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Sex-dependent PD-L1/sPD-L1 trafficking in human endothelial cells in response to inflammatory cytokines and VEGF

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ABSTRACT

Programmed cell death 1 ligand 1 (PD-L1) expressed in non-immune cells is involved in immune-mediated tissue damage in the context of inflammatory conditions and tumor immune escape. Emerging evidence suggests soluble (s)PD-L1 as a marker of inflammation. Based on well-established sex-specific differences in immunity, we tested the novel hypotheses that (i) endothelial cell PD-L1 is modulated by inflammatory cytokines and vascular endothelial growth factor (VEGF) in a sex-specific fashion, and (ii) the endothelium is a source of sPD-L1. After exposure of human umbilical vein endothelial cells (HUVECs) to lipopolysaccharide, interleukin (IL)1β or VEGF for 24 h, total PD-L1 levels were upregulated solely in cells from female donors, while being unchanged in those from male donors. Accordingly, exposure to synovial fluids from patients with inflammatory arthritis upregulated PD-L1 levels in HUVECs from female donors only. Membrane PD-L1 expression as measured by flow cytometry was unchanged in response to inflammatory stimuli. However, exposure to 2 ng/mL IL-1β or 50 ng/mL VEGF time-dependently increased sPD-L1 release by HUVECs from female donors. Treatment with the metalloproteinase (MMP) inhibitor GM6001 (10 μM) prevented IL-1β-induced sPD-L1 release and enhanced membrane PD-L1 levels. The anti-VEGF agents bevacizumab and sunitinib reduced both VEGF-induced PD-L1 accumulation and sPD-L1 secretion. Thus, inflammatory agents and VEGF rapidly increased endothelial PD-L1 levels in a sex-specific fashion. Furthermore, the vascular endothelium may be a sPD-L1 source, whose production is MMP-dependent and modulated by anti-VEGF agents. These findings may have implications for sex-specific immunity, vascular inflammation and response to anti-angiogenic therapy.

1. Introduction

The endothelium plays an important role in initiating and shaping the immune response [1]. Besides regulating the movement of leukocytes to sites of inflammation, endothelial cells (ECs) may serve as antigen-presenting cells. Emerging evidence also highlights the role of EC programmed death ligand 1 (PD-L1) in the control of immune cell activation [2]. PD-L1 is the first functionally characterized ligand of the immune receptor PD-1, and the PD-L1/PD-1 axis is a critical determinant of immune homeostasis by serving as a negative regulator of immune response.

The expression of PD-L1 on non-hematopoietic cells including ECs is important to control T cell activation, tolerance, and immune-mediated tissue damage in the context of inflammatory conditions [3–5]. Indeed, PD-L1 is increased in response to inflammatory stimuli such as interferons (IFNs) and tumor necrosis factor (TNF) in ECs of different origin and suppresses T cell activation, an effect that is opposed by anti-PD-L1 agents [6,7]. Consistently, recent work in heart transplantation recipients showed that abrogation of PD-L1 expression in graft ECs leads to acute rejection and reduced graft survival, supporting a critical role for EC PD-L1 in the control of immune-mediated inflammatory injury [8]. PD-L1 is also overexpressed in tumor-associated vessels, where it contributes to the immunological escape of neoplastic tissues from adaptive anticancer responses [9,10] and possibly to immunotherapy resistance [11]. Notably, in addition to promoting angiogenesis, VEGF exerts an immunosuppressive role through several mechanisms including increased PD-1 expression on immune cells [12]. In line with this evidence, anti-VEGF agents show immune-supportive properties [13].

Recent work from our group highlighted a sexual dimorphism in the angiogenic response of human ECs exposed to synovial fluids from

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Nomenclature

FBS	fetal bovine serum
IL-1	Interleukin 1
IL-6	Interleukin 6
IFNγ	Interferon gamma
MMP	Matrix metalloproteinase
TNFα	Tumor necrosis factor alpha

patients with rheumatoid arthritis (RA), containing higher levels of inflammatory cytokines and VEGF [14]. However, whether (i) VEGF plays a role in regulating endothelial immune checkpoint expression, and (ii) EC PD-L1 is involved in sex differences in the immune response remains to be determined.

Recently, PD-L1 has been reported to occur in soluble forms that may be generated by proteolytic cleavage of the membrane-bound form through proteases such as metalloproteinases (MMPs), which are overexpressed in an inflammatory microenvironment [15,16]. Soluble (s) PD-L1 levels were also found to be higher in serum of pregnant with respect to non-pregnant women and to increase throughout gestation. In the latter setting sPD-L1 contributes to suppress maternal immunity [17]. Although its functional role has not been completely clarified, sPD-L1 likely behaves as an immunosuppressive molecule mimicking the effects of PD-L1. Thus, sPD-L1 could represent a general marker of inflammatory status in the context of overactive immune-mediated inflammatory responses. Remarkably, sPD-L1 has been detected in cancer cell supernatants and in plasma from patients with cancer or chronic inflammatory diseases including diabetes and acute coronary syndrome, where sPD-L1 levels are associated with disease severity and poor prognosis [18–21]. However, it is unclear whether and to what extent ECs are a source of circulating sPD-L1.

In this study, we assessed (i) the expression and release of PD-L1/sPD-L1 in response to inflammatory cytokines, including IL-1β, IL-6 and the growth factor VEGF in sex-differentiated human endothelial cells, and (ii) the regulation of sPD-L1 secretion by MMP inhibitors and anti-VEGF agents. We provide the first evidence that not only cytokines but also VEGF rapidly increased PD-L1 expression by female but not male ECs. Even more importantly, ECs from female donors are a source of sPD-L1, whose production is dependent on MMP activity and may be modulated by sunitinib and bevacizumab.

2. Materials and methods

2.1. Chemicals

Trypan Blue, 3–4,5 dimethylthiazol-2-yl-2,5 diphenyltetrazolium bromide (MTT), and IL-1β were from Roche (Basel, Switzerland). Bevacizumab was kindly provided by the Aviano Cancer Reference Centre. GM6001 and sunitinib were purchased from Aurogene S.r.l and added 1 h before the stimulus. VEGF and IL-6 were from ImmunoTools (Friesoythe, Germany). LPS was from InvivoGen.

2.2. Cell culture

HUVECs were isolated from human umbilical cords collected after delivery from full-term normal pregnancies at the Obstetrics and Gynecological Unit of Padua University Hospital as previously described [22]. The mothers gave their informed consent, and collected cords were identifiable only by sex. The procedure was approved by the Padua University Hospital Ethics Committee (Comitato Etico per la Sperimentazione Clinica della Provincia di Padova, N.0038309, 22/06/2017). Briefly, endothelial cells were isolated by collagenase digestion of the umbilical vein interior (0,01% collagenase in PBS for 15 min at 37 °C). The cell suspension was centrifuged at 300 *g* for 5 min and the pellet was resuspended in M199 medium (Invitrogen, Thermofisher) supplemented with 15% fetal bovine serum (FBS, Invitrogen), gentamicin (40 μg/mL, Invitrogen), endothelial cell growth supplement (ECGS, 100 μg/mL), and heparin (100 IU/mL, Sigma-Aldrich). HUVECs were grown at 37 °C and 5% CO_2, and reached confluence after 3–4 days. Images obtained with bright-field microscope (Nikon Eclipse-Ti) equipped with a digital camera showed that cultured HUVECs grew as monolayers of closely opposed, polygonal large cells (cobblestone morphology). Endothelial cell purity was assessed in random preparations by measuring the expression of the surface marker CD31 (CD31-PE, 0.0125 mg/mL; BD Bioscience) by flow cytometry as described in Section 2.4 below.

Cells were obtained from $n = 5$ male and $n = 5$ female donors; one cell preparation was derived from each donor. HUVECs were used from passage 2–5, and cells from male and female donors at the same passage were used in each independent experiment. The experiments were performed using M199 medium supplemented with 5% FBS, 40 μg/mL gentamicin, 100 μg/mL ECGS and 100 IU/mL heparin. Selected experiments with synovial fluids were carried out in serum-free M199 supplemented with heparin.

2.3. Synovial fluids

Synovial fluids (SFs) were collected by arthrocentesis from swollen knees of untreated patients with rheumatoid arthritis (RA), psoriatic arthritis (PsA) and non-inflammatory arthritis (osteoarthritis, OA) attending the outpatients' clinic of the Rheumatology Unit at Padua University Hospital. The primary purpose for joint aspiration was therapeutic relief and/or diagnosis. Discarded samples were studied under protocols including written informed consent and approved by the local Institutional Review Board. The investigation conformed to the principles outlined in the Declaration of Helsinki. SFs were obtained from 15 patients (6 RA, 6 PsA and 3 OA) with mild-to-moderate disease who presented after January 2019 due to acute pain. Patients already on treatment with glucocorticoids and/or anti-cytokine drugs were excluded from the study. Routine SF analysis consisted of total and differential white blood cell (WBC) count by light microscopy using a Bürker counting chamber and pre-stained slides for cell morphology (Testsimplets®), respectively. All SFs tested from RA and PsA donors were characterized by a high inflammatory index (WBC > 2000) and the absence of crystals and pathogens. SFs were centrifuged at 3000 rpm for 10 min and stored at − 20 °C until further analysis.

2.4. Flow cytometry

Surface PD-L1 (CD274) expression was analyzed by flow cytometry. HUVECs (3×10^5 cells/well) were seeded in 35-well plates and treated as indicated in the Results. In selected experiments, cells were treated with the broad-spectrum MMP inhibitor GM6001 {(2 R)-N4-Hydroxy-N1-[(1 S)− 1-(1 H-indol-3-ylmethyl)− 2-(methylamino)− 2-oxoethyl]− 2-(2-methylpropyl) butanediamide} for 30 min before adding the stimulus. At the end of stimulations, HUVECs were washed and harvested by gently scraping the culture plates with 1 mL of PBS containing 5 mM EDTA and 2% FBS. Cells were centrifuged and resuspended in PBS supplemented with 5% FBS. Thereafter, 100 μL of either FITC-labeled mouse anti-human PD-L1 or FITC-labeled mouse IgG1 isotype control (BD Pharmingen) were added at a concentration of 20 μg/10^6 cells and incubated at room temperature for 20 min in the dark. The reaction was stopped by adding 200 μL of 5% FBS in PBS. Analyses were performed on a FACSCanto II flow cytometer (BD Biosciences), recording at least 10,000 events for each sample. Data were analyzed using the FACS Diva software (BD Biosciences). Isotype-matched controls were used as baseline reference. Typically, < 2% positive cells were allowed beyond the statistical marker in appropriate controls.

2.5. Western blot

HUVECs (3 $\times 10^5$cells/well) were seeded in 35-mm dish and treated as indicated in Results. Cells were lysed with 80 μL lysis buffer (phosphate-buffered saline supplemented with 1.2% Triton X-100, 1X Roche cOmplete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), 2.5 mM NaF, 2 mM $Na_4P_2O_7$ (Sigma-Aldrich), 4 mM Na orthovanadate and 1 mM phenylmethanesulfonylfluoride). After centrifugation at 10,000 rpm for 15 min, supernatants were collected. Protein quantification was performed using a BCA assay kit (Euroclone, Milan, Italy). Proteins (40 μg) were separated on SDS-PAGE and transferred onto Amersham Hybond-P polyvinylidene difluoride membranes. Membranes were then blocked and probed using the anti-PD-L1 rabbit primary monoclonal antibodies (Abcam, Cambridge, UK) and anti-GAPDH polyclonal antibody. After washing, membranes were incubated with rabbit secondary horseradish peroxidase–conjugated antibodies (Vector Laboratories, Burlingame, CA, USA). Bands were detected by chemiluminescence using Western bright™ Quantum (Advansta, Menlo Park, CA, USA). Images were acquired by the Alliance mini HD9 Imaging System (Uvitec, Cambridge, United Kingdom). Densitometry analysis of bands was performed using the Image J version 1.47 software (U.S. National Institutes of Health). Results are expressed as percentage of controls and represent the mean values of 3–5 independent experiments.

2.6. ELISA assay

sPD-L1 in SF samples and HUVEC supernatants treated as described in the Results was measured using the Human PD-L1 DuoSet ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Since the lower limit of detection of this assay was 39.1 pg/mL, the protocol was optimized with respect to antibody concentration. A standard curve prepared with human recombinant B7-H1 (PD-L1) (0.97–500 pg/mL) was used for calculation of absolute sPD-L1 levels in samples.

2.7. Statistical analysis

All experiments were performed in at least 3 independent replicates; results are presented as mean values, with error bars representing the standard deviation (SD). Statistical analysis was performed using Graph Pad Prism 6 (Graph Pad Software Inc., La Jolla, CA, USA). Unpaired, two-tails Student's *t*-test was used to compare the means of two independent groups. One-way ANOVA followed by Tukey's *post hoc* test was used for multiple comparisons. Pearson's correlation analysis was used to determine associations. A p value of < 0.05 was considered statistically significant. Results have been reported according to current statistical nomenclature including *p*-values, degrees of freedom (df), F- and/or t- values.

3. Results

3.1. PD-L1 expression in response to inflammatory stimuli and VEGF by HUVECs is sexually dimorphic

Inflammatory stimuli regulate the expression of several immunoregulatory molecules in different endothelium models in a cell- and time-dependent manner, thus allowing fine tuning of the immune response [6,7,23,24]. Recent evidence shows that VEGF upregulates the expression of immune checkpoints in T lymphocytes [12], but whether this occurs in HUVECs remains unknown.

We first showed that pooled HUVECs (i.e. not differentiated by sex) expressed basal levels of PD-L1 as measured by Western blot that were significantly increased following 24-h treatment with LPS (10 ng/mL) and IL-1β (2 ng/mL), but not IL-6 (50 ng/mL), where a trend to an increase was observed (Fig. 1 A). Next, we measured PD-L1 levels in HUVECs stratified by sex. PD-L1 increased in HUVECs from female donors (fHUVECs) exposed to LPS, IL-1β and IL-6 as compared with baseline (Fig. 1B) but were unchanged in HUVECs from male donors mHUVECs (Fig. 1 C). Similar to what observed in response to inflammatory stimuli, VEGF increased PD-L1 levels with respect to baseline in fHUVECs (Fig. 2 A) but not in mHUVECs (Fig. 2B).

To further explore the mechanisms underlying the sex difference in PD-L1 expression in response to various stimuli, we assessed baseline PD-L1 levels. As shown in Fig. 3, the amount of basal PD-L1 was higher in mHUVECs than in fHUVECs, in line with a lower basal reactivity of the male immune system. Finally, we tested the time course of PD-L1 accumulation in fHUVEC in response to IL-1β and VEGF. After stimulation with 2 ng/mL IL-1β, PD-L1 accumulation in fHUVECs increased in a time-dependent manner (6–24 h), peaking at 24 h (Fig. 4 A). PD-L1 was also increased upon treatment with VEGF for 3–24 h (Fig. 4B), being statistically different with respect to controls already after 6 h.

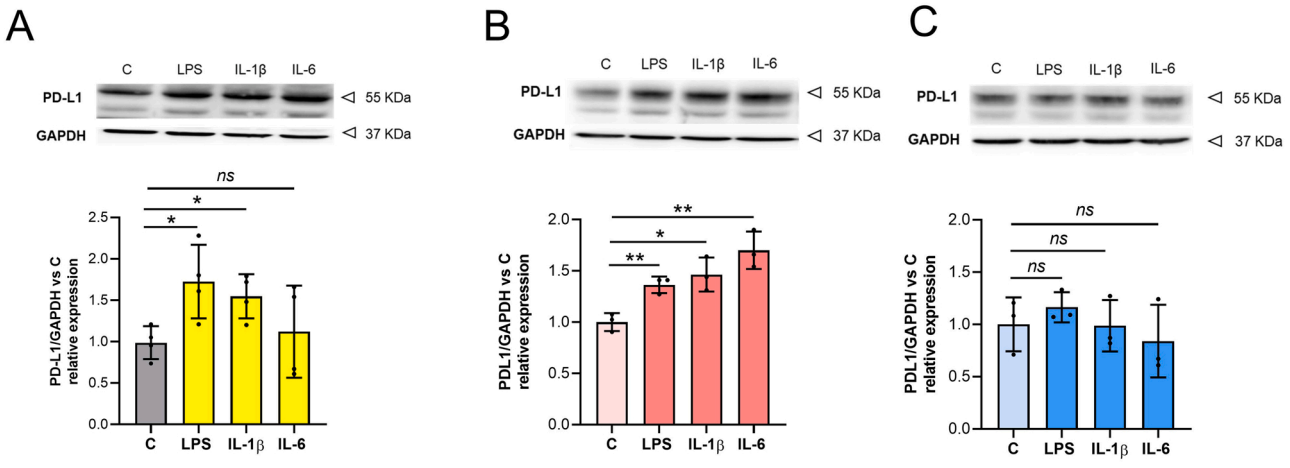

Fig. 1. PD-L1 levels in pooled, male or female HUVECs stimulated with proinflammatory mediators. HUVECs from pooled (i.e. not differentiated by sex, **A**), female (**B**) and male (**C**) donors (3 $\times 10^5$ cells) were seeded in 35-mm dishes in complete culture medium; the next day cells were treated with LPS (10 ng/mL), IL-1β (2 ng/mL), or IL-6 (50 ng/mL) in complete medium supplemented with 5% FBS for 24 h. *Upper panels*: Representative Western blots showing PD-L1 immunodetection; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. *Lower panels*: bar graphs show densitometric analysis of bands, normalized to GAPDH levels. Values in the control (C) group were set as 1 (relative expression). Data are expressed as mean ± SD. Panel **A**: $n = 4$ independent experiments, degrees of freedom (df)= 6, t > 3.032, *$p < 0.05$; panel **B**: $n = 3$, df= 4, t > 4.269, * * $p < 0.01$; panel **C** $n = 3$: df= 4, t < 0.9574, *ns*, nonsignificant; *t*-test.

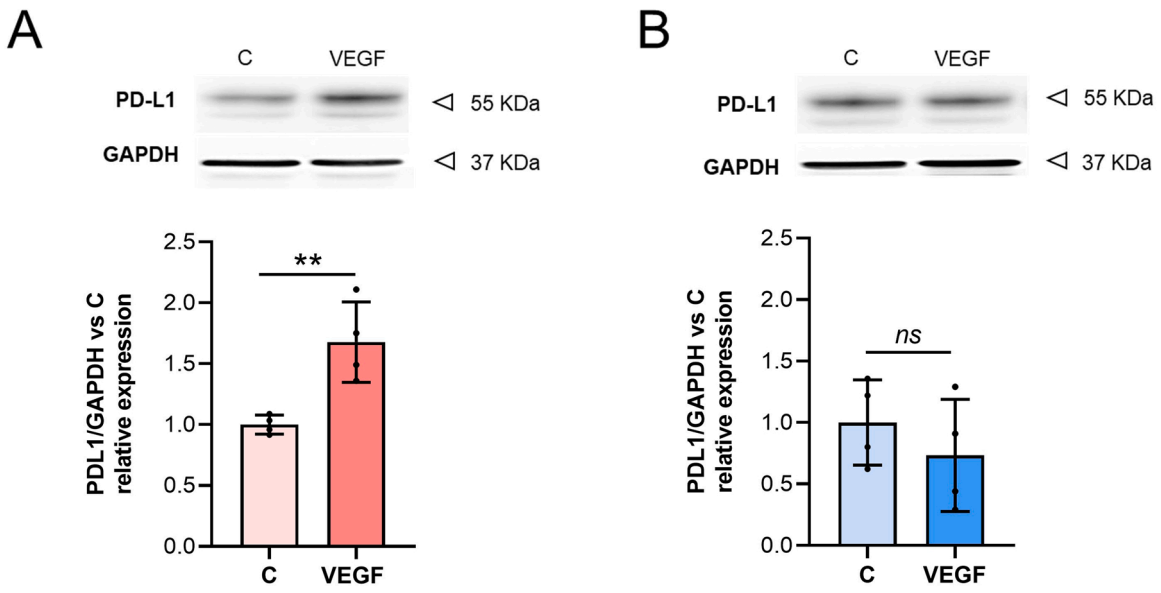

Fig. 2. PD-L1 levels in HUVECs from male and female donors exposed to VEGF. HUVECs (3×10^5 cells) from female (**A**) and male (**B**) donors were seeded in 35-mm dishes in complete culture medium; the next day cells were treated with VEGF (50 ng/mL) in complete medium supplemented with 5% FBS for 24 h. *Upper panels*: Representative Western blot showing PD-L1 immunodetection; GAPDH was used as a loading control. *Lower panels*: Bar graph shows densitometric analysis of bands, normalized to GAPDH levels. Values in the control (C) group were set as 1 (relative expression). Data are expressed as mean ± SD. Panel **A**: $n = 4$ independent experiments, degrees of freedom (df)= 6, t = 3.988, * $*p < 0.01$; panel **B**: $n = 4$, df= 6, t = 0.9348, *ns*, nonsignificant; *t*-test.

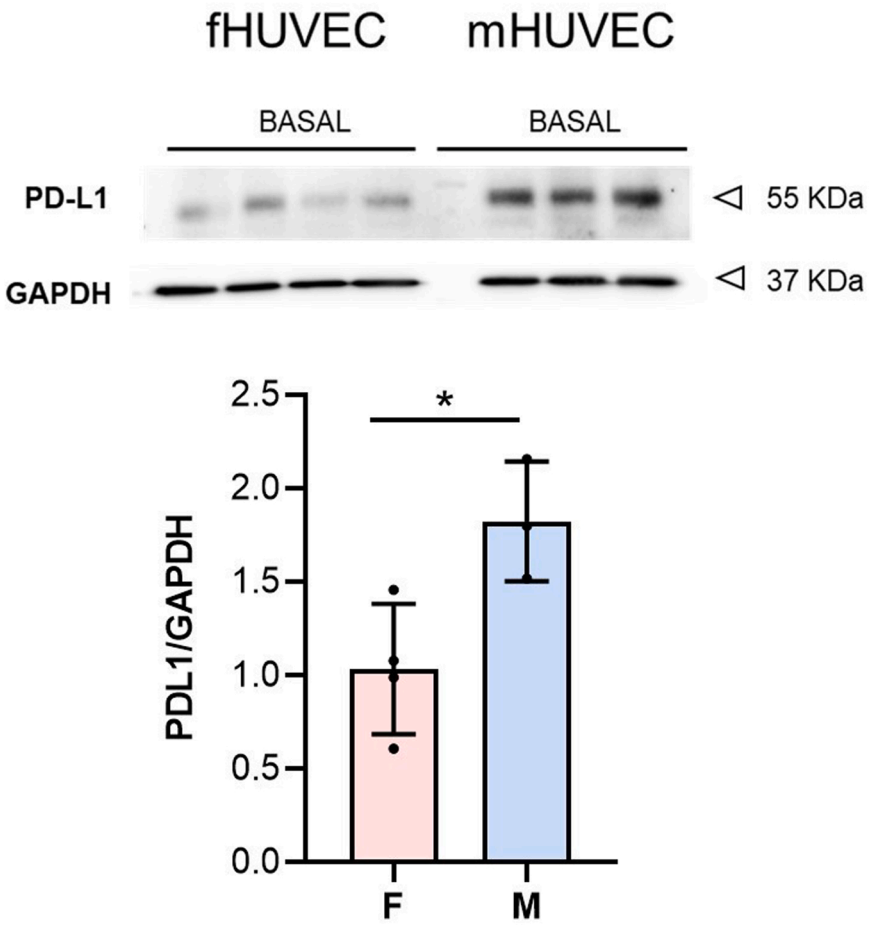

Fig. 3. Gender differences in baseline PD-L1 levels. HUVECs from female (fHUVECs) and male (mHUVECs) donors (both 3×10^5 cells) were seeded in 35-mm dishes in complete culture medium; the next day medium was changed to complete medium with 5% FBS for 24 h. *Upper panel*: Representative Western blot showing PD-L1 immunodetection; GAPDH was used as a loading control. *Lower panel*: The bar graph shows densitometric analysis of bands, normalized to GAPDH levels. Data are expressed as mean ± SD of $n = 3$ male (M) and 4 female (F) donors. Df= 5, t = 3.067, $*p < 0.05$, *t*-test.

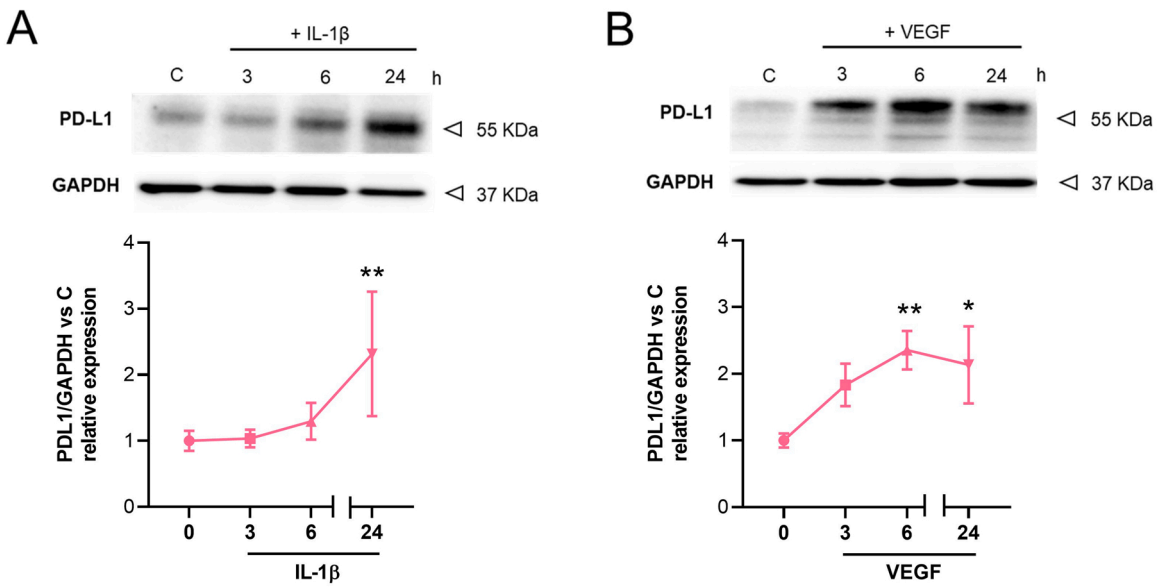

Fig. 4. Time course of PD-L1 expression in HUVECs from female donors exposed to IL-1β and VEGF. A. HUVECs (3×10^5 cells) from female donors were seeded in 35-mm dishes in complete culture medium and treated with 2 ng/mL IL-1β (**A**) or 50 ng/mL VEGF (**B**) in complete medium supplemented with 5% FBS for 3–24 h. *Upper panels*: Representative Western blot showing PD-L1 immunodetection; GAPDH was used as a loading control. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels. Baseline values were set as 1 (relative expression). Data are expressed as mean ± SD. Panel **A**: $n = 5$ independent experiments, df= 19, F= 7.586, * *$p < 0.01$ vs control (C); panel **B**: $n = 3$, df= 11, F= 7.971, * * $p < 0.01$, *p < 0.05 vs control (C); one-way ANOVA, Tukey's *post hoc* test.

Overall, PD-L1 expression was increased in response to inflammatory stimuli (LPS, IL-1β, IL-6) and VEGF in a sex-specific manner, suggesting a feedback compensatory mechanism to control the greater immune reactivity that makes women more prone to autoimmune diseases.

3.2. Membrane PD-L1 expression was unchanged in response to inflammatory stimuli

PD-L1 located in the membrane (mPD-L1) of ECs binds to the PD-1 receptor, which is highly expressed in immune cells including monocytes. This interaction delivers immunosuppressive signals that control inflammation and prevent immune-mediated tissue damage [5,25].

We assessed mPD-L1 expression by flow cytometry in pooled HUVECs challenged with IL-1β (2 ng/mL) or IFN-γ (1000 IU/mL), a recognized trigger of mPD-L1 expression in ECs [7,24]. Unexpectedly, mPD-L1 levels were unchanged after exposure to IFN-γ or IL-1β for 24 h as compared with basal levels (Fig. 5). We then investigated potential gender differences in mPD-L1 levels at baseline (*i.e.*, in the absence of any stimulus). In line with what observed for baseline PD-L1 levels, mPD-L1 was detected in unstimulated cells, with a trend to higher levels in mHUVECs versus fHUVECs (Supplementary Figure 1).

Combined with those of Figs. 1–4, these results suggest that PD-L1 shuttles between the intracellular compartment and the cell membrane, raising the hypothesis that it is rapidly released into the medium in a soluble form.

3.3. Soluble PD-L1 is released under inflammatory conditions and in response to VEGF in a sex-specific manner

Soluble forms of PD-L1 are detected in plasma from patients with chronic inflammation, and there is evidence that it can be generated by proteolysis of membrane-bound protein [15,19]. However, the role of endothelium in sPD-L1 production remains unexplored.

We investigated whether IL-1β and VEGF treatment triggered PD-L1 release from ECs. Supernatants of HUVECs differentiated by sex were assayed for sPD-L1 concentration using ELISA kits. sPD-L1 levels increased upon treatment of fHUVECs with both IL-1β and VEGF for 24 h (Fig. 6 A). Conversely, sPD-L1 released by both IL-1β- and VEGF-stimulated mHUVECs was unchanged with respect to control (Fig. 6 A). sPD-L1 released by IL-1β- and VEGF-stimulated fHUVECs increased in a time-dependent manner, reaching statistical significance compared to unstimulated cells at 24 h (Fig. 6B).

Overall, these data support the hypothesis that ECs are a source of sPD-L1 and may therefore behave as an immunological barrier.

3.4. Modulation of PD-L1 trafficking by protease inhibitors and anti-VEGF agents

sPD-L1 can be generated at least in part by proteolysis of mPD-L1 via metalloproteinases (MMPs), whose activity is increased in response to inflammatory cytokines or controlled by anti-inflammatory agents in various cell types including ECs [26].

To assess whether sPD-L1 release by ECs involved MMPs, fHUVECs were treated with 2 ng/mL IL-1β in the presence or absence of the MMP inhibitor GM6001 (10 μM) for 3–24 h. The time course of PD-L1 accumulation as evaluated by Western blot was not affected by this compound (Fig. 7 A). However, GM6001 significantly reduced the cumulative IL-1β-induced increase in sPD-L1 after 24 h (Fig. 7B), suggesting that proteolytic cleavage of membrane PD-L1 occurred over time in an inflammatory microenvironment. To provide further evidence of MMP involvement in the release of sPD-L1 from membrane-bound protein, we challenged fHUVECs with IL-1β in the presence or absence of GM6001 (10 μM) and measured mPD-L1 by flow cytometry. As shown in Fig. 7 C, cells pretreated with the MMP inhibitor did express higher PD-L1 levels with respect to untreated cells. Subsequently, to better characterize the immunoregulatory mechanisms of VEGF, we investigated the effect of anti-VEGF agents on PD-L1 expression and release. VEGF-induced PD-L1 accumulation as well as sPD-L1 release were inhibited by pretreatment with either 6.7 μM bevacizumab (Fig. 8 A, B) or 0.1–2 μM sunitinib (Fig. 8 C, D), respectively. Sunitinib treatment under these conditions did not affect HUVEC viability (Supplemental Fig. 2).

Collectively, these data show that PD-L1 was increased and rapidly released by fHUVECs in response to various stimuli, suggesting that VEGF signaling plays an immunomodulatory role in the endothelium.

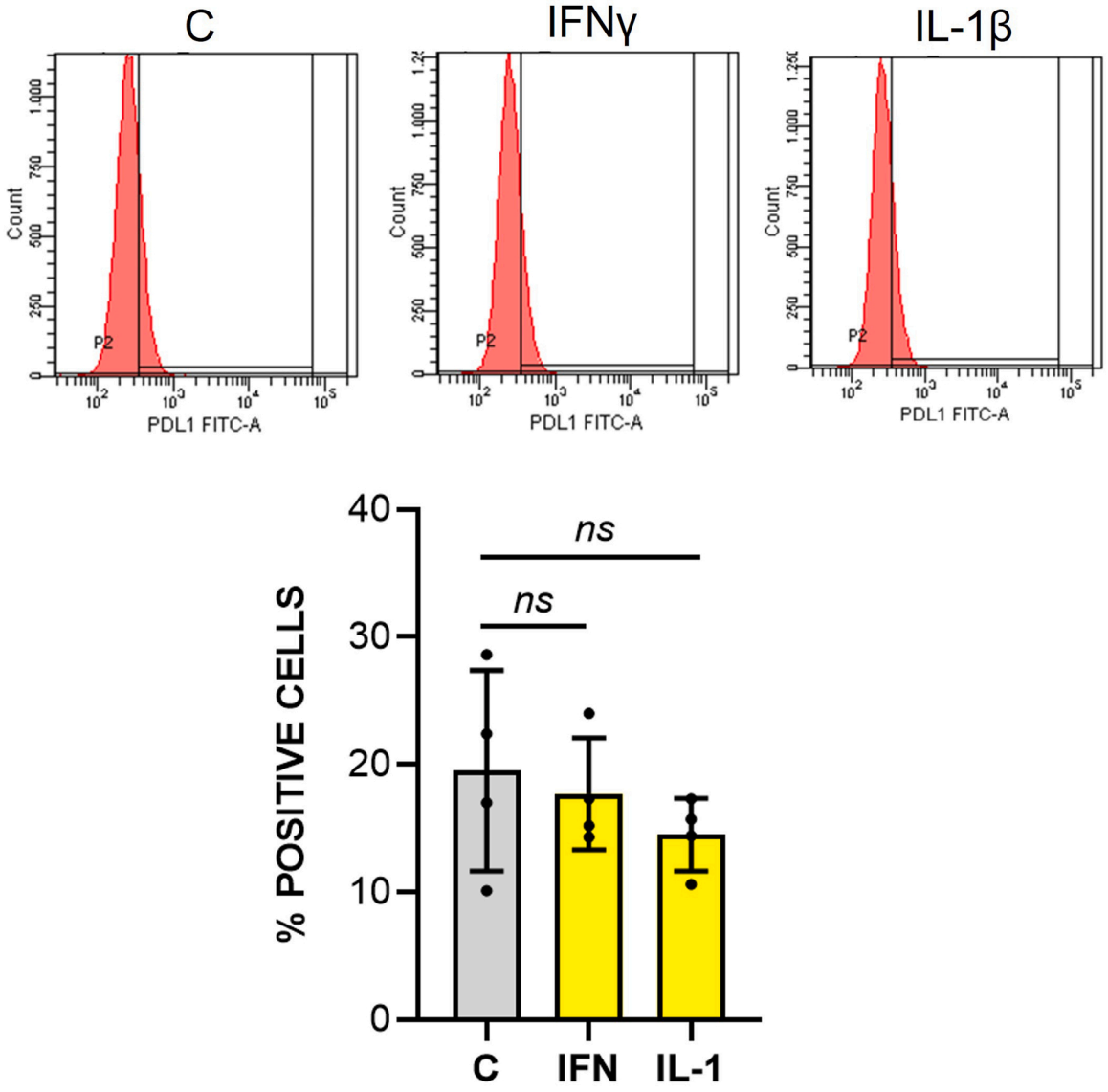

Fig. 5. Membrane PD-L1 expression in response to inflammatory stimuli. HUVECs (3×10^5 cells) were seeded in 35-mm dishes in complete culture medium; the next day cells were treated with IFN-γ (1000 IU/mL) or IL-1β (2 ng/mL) for 24 h in complete medium supplemented with 5% FBS and analyzed by flow cytometry as described in Materials and Methods. C=control. *Upper panel*: Representative flow cytometry analysis including isotype control. *Lower panel*: Bar graph showing levels percent of mPD-L1-expressing (positive) cells. Data are mean ± SD of $n = 4$ independent experiments. Df= 6, t = <1.200, *ns*, nonsignificant; *t*-test.

3.5. Synovial fluids from arthritis patients increased PD-L1 levels and were a source of sPD-L1

We previously showed that SFs from patients with inflammatory arthritis contain higher levels of inflammatory cytokines including IL-1β and IL-6 compared with SFs from OA patients [14].

Therefore, to mimic an inflammatory microenvironment *in vivo*, we exposed HUVECs to SFs from RA or PsA patients. Consistent with the sexually dimorphic PD-L1 accumulation observed in the presence of single inflammatory cytokines, PD-L1 accumulation increased in fHUVECs but not in mHUVECs (Fig. 9 A, B). We next measured sPD-L1 concentration in SFs from RA, PsA and OA patients, and found that sPD-L1 levels were higher in SFs from RA and PsA with respect to those from OA patients. sPD-L1 levels in inflamed SFs were about 10-fold higher with respect to those in IL-1β-stimulated ECs, suggesting that cells other than ECs contribute to its release into the inflammatory microenvironment. Accordingly, we also found a positive correlation between sPD-L1 levels and WBC count (Fig. 9 C), suggesting a potential role for sPD-L1 as a marker of inflammatory status.

4. Discussion

Due to their location, ECs interact with immune cells in the circulation and may actively participate in the regulation of immune responses through specific immune checkpoints such as PD-L1 [2,27]. The immunoregulatory properties of ECs are strictly dependent on the microenvironment. Previous work showed that in the context of inflammation PD-L1 is induced as a suppressive signal on different cell types including ECs [24,28,29]. In this study, we provide evidence that PD-L1 expression in human ECs increased in response not only to inflammatory stimuli but also to VEGF. We also highlight for the first time that ECs express and release a soluble form of PD-L1 in a sex-dependent manner via an MMP-dependent mechanism that may be controlled by anti-VEGF agents.

Gender differences can be identified at multiple levels of the immune response [30]. We first considered total PD-L1 levels and found that, in response to either LPS or inflammatory cytokines such as IL-1β and IL-6, PD-L1 was expressed in a sexually dimorphic manner, being enhanced only in HUVECs from female donors. Along with the difference in basal

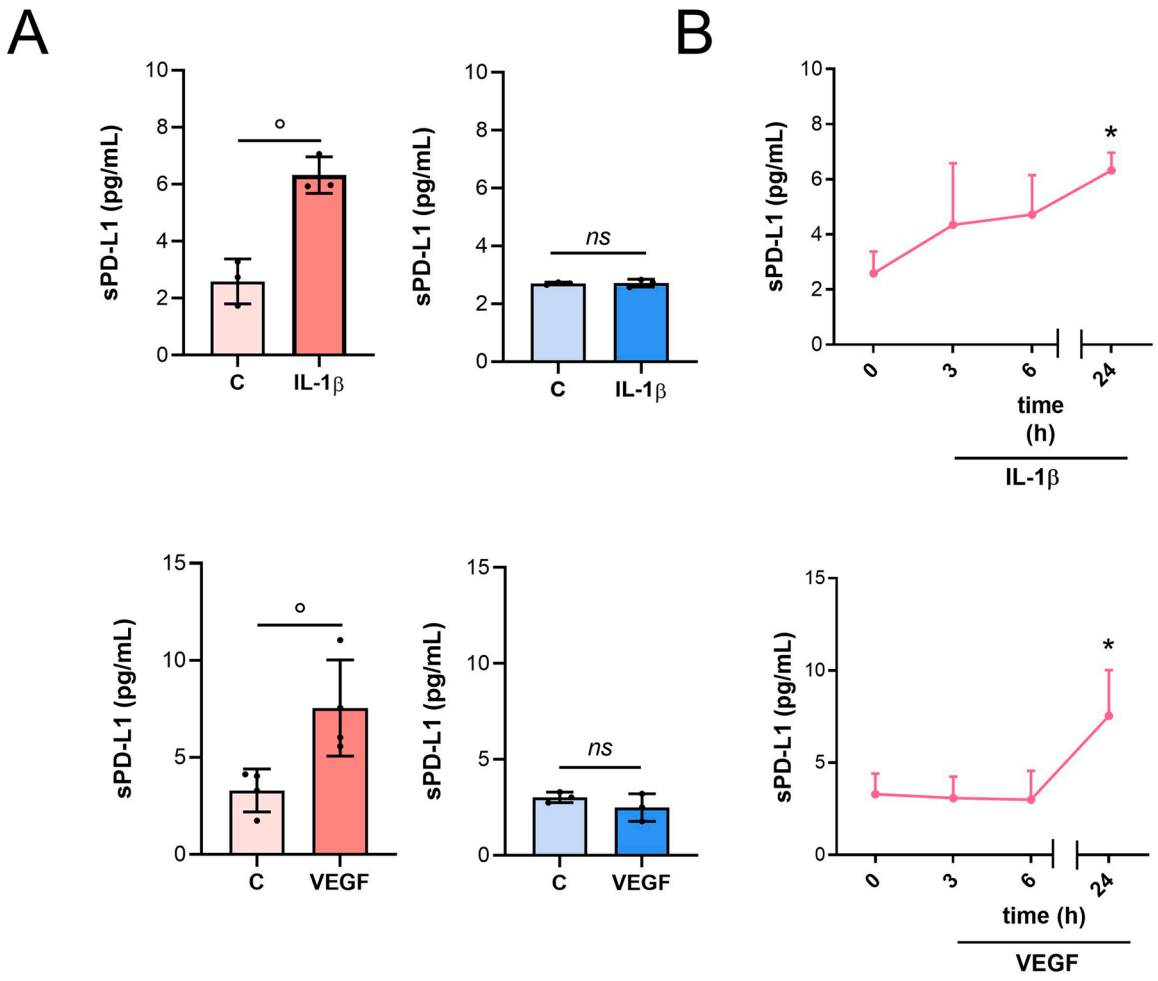

Fig. 6. IL-1β- and VEGF-stimulated PD-L1 release from HUVECs. HUVECs (3×10^5 cells) from male (blue) and female (pink) donors were seeded in 35-mm dishes in complete culture medium. Cells were then stimulated in 1 mL of complete medium supplemented with 5% FBS with 2 ng/mL IL-1β (*upper panels*) or 50 ng/mL VEGF (*lower panels*) for 24 h (**A**) or the indicated times (**B**). Cell culture supernatants were collected and assayed for sPD-L1 using ELISA kits as described in Materials and Methods. (**B**) Curves show the concentration of sPD-L1 (pg/mL) released by HUVECs from female donors upon treatment with IL-1β *(upper panels)* or VEGF *(lower panels)* at different time points as indicated. Data are expressed as mean ± SD. Panel **A**: female IL-1β *n=3* independent experiments, *df=4, t= 6.370,* °$p < 0.05$; female VEGF *n=4, df=6, t=3.131,* °$p<0.05$; male *n=3, df= 4, t< 1.195, ns,* nonsignificant; *t*-test. Panel **B**: IL-1β *n*=3, df= 11, F= 3.497, * $p < 0.05$ vs baseline (t_0); VEGF *n*=4, df= 15, F=7.043, *$p<0.05$ vs baseline (t_0); one-way ANOVA, Tukey's post-hoc test.

PD-L1 expression, which turned out to be higher in HUVECs from male donors, our findings may contribute to explain the donor variability in PD-L1 protein amount reported by Mazanet and Hughes [24]. In addition, upregulation of PD-L1 levels by IL-1β and IL-6 in ECs is in line with previous observations in cancer cells, and supports the use of combined therapies with anti-cytokine agents [31–33]. ECs may contribute to sex differences in the immune response under physiological as well as pathological conditions [30,34]; therefore, it is conceivable that EC PD-L1 expressed over time in response to various inflammatory stimuli provides a feedback compensatory mechanism to control an excessive immune response in female. Several *in vivo* studies support the view that EC PD-L1 is induced as an immunosuppressive signal. In particular, Grabie and colleagues [35] showed that EC PD-L1 has an important role in protecting the heart from immune-mediated inflammatory injury in a murine model of T lymphocyte-mediated myocarditis. More recently, PD-L1 of endothelial origin has been shown to be inversely related with the infiltration of graft infiltrating leukocytes in heart transplant patients undergoing surveillance endomyocardial biopsies [8]. In the context of cancer, the overexpression of EC PD-L1 restricts T cell infiltration within tumors, thus contributing to create an immunosuppressive barrier [11]. However, the functional role of EC PD-L1 *in vivo* should be investigated from a gender perspective in future studies.

VEGF plays and important role in the regulation of EC functions, including angiogenesis. We and others have previously shown sex-differences in the angiogenic response of ECs exposed to VEGF and other growth factors or cytokines [14,36,37]. In addition, VEGF exerts immunosuppressive functions [12,38], and a positive correlation between VEGF and PD-L1 expression has been described in cancer cells [38]. We herein report that VEGF induced rapid and sustained increase of PD-L1 accumulation in female HUVECs only, an effect that was reversed by antiangiogenic agents such as bevacizumab and sunitinib. These data reveal a previously unexplored immunomodulatory effect of VEGF, and support combined targeting of angiogenic and PD-L1 pathways to enhance anticancer immunity [13]. Hence, the set of agents that turn female ECs into an immune suppressive barrier by inducing PD-L1 include VEGF, which likely synergizes with inducible cytokines released by inflamed ECs to dampen blood vessel inflammation or, more generally, to control blood vessel function [39]. Considering that EC PD-L1 has been shown to promote angiogenesis [40], the sexual dimorphism in PD-L1 accumulation reported in our study might also account, at least in part, for the well-recognized higher angiogenic potential of female *vs* male endothelium [14,36,37]. In the setting of cancer, VEGF-induced EC PD-L1 expression may contribute to shape a sexually dimorphic tumor microenvironment. Accordingly, Conforti et al. [41] recently showed

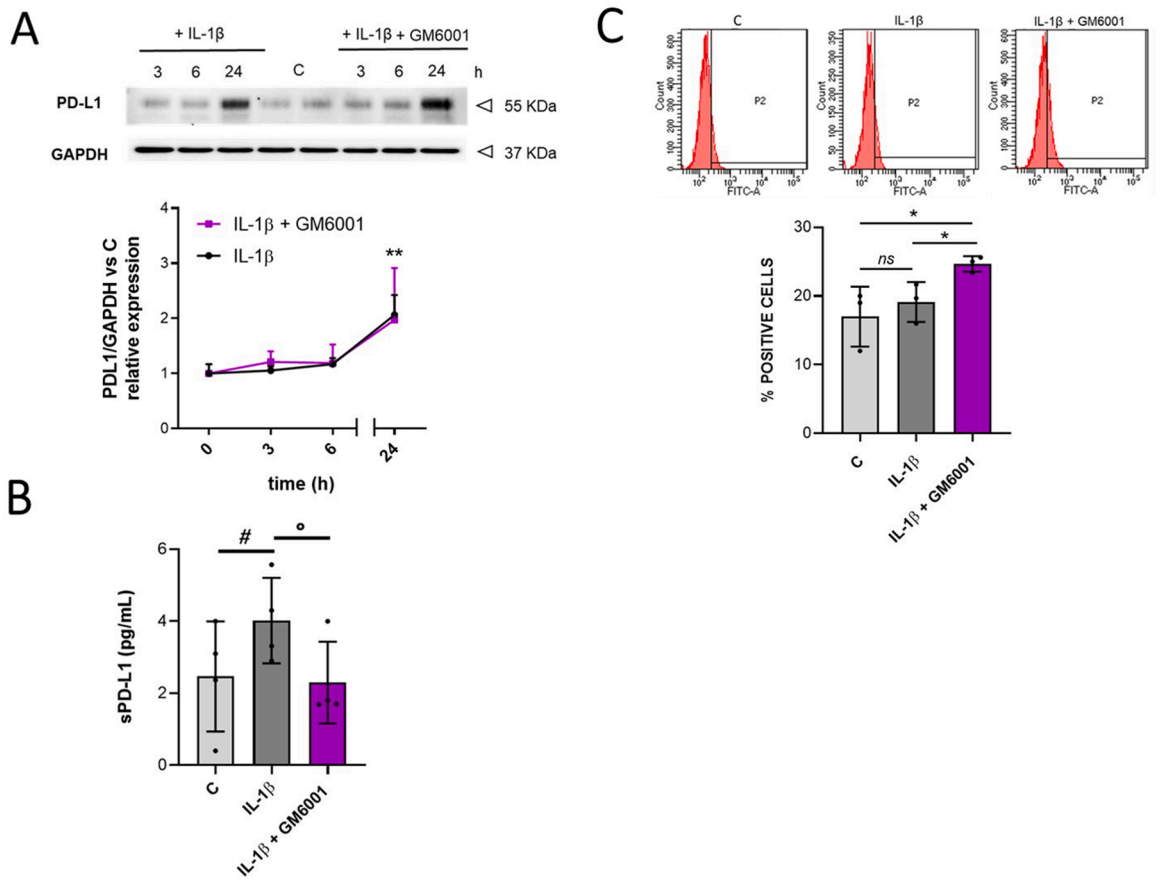

Fig. 7. Modulation of PD-L1 trafficking by protease inhibitors in HUVECs from female donors. HUVECs from female donors (3×10^5 cells) were seeded in 35-mm dishes in complete culture medium. Cells were then stimulated with IL-1β (2 ng/mL) in 1 mL of complete medium supplemented with 5% FBS in the presence or absence of the MMP inhibitor GM6001 (10 μM) added 30 min before the stimulus. Cells were harvested after 24 h and analyzed by Western blot (**A**) or flow cytometry (**C**) as described in Materials and Methods. **A.** *Upper panel*: Representative Western blot showing PD-L1 immunodetection; GAPDH was used as a loading control. *Lower panel*: Densitometric analysis of bands, normalized to GAPDH levels; baseline values were set as 1 (relative expression). Data are expressed as mean ± SD of $n = 3$ independent experiment**.** **A**; time factor dfn/dfd= 3/16, F= 8352, * * $p < 0{,}01$; treatment factor/dfd= 1/16, F= 0.01744, nonsignificant, *ns*; two-way ANOVA. **B.** Cell culture supernatants were collected after 24 h and analyzed for sPD-L1 concentration using an ELISA kit. C, control. Data are expressed as mean ± SD of $n = 4$ independent experiments; df= 6, $t > 2.453$, # $p < 0.05$ *vs* C, °$p < 0.05$ *vs* IL-1β; *t*-test. **C.** *Upper panel*: Representative flow cytometry analysis including isotype control. *Lower panels*: Bar graph showing percent of mPD-L1-expressing (positive) cells. Data are mean ± SD of $n = 3$ independent experiments; df= 4, $t > 2.948$, * $p < 0.05$; $t = 0.70$, *ns*, nonsignificant; *t*-test.

higher expression of immune checkpoints including PD-L1 in stromal cells from female tumor samples, although the specific cell type has not been characterized. Our data suggest a role for ECs in sex-based differences in the anticancer immune response and immune evasion mechanisms. However, whether the present findings are related to the reported male advantage and to sex-specific cardiovascular adverse effects in response to treatment with immune checkpoint inhibitors [42–45] requires further investigation.

Although several lines of evidence suggest a functional role for intracellular/nuclear PD-L1, this protein is generally considered to be a surface-expressed molecule. Indeed, PD-L1 can recycle from the cytoplasm to the cell membrane, where it exerts immune-suppressive functions by interacting with PD-1 on immune cells [31]. Unexpectedly and in contrast to the literature, we found that membrane PD-L1 levels did not change following exposure to inflammatory stimuli in HUVECs from both female and male donors. To address this discrepancy, we tested the hypothesis that membrane PD-L1 would be rapidly released as a soluble form through the action of MMPs, whose activity is known to be enhanced by inflammatory cytokines and VEGF [46,47]. We first observed a time-dependent increase in sPD-L1 levels in the medium of cells exposed to either IL-1β or VEGF, suggesting that intracellular PD-L1 can be recycled from endosomes to the cell membrane and then rapidly released into the culture medium. As observed for total PD-L1, sPD-L1 levels increased only in HUVECs from female donors. We also observed that the broad-spectrum MMP inhibitor GM6001 reduced sPD-L1 release from fHUVECs, while increasing mPD-L1 in IL-1β-treated cells. Although we cannot exclude that sPD-L1 in cell culture supernatants was generated from PD-L1 on the surface of exosomes or by alternative splicing of PD-L1 pre-mRNA, our data point to MMPs as major determinants of sPD-L1 release by ECs [15]. To the best of our knowledge, this is the first evidence that ECs are a source of sPD-L1. Considering that ECs cover a surface area of 1000 square meters [48], if confirmed in models of mature endothelium including coronary or aortic ECs, the release of sPD-L1 through activated MMPs could represent a mechanism whereby the endothelium regulates the activation of the immune system in the circulation.

The exact biological function of sPD-L1 remains largely unknown, but it is broadly predicted to act as a negative regulatory signal [15]. Elevated sPD-L1 levels as those found in pregnant women sera play a role in suppressing the maternal immune reaction to alloantigen, placenta and fetus [17,49]. Although the placenta has been long considered the main source of sPD-L1 [50], our data suggest that the fetal endothelium (*i.e.* HUVECs) may also contribute to detectable sPD-L1 levels in pregnant women serum. sPD-L1 may represent a general marker of inflammatory status. Of note, higher serum sPD-L1 levels have been measured in patients with diabetes complicated with

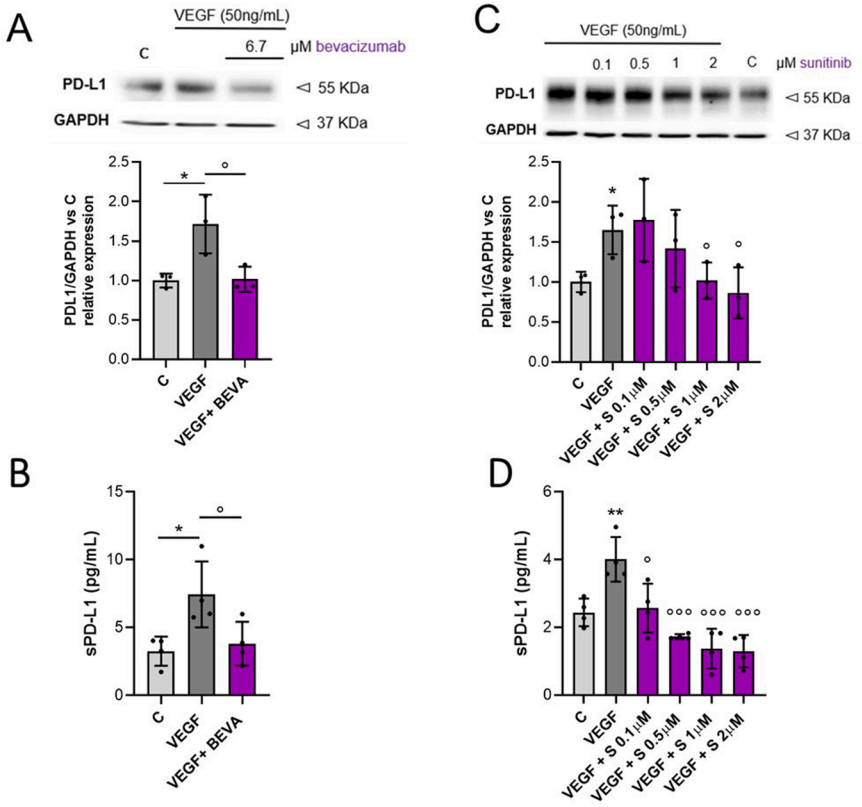

Fig. 8. Modulation of PD-L1 trafficking by anti-VEGF agents in HUVECs from female donors. HUVECs from female donors (3×10^5 cells) were seeded in 35-mm dishes in complete culture medium. Cells were then stimulated with VEGF (50 ng/mL) in complete medium supplemented with 5% FBS in the presence or absence of 6.7 μM bevacizumab (beva; **A, B**) or 0.1–2 μM sunitinib (S; **C, D**). **A, C:** Cells were harvested after 24 h and analyzed by Western blot. *Upper panels:* Representative Western blot showing PD-L1 immunodetection; GAPDH was used as a loading control. *Lower panels*: Densitometric analysis of bands, normalized to GAPDH levels, values in the control (C) group were set as 1 (relative expression). Data are expressed as mean ± SD of $n = 3$ independent experiments. Panel **A**: df= 4, t = 3.253, $^{*}p < 0.05$ *vs* C; t = 3.003, $^{\circ}p < 0.05$ *vs* VEGF; t-test. Panel **C**: df= 4:, t = 3.417, *p < 0.05 vs C; t > 2.902, °p < 0.05 vs VEGF; *t*-test. **B, D:** Cell culture supernatants were collected after 24 h and assayed for sPD-L1 concentration using an ELISA kit. Data are expressed as mean ± SD of $n = 4$ independent experiments. Panel **B**: df= 6, t = 3.153, $^{*}p < 0.05$ *vs* C; t = 2.493, $^{\circ}p < 0.05$ *vs* VEGF. Panel **D**: df= 6, t = 4.056, $^{**}p < 0.01$ *vs* C; t = 2.955, $^{\circ}p < 0.05$ *vs* VEGF; t > 5.980, $^{\circ\circ\circ}p < 0.001$ vs VEGF; *t*-test.

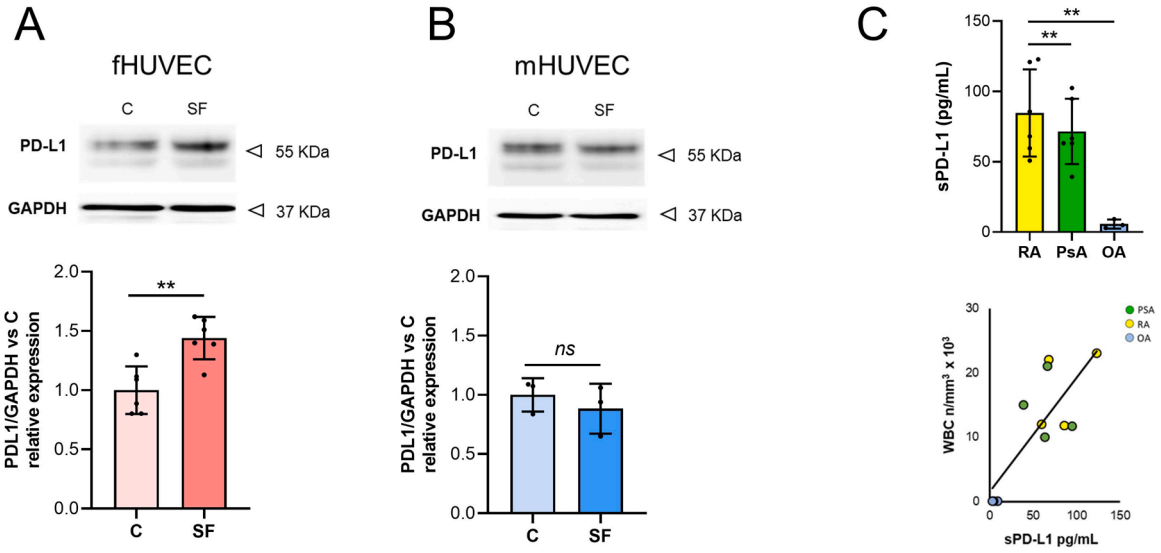

Fig. 9. PD-L1 accumulation in HUVECs exposed to synovial fluids from patients with inflammatory arthritis. HUVECs (3×10^5 cells) from female (**A**) and male (**B**) donors were seeded in 35-mm dishes in complete culture medium, and exposed to synovial fluid (SF) from patients with rheumatoid arthritis (RA) or psoriatic arthritis (PsA) in M199 medium supplemented with 100 IU/mL heparin for 24 h. **A, B.** *Upper panels*: Representative Western blot showing PD-L1 immunodetection in fHUVEC and mHUVEC, respectively; GAPDH was used as a loading control. *Lower panels*: The bar graph shows densitometric analysis of bands, normalized to GAPDH levels. Values in the control (C) group were set as 1 (relative expression). Data are expressed as mean ± SD. Panel **A**: n=6, df= 10, t=4.006, $^{**}p<0.01$; *t*-test; panel **B**: n=3, df= 4, t=0.7959, *ns*, nonsignificant; *t*-test. **C.** *Upper panel*: Concentration of sPD-L1 in SFs from RA ($n = 6$), PsA (n=6) and osteoarthritis (OA, $n = 3$) patients; df= 14, F= 10.44, $^{**}p< 0.01$; ANOVA, Tukey's post-hoc test. *Lower panel*: Correlation between WBC and sPD-L1 in SFs from RA, OA and PsA patients. The concentration of sPD-L1 was associated with WBC infiltration (n= 11, Pearson correlation coefficient = 0.8, $p<0.01$). WBC, white blood cells.

atherosclerotic disease compared to healthy subjects, possibly reflecting vascular inflammation [20]. More recently, Fujisue et al. [19] found increased sPD-L1 levels in patients with acute coronary syndrome. Since MMPs play a critical role in inflammatory processes including plaque rupture, it is conceivable that sPD-L1 is released by dysfunctional endothelium in this setting. In addition, serum sPD-L1 levels have been related to systemic inflammation in RA [21]. Accordingly, we found higher sPD-L1 levels in the synovial fluids of patients with RA and PsA with respect to those from OA patients [14]. Furthermore, when HUVECs were exposed to SFs from patients with RA or PSA, total PD-L1

levels increased in fHUVECs but not in mHUVECs, suggesting that ECs are a source of synovial sPD-L1 as well. Whether sPD-L1 is involved in the sexually dimorphic response to anti-PD-L1 immunotherapy remains to be clarified.

Among the limitations of the present study, experiments were carried out in HUVECs, thus the question remains whether the reported sex differences are restricted to this cell sub-type or other ECs show the same profile. Further, whether these gender differences are apparent in other cell types expressing PD-L1 remains to be explored. Finally, the relative contribution of sex hormones, namely estrogens, to the sexually dimorphic PD-L1 trafficking deserves to be explored. Few *in vitro* studies show that estrogen increases the expression of immune checkpoints including PD-L1 in cancer and immune cells [51,52]. In humans, Shen et al. showed that PD-L1 but not PD-1 is increased on $CD8^+$ T cells from premenopausal women and is selectively responsive to estradiol, suggesting that PD-L1 is involved in the differential regulation of female reproductive tract immune responses between pre- and postmenopausal women [53].

In conclusion, the present study expands knowledge on the sexual dimorphism of endothelial function and proposes ECs as a novel source of sPD-L1, which is released in a sex-dependent manner by proteolytic cleavage in response to cytokines as well as VEGF. These findings may pave the way to further analyses of sPD-L1 as a sex-specific biomarker with implications in (i) pathological conditions fostered by sustained inflammation such as atherosclerotic cardiovascular disease, rejection surveillance and inflammatory arthritis, and/or (ii) resistance to immune therapies.

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CRediT authorship contribution statement

Chiara Baggio: Methodology, Investigation, Formal analysis, Visualization. **Giovanni E. Ramaschi:** Methodology, Investigation, Visualization. **Francesca Oliviero:** Methodology, Resources. **Roberta Ramonda:** Methodology, Resources. **Paolo Sfriso:** Methodology, Resources. **Lucia Trevisi:** Methodology, Formal analysis, Visualization. **Andrea Cignarella:** Formal analysis, Writing – review & editing, Visualization, Funding acquisition. **Chiara Bolego:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Supervision, Project administration, Funding acquisition.

Conflict of interest statement

None.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114670.

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