circulating MDSCs from patients with PAH.

Animal studies were conducted in accordance with the University of Florida Institutional Animal Care and

Use Committee. We used wild-type (WT) C57BL/6 mice (males and females, aged 12–16 weeks; Jackson Laboratories) for these studies. Experimental mice underwent chronic hypoxia exposure, FiO₂ 10% for 28 days, versus normoxia exposure in controls. Mice were analyzed for invasive measurement of right ventricular systolic pressure (RVSP; mmHg), and assessment of right ventricle to left ventricle plus septal mass ratio (%), as previously described.⁴ Sample preparation, flow cytometric analysis, and antibody information have previously been described,³ except for the addition of anti-PD-L1 antibody (BV785, rat; BioLegend Cat. no. 124331) and anti-PD-1 antibody (PE-CF594, hamster; BD Cat. no. 56523).

We defined the circulating MDSC immunophenotype in the peripheral blood mononuclear cell (PBMC) samples from a cohort of patients with PAH⁵ (n = 24), and healthy controls⁶ (n = 7) without diagnosed pulmonary or cardiovascular disease. Absolute cell counts from PBMC samples were unavailable and therefore all data are limited to reporting as proportional analysis. PBMC were chosen for analysis based upon previous reports of MDSC sub-population characterization using these kinds of samples.⁷ All studies were approved by the University of Florida and Vanderbilt University Institutional Review Boards. As per the above, sample preparation, flow cytometric analysis, and antibody information have previously been described,³ except for

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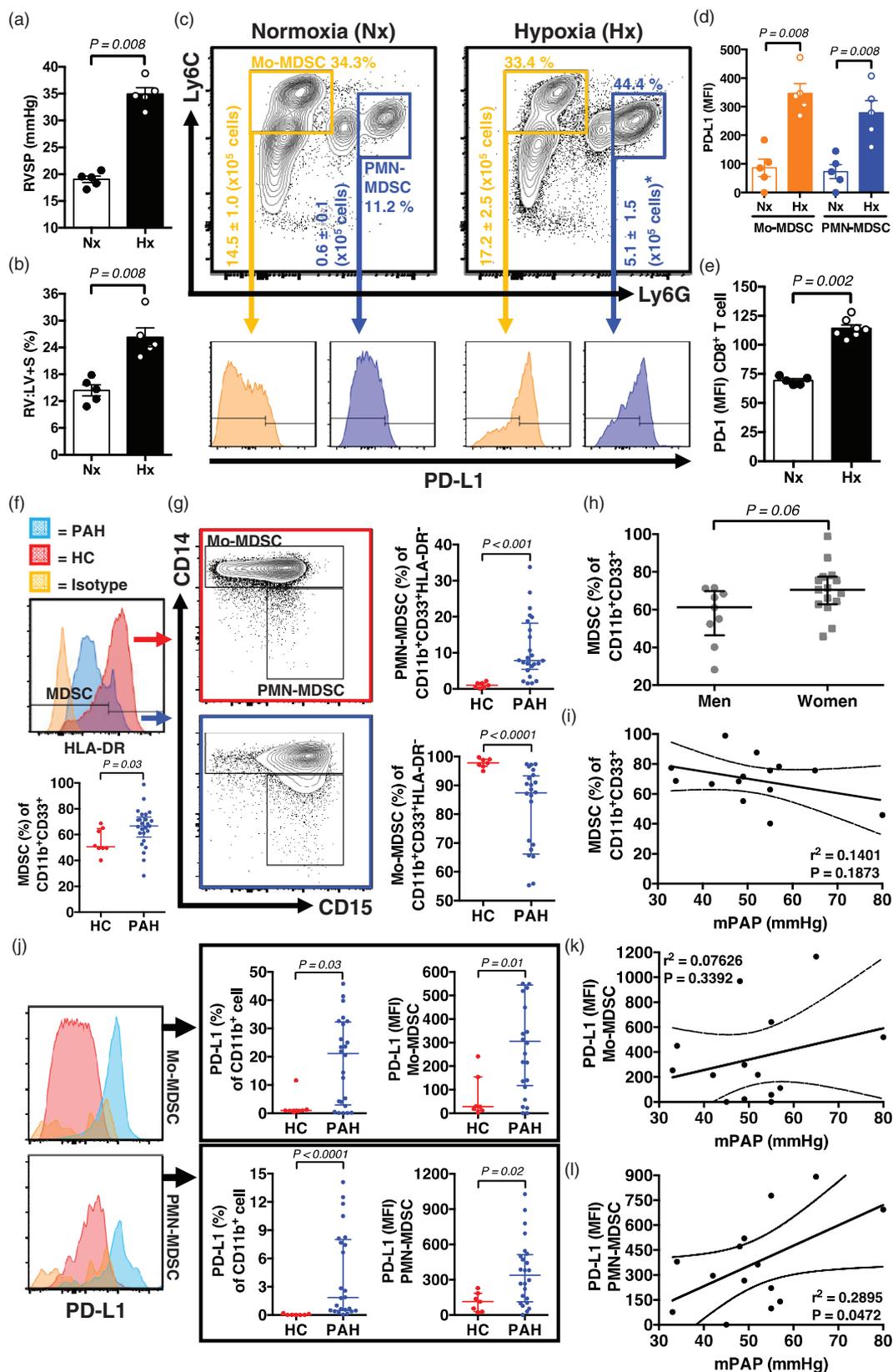


Fig. 1. (a) Right ventricular systolic pressure (RVSP; mmHg) in WT C57BL/6 mice exposed to hypoxia (Hx; FiO₂ 10% for 28 days) compared to normoxia (Nx)-exposed controls, and (b) associated right ventricle hypertrophy as assessed by right ventricle to left ventricle plus septal mass ratio (RV:LV + S; %). (c) Monocytic myeloid-derived suppressor cells (Mo-MDSC; CD45⁺CD11b⁺CD11c⁺Ly6C^{hi}Ly6G^{lo} cells) and polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC; CD45⁺CD11b⁺CD11c^{lo}Ly6C^{lo}Ly6G^{hi} cells) in lungs of Nx and Hx treated mice (% of (Continued).

addition of anti-PD-L1 antibody (BV 605, mouse CD274; BioLegend Cat. no. 124321). The expression of activation markers is presented as median fluorescence intensity (MFI). Data were analyzed using FlowJo software (Tree Star). Statistical analysis was performed using Prism 6.0 (GraphPad Software). Data were compared using ANOVA with Bonferroni post-test for multiple comparisons or Spearman rank coefficient analysis, as indicated. A P value < 0.05 was considered significant.

In order to assess for PD-L1 expression on MDSC, we first exposed WT mice to chronic hypoxia, to induce PH and right ventricular hypertrophy (Fig. 1a and 1b). We next confirmed our previously reported observation of increased MDSC, mainly PMN-MDSC, accumulation within the lung of hypoxic mice (Fig. 1c), noting an increase in PD-L1 expression on both Mo-MDSC and PMN-MDSC sub-populations under hypoxia (Fig. 1d, populations are defined in the figure legend). A similar distribution pattern of MDSC sub-groups was noted in the bone marrow of exposed mice, as well (data not shown). It is known that PD-1 is induced on CD8⁺ T cells by hypoxia.⁸ Therefore, we looked for PD-1 expression on these T lymphocytes in our model, finding a significant elevation within the lung samples from the hypoxia-treated group versus the controls (Fig. 1e). These findings indicate that PD-1/PD-L1 signaling may be biologically relevant in models of PH.

We next verified that MDSC-like cells, defined as CD11b⁺CD33⁺HLA-DR⁻, were elevated in patients with PAH (Fig. 1f). Moreover, the ratio of cells with PMN-MDSC surface markers (CD14⁻CD15⁺) were elevated in patients with PAH, while Mo-MDSCs (CD14⁺) were decreased (Fig. 1g). Since the study by Yeager et al. found an increase in circulating MDSC in female versus male PAH patients,² we subsequently analyzed for MDSC percentage by sex in our cohort. In our patient population, however, women and men with PAH did not have a significant difference in the circulating MDSC as a proportion of CD11b⁺CD33⁺ cells, although there was a strong trend ($P = 0.06$) toward agreement with the higher female predisposition to PAH (Fig. 1h). Additionally, we found no relationship between percentage of MDSC and the available mean pulmonary artery pressures (mPAP; mmHg), analyzed

near or at the time of PBMC collection (Fig. 1i). Examining MDSC sub-groups for PD-L1 expression, we found that the population of PD-L1-expressing MDSC was higher in patients with PAH compared to controls; in addition, the surface expression of PD-L1 was higher in both of the MDSC sub-populations of PAH patients compared to healthy controls (Fig. 1j). Although there was no significant correlation between PD-L1 expression on Mo-MDSC and mPAP (Fig. 1k), there was a significant positive correlation between PMN-MDSC expression of PD-L1 with severity of PH (Fig. 1l). Similar to our murine experiments, the clinical data indicate that PD-1/PD-L1 may play a role in development of PH, although a harmful or protective effect cannot be determined yet.

The findings in the current study are consistent with the hypothesis that MDSC, in combination with PD-L1 expression, can possibly mediate inflammation related to chronic vasculopathy. This observation has the potential to be explored further in the hope of identifying therapeutic targets to influence development of vascular remodeling in pulmonary vessels, such as examining the role of PD-1 associated CD28-dependent pathways in rescuing T cell exhaustion in PH.⁹ Additionally, given the role chemokine receptors such as CXCR2 are known to play in the pathogenesis of PAH,^{10,11} and the potential for synergism between these receptors and PD-1/PD-L1 expression,¹² their analysis in circulating subsets of MDSC may deserve more attention as putative inflammatory markers of disease.¹³

Based on our observational findings alone, we can draw no conclusions on the role of either MDSCs or PD-L1 expression in PAH. However, one can speculate on these current findings, especially in light of the relationship between MDSC and polarized T-lymphocyte populations in PAH models of disease.¹⁴ In particular, changes in inducible Treg function – through regulation of transcriptional factor FoxP3 – can result in a deleterious positive feedback loop leading to aberrant MDSC regulation of other potentially maladaptive immune cells, such as $\gamma\delta$ T cells, or uninhibited production of growth factors known to contribute to PAH, such as transforming growth factor beta (TGF- β).¹⁵ In this context, caution must be exercised in the interpretation of our findings. The primary study to date examining

Fig. 1. Continued

CD11b⁺ cells and absolute cells per lung; * $P < 0.05$, comparing hypoxia and normoxia exposure), with corresponding histogram of PD-L1 expression per sub-population; the latter quantified in (d). Representative flow plots displayed. $n = 5$ mice (3 male and 2 female) per group. (e) PD-1 expression on CD8⁺ T cells in lungs of Nx- vs. Hx-exposed WT mice. $n = 7$ mice (4 male and 3 female) per group. Data are shown as mean and SEM. (f) Changes in percentage of live CD11b⁺CD33⁺HLA-DR⁻ cells (myeloid-derived suppressor cells, MDSC) in human healthy control (HC; $n = 7$) and pulmonary arterial hypertension (PAH, $n = 24$) peripheral blood mononuclear cell (PBMC) samples. (g) Differences in percentage of CD11b⁺CD33⁺HLA-DR⁻CD14⁻CD15⁺ cells (polymorphonuclear MDSC, PMN-MDSC) and CD11b⁺CD33⁺HLA-DR⁻CD14⁺ cells (monocytic MDSCs, Mo-MDSC) between HC and PAH groups. (h) MDSC (% of CD11b⁺CD33⁺ cells) in men ($n = 9$) and women ($n = 15$) with PAH. (i) MDSC (% of CD11b⁺CD33⁺ cells) in patients with PAH correlated with mean pulmonary artery pressure (mPAP; mmHg). (j) Changes in percent expressing cells, and expression (median fluorescence intensity, MFI), of programmed death-ligand 1 (PD-L1) of MDSC sub-populations, Mo-MDSC and PMN-MDSC between HC and PAH patient peripheral blood samples. PD-L1 expression correlated with mPAP in (k) Mo-MDSC and (l) PMN-MDSC. Representative flow plots displayed. Data are shown as median and IQR. Spearman rank correlation analysis was performed between variables, as indicated.

checkpoint PD-L1 expression, mainly by endothelial cells, suggests that inhibition reverses protection conferred by functional Treg adoptive transfer, fully replicating the phenotype of severe PH associated with Treg deficiency, in the SU5416 and chronic hypoxia rat model.¹⁶

The chronic hypoxia model is, of course, severely limited in replicating the pathology of human PAH. However, the model is helpful in recapitulating key components of pulmonary hypertensive lung changes, such as immune cell oxidative stress, endothelial cell injury, and a distinct type of pulmonary inflammatory response. Additionally, chronic hypoxia results in muscularization of the media, with resulting PA lumen narrowing and associated RV hypertrophy, although this is reversible upon re-exposure of mice to normoxia. While we are incapable of drawing conclusions comparing the functional capabilities of MDSC from hypoxia-exposed mice to those from patients with PAH, chronic hypoxia itself is known to have effects on accumulation of MDSC within tissue, resulting in pathologic changes to the microenvironment.¹⁷

Although our study did not functionally define MDSC, the exclusion of mature granulocytes by density fractionation before our analysis makes contamination of our samples by mature neutrophils unlikely.¹⁸ Moreover, neutrophils that significantly express PD-L1 display an MDSC-like suppressive phenotype, regardless,¹⁹ and have been demonstrated to be transcriptionally distinct from inflammatory neutrophils.²⁰ While all of our analysis was uniformly performed on thawed PBMC samples, we cannot exclude biologically relevant differences in MDSC composition compared to analysis performed on fresh peripheral blood.²¹

In conclusion, we posit that PD-L1 is elevated on MDSC from patients with PAH and that the proportion of MDSC subsets is shifted in disease. These anomalies may play a role in the chronic inflammatory process leading to pulmonary vascular remodeling and the progression of PAH. Conveniently, this hypothesis can be tested in part through manipulation of the signaling pathway in the chronic hypoxia murine model of PH. While future studies are necessary to prove or disprove their role in disease pathology, it is a provocative area of research given the large amount of therapeutic resources now available in the field.

Conflict of interest

The authors declare that there is no conflict of interest.

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References

1. Marsh LM, Jandl K, Grunig G, et al. The inflammatory cell landscape in the lungs of patients with idiopathic pulmonary arterial hypertension. *Eur Respir J* 2018; 51: 1701214.
2. Yeager ME, Nguyen CM, Belchenko DD, et al. Circulating myeloid-derived suppressor cells are increased and activated in pulmonary hypertension. *Chest* 2012; 141: 944–952.
3. Bryant AJ, Shenoy V, Fu C, et al. Myeloid-derived suppressor cells are necessary for development of pulmonary hypertension. *Am J Respir Cell Mol Biol* 2018; 58: 170–180.
4. Bryant AJ, Carrick RP, McConaha ME, et al. Endothelial HIF signaling regulates pulmonary fibrosis-associated pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2016; 310: L249–262.
5. Hemnes AR, Trammell AW, Archer SL, et al. Peripheral blood signature of vasodilator-responsive pulmonary arterial hypertension. *Circulation* 2015; 131: 401–409. (discussion 409).
6. Krotova K, Marek GW, Wang RL, et al. Alpha-1 antitrypsin-deficient macrophages have increased matriptase-mediated proteolytic activity. *Am J Respir Cell Mol Biol* 2017; 57: 238–247.
7. Sacchi A, Tumino N, Grassi G, et al. A new procedure to analyze polymorphonuclear myeloid derived suppressor cells in cryopreserved samples cells by flow cytometry. *PLoS One* 2018; 13: e0202920.
8. Cubillos-Zapata C, Avendano-Ortiz J, Hernandez-Jimenez E, et al. Hypoxia-induced PD-L1/PD-1 crosstalk impairs T-cell function in sleep apnoea. *Eur Respir J* 2017; 50: 1700833.
9. Kamphorst AO, Wieland A, Nasti T, et al. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. *Science* 2017; 355: 1423–1427.
10. Burton VJ, Ciucan LI, Holmes AM, et al. Bone morphogenetic protein receptor II regulates pulmonary artery endothelial cell barrier function. *Blood* 2011; 117: 333–341.
11. Burton VJ, Holmes AM, Ciucan LI, et al. Attenuation of leukocyte recruitment via CXCR1/2 inhibition stops the progression of PAH in mice with genetic ablation of endothelial BMPR-II. *Blood* 2011; 118: 4750–4758.
12. Highfill SL, Cui Y, Giles AJ, et al. Disruption of CXCR2-mediated MDSC tumor trafficking enhances anti-PD1 efficacy. *Sci Transl Med* 2014; 6: 237ra267.
13. Yamauchi Y, Safi S, Blattner C, et al. Circulating and tumor myeloid-derived suppressor cells in resectable non-small-cell lung cancer. *Am J Respir Crit Care Med* 2018; 198: 777–787.
14. Tamosiuniene R, Tian W, Dhillon G, et al. Regulatory T cells limit vascular endothelial injury and prevent pulmonary hypertension. *Circ Res* 2011; 109: 867–879.
15. Lee CR, Kwak Y, Yang T, et al. Myeloid-derived suppressor cells are controlled by regulatory T cells via TGF-beta during murine colitis. *Cell Rep* 2016; 17: 3219–3232.
16. Tamosiuniene R, Manouvakhova O, Mesange P, et al. Dominant role for regulatory T cells in protecting females against pulmonary hypertension. *Circ Res* 2018; 122: 1689–1702.

17. Jayaprakash P, Ai M, Liu A, et al. Targeted hypoxia reduction restores T cell infiltration and sensitizes prostate cancer to immunotherapy. *J Clin Invest* 2018; 128: 5137–5149.
18. Bian Z, Shi L, Venkataramani M, et al. Tumor conditions induce bone marrow expansion of granulocytic, but not monocytic, immunosuppressive leukocytes with increased CXCR2 expression in mice. *Eur J Immunol* 2018; 48: 532–542.
19. Langereis JD, Pickkers P, de Kleijn S, et al. Spleen-derived IFN-gamma induces generation of PD-L1(+)-suppressive neutrophils during endotoxemia. *J Leukoc Biol* 2017; 102: 1401–1409.
20. Fridlender ZG, Sun J, Mishalian I, et al. Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. *PLoS One* 2012; 7: e31524.
21. Kotsakis A, Harasymczuk M, Schilling B, et al. Myeloid-derived suppressor cell measurements in fresh and cryopreserved blood samples. *J Immunol Methods* 2012; 381: 14–22.