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Propranolol reduces IFN-γ driven PD-L1 immunosuppression and improves anti-tumour immunity in ovarian cancer

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ABSTRACT

The immune system plays an important role in controlling epithelial ovarian cancer (EOC). EOC is considered to be a “cold tumour,” a tumour that has not triggered a strong response by the immune system. However, tumour infiltrating lymphocytes (TILs) and the expression of programmed cell death ligand (PD-L1) are used as prognostic indicators in EOC. Immunotherapy such as PD-(L)1 inhibitors have shown limited benefit in EOC. Since the immune system is affected by behavioural stress and the beta-adrenergic signalling pathway, this study aimed to explore the impact of propranolol (PRO), a beta-blocker, on anti-tumour immunity in both in vitro and in vivo EOC models.

Noradrenaline (NA), an adrenergic agonist, did not directly regulate PD-L1 expression but PD-L1 was significantly upregulated by IFN-γ in EOC cell lines. IFN-γ also increased PD-L1 on extracellular vesicles (EVs) released by ID8 cells. PRO significantly decreased IFN-γ levels in primary immune cells activated ex vivo and showed increased viability of the CD8+ cell population in an EV-immune cell co-incubation. In addition, PRO reverted PD-L1 upregulation and significantly decreased IL-10 levels in an immune-cancer cell co-culture. Chronic behavioural stress increased metastasis in mice while PRO monotherapy and the combo of PRO and PD-L1 inhibitor significantly decreased stress-induced metastasis. The combined therapy also reduced tumour weight compared to the cancer control group and induced anti-tumour T-cell responses with significant CD8 expression in tumour tissues. In conclusion, PRO showed a modulation of the cancer immune response by decreasing IFN-γ production and, in turn, IFN-γ-mediated PD-L1 overexpression. The combined therapy of PRO and PD-(L)1 inhibitor decreased metastasis and improved anti-tumour immunity offering a promising new therapy.

1. Introduction

Epithelial ovarian cancer (EOC) is the sixth most common cancer among women, with an estimation of 7300 women diagnosed each year in the UK (UK, 2020). Although recent advances in treatments and survival for other types of cancer, EOC remains one of the deadliest (Torre, 2018). In addition to the high mortality rate due to the advanced stage at diagnosis, patients suffer from severe symptom burden, enormous loss of quality of life, high levels of stress, and depressive symptoms (Bodurka-Bevers, 2000). Stress activates a series of biological responses acting on the hypothalamic-pituitary (HPA) axis and the sympathetic nervous system (SNS) (Habib et al., 2001). The biological stress effectors are hormones such as noradrenaline (NA) and cortisol, which can directly affect the tumour cells. There has been much interest in adrenergic stress mediated by NA binding the β2-adrenoceptor which are crucial in the modulation of tumour-related processes such as

Abbreviations: EOC, Epithelial Ovarian Cancer; SNS, Sympathetic Nervous System; NA, Noradrenaline; PRO, Propranolol; PD-1, Programmed cell Death protein 1; PD-L1, Programmed cell Death protein Ligand 1; IFN-γ, Interferon gamma; EVs, Extracellular vesicles; VEGF, Vascular Endothelial Growth Factor; TILs, Tumour Infiltrating Lymphocytes; IL-10, Interleukin 10; HPA, Hypothalamic-Pituitary Axis; TME, Tumour Microenvironment.

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Adrenergic stress has been shown to impact EOC in an orthotopic mouse model of ovarian cancer regulating the expression of proteins such as the vascular endothelial growth factor (VEGF) which is important for tumour growth and metastasis (Thaker, 2006). Furthermore, mice undergoing a chronic restraint stress procedure showed an increase in tumour burden, metastatic nodules, and angiogenesis (VEGF) (Thaker, 2006). Interestingly, the effects of an adrenergic stimulus were abrogated by using propranolol (PRO), a non-selective beta-blocker (Thaker, 2006). PRO also induces anti-tumour immunity in a model of spontaneous melanoma (Jean Wrobel, 2016).

Beta-blockers have shown potential in anti-cancer therapy in non-small-cell lung cancer (Wang, 2013), breast cancer (Hiller, 2020) and prostate cancer (Lu, 2015). A small retrospective study on ovarian cancer was carried out in 2012, observing that beta-blocker use influenced the survival rate. In particular, the progression-free survival was 27 months for beta-blocker users compared to 17 months for non-users (Diaz et al., 2012). The clinical impact of non-selective beta-blockers such as PRO was studied in a multi-centre review of patients with EOC. Patients receiving non-selective beta-blockers during chemotherapy had longer overall survival than non-users (Watkins, 2015). Additionally, a prospective clinical trial demonstrated the feasibility of adding PRO to EOC-directed chemotherapy, improved quality of life, and decreased inflammatory markers (Ramonetta, 2019).

The immune system is not only affected by stress hormones but also plays an important role in activating and suppressing mechanisms controlling EOC (Black, 1994). Although EOC, along with other solid tumours, can be termed a ‘cold cancer’ the trafficking of TILs into the tumour mass and the expression of the programmed cell death protein ligand 1 (PD-L1) are prognostic indicators in EOC (Santoiemma and Sood, 2010; Sood, 2006). TILs influence the pathogenesis and progression of the disease and correlate with the survival of ovarian cancer patients (Curiel, 2004; Zhang, 2003). High numbers of CD8+ cytotoxic T cells are associated with survival benefits and favourable outcomes, while infiltration by regulatory T cells (Tregs) predicts a reduced survival of EOC patients (Curiel, 2004; Sato, 2005).

Reciprocal interactions between cancer and immune cells create a local microenvironment that promotes tumour growth. Extracellular vesicles (EVs) such as exosomes, microvesicles, and large oncosomes are small (30 nm-10 μm) lipid ‘capsules’ that are involved in tumour-immune cell communication by shuttling signalling cargo and other molecules between cells (Wendler, 2017). These circulating EVs can transport important molecules such as PD-L1 to other areas of the body to prime metastatic niches in otherwise healthy tissue, which is ultimately detrimental to the patient. Cancer cells have been shown to use EVs-transmitted molecules to escape the immune system and T cell-derived exosomes containing microRNAs can suppress further immune responses Wendler et al., 2016.

Interferon (IFN)-γ is a complex signalling molecule with numerous roles underlying cancer immunology. Historically, with its anti-angiogenic, pro-apoptotic and immune-stimulating effects, IFN-γ has been considered a key regulator in anti-tumour immunity (Tao, 2001). Many clinical trials and immunotherapy approaches have been tested to strengthen IFN-γ-mediated immunity for cancer with limited success. The current literature suggesting the pro-tumourigenic effects of IFN-γ, and with its reported role of inducing PD-L1 expression on cancer cells highlights the complexity of this cytokine in the tumour microenvironment (TME) (Castro, 2018; Abiko, 2015; Jorgovanovic, 2020). Similarly, IL-10 dictates the T cell activation to either pro-tumour or anti-tumour phenotype (Challagundla, 2022).

The programmed death-ligand 1 (PD-L1) axis has been shown to play a pivotal role in cancer immunology. Programmed death-1 (PD-1) is a cell surface receptor which is involved in regulating T cell exhaustion. PD-1 binds to its ligand, programmed death-ligand 1 (PD-L1), activating downstream signalling pathways and inhibiting T cell activation. Increased PD-L1 expression on tumour cells and antigen-presenting cells in the TME facilitates tumour immune escape. PD-L1 is expressed in numerous cancers (Majidpoor and Mortezaee, 2021), and PD-1 is overexpressed in T cells presenting an exhausted phenotype (Tsai and Hsu, 2017). The molecular mechanism through which cancer cells escape the immune response can be due – among other processes - to PD-1/PD-L1 interaction which leads to immunosuppression (Abiko, 2015). PD-L1 expression on cancer cells can be induced by IFN-γ (Abiko, 2015; Bellucci, 2015; Mimura, 2018), suggesting that PD-1/PD-L1 signalling is inducible depending on the immune TME. The role of the PD-1/PD-L1 axis in cancer immunology has promoted a new branch of immune-based cancer therapies against PD-1 and PD-L1; a strategy to prevent immunosuppression and improve the immune response against cancer (Bellucci, 2015). However, knowledge of the molecular mechanisms at the basis of PD-L1 expression on cold cancers and the role of PD-1/PD-L1 interaction in shaping the TME is still deficient. Furthermore, unfortunately, there are numerous negative trials with PD-1/PD-L1 inhibitors in both the upfront and recurrent EOC setting (Matulonis, 2019; Moore, 2021). In this study, we explored the impact of adrenergic stress and its blockade with PRO on the expression of PD-L1 on ovarian cancer cells both in vitro and in vivo and examined PD-L1 immunotherapy in combination with propranolol on tumour burden, metastasis, and immunity.

2. Materials and methods

2.1. Cell culture

The mouse ID8 cell line was kindly provided by Dr. Premal Thaker, Washington University School of Medicine, St. Louis, USA. ID8 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) with 4 % Foetal Bovine Serum (Gibco) and a supplement of Insulin-Transferrin-Sodium Selenite (Sigma-Aldrich). Human ovarian cancer cell lines SKOV3ip1 and OVCAR8 were kindly donated by MD Anderson Cancer Center, Houston, USA. SKOV3ip1 cells were grown in McCoy’s 5a medium modified (Gibco) and 10 % Foetal Bovine Serum (Gibco). OVCAR8 was grown in RPMI 1640 medium with 10 % Foetal Bovine Serum (Gibco).

2.2. Splenocyte isolation

We used splenocytes as they reflect the immune system consisting of antigen-presenting cells, as well as B and T lymphocytes. In addition, the spleen is now viewed as the prominent site of extramedullary hematopoiesis in cancers (Wu et al., 2020). Furthermore, evidence suggests that the bone marrow, blood, spleen and draining lymph node form an immunological network in constant communication in the TME (Ham Galvez et al., 2021). Murine spleens were removed and processed into single-cell suspensions (Flint, 2005; Flint, 2011) and centrifuged with Red Blood Cell Lysis buffer. Cells were grown in RPMI supplemented with 10 % FBS and cell viability was assessed by trypan blue dye exclusion. Cells were 95–98 % viable for all experiments.

2.3. Cell and splenocyte treatments

Ovarian cancer cell lines were treated with 1 μM NA (Sigma Aldrich) or 1 μM PRO (Sigma Aldrich) either alone or 30 min before NA (PRO/NA) to efficiently inactivate the β-adrenergic receptors for 24 h. Cells were treated with recombinant IFN-γ (Peprotech) at a concentration of 20 ng/ml or lower concentrations depending on the experiment. Splenocytes were activated ex vivo with Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich at a concentration of 50 ng/ml) and Ionomycin (Sigma Aldrich at a concentration of 1 μg/ml) for 24 h. Treatments with NA (1 μM) or PRO (1 μM) plus NA were added to the activation mixture.
2.4. Trans-well co-culture

To avoid immune rejection, cancer cells and splenocytes derived from the same species and strain (C57BL/6 mouse) and splenocytes were co-cultured with ID8 cancer cells. Splenocytes were activated with PMA and Ionomycin with or without the addition of NA or PRO and NA. After 24 h, cells were harvested and centrifuged at 300 \( \times \) 10^3 g for 10 min. The media was saved for further analysis and cells were resuspended in fresh growth media and seeded in a 6-well plate with 2 \( \times \) 10^6 splenocytes per well. An insert with 8 \( \mu \)m pores (MERCK Millicell Cell Culture Insert) was placed in each well and 50,000 ID8 cells were seeded with 1 ml of ID8 complete growth media. Cells in co-culture were incubated at 37 \( ^\circ \) C, 5 % CO\(_2\) for 24 h.

2.5. EV separation

EVs were separated from conditioned media by differential ultracentrifugation. The conditioned media was centrifuged at 500V for 5 min, the pellet was discarded, and the supernatant was then centrifuged at 2000g for 20 min. The supernatant was retained and subsequently centrifuged at 10,000g for 30 min in an Optima LE 80-k ultracentrifuge, Type 70 Ti rotor, polypropylene centrifuge 25 \( \times \) 89 mm tubes, Beckman Coulter. The supernatant was filtered through a 0.2 \( \mu \)m pore filter and centrifuged at 100,000g for 90 min to pellet EVs. The final pellet was resuspended in 100 \( \mu \)L sterile PBS and centrifuged again at 100,000g for 90 min. The final pellet was resuspended in 100 \( \mu \)L sterile PBS and stored at -80 \( ^\circ \) C.

2.6. Nanoparticle tracking analysis (NTA)

EV fractions were analysed by NTA using the Nanosight NS300 (Malvern Instruments) and NTA 3.2 software. The instrument was set up with an sCMOS camera and a blue 488 laser. EVs were diluted 1:250 in PBS and characterised by NTA using the following parameters: Infusion rate: 70 AU, detection threshold: 5AU, screen gain: 1.0 AU and camera level: 16 AU.

2.7. EV culture with splenocytes

EVs from ID8 cells treated with NA, PRO or IFN-\( \gamma \) were incubated with splenocytes in triplicate at a concentration of 20 \( \mu \)g/mL. The cells and EVs were incubated for 72 h, and the immune cells were washed twice with PBS. The immune cells were then analysed using flow cytometry.

2.8. Western blot

Proteins were extracted by resuspension of cells in RIPA buffer. Samples were resolved on SDS-PAGE gels (12 % resolving and 4.5 % stacking) and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5 % BSA in TBST and incubated with the following primary antibodies; PD-L1 1:1000 (Abcam (Zhang, 2021) for band size), CD9 1:1000, CD81 1:1000, ALIX 1:1000, Calnexin 1: 1000, HSP70 1:1000, GM130 1:1000, GAPDH 1:1000 and ADRB2 1:1000 in TBST (Thermo Scientific, UK) and \( \beta \)-actin 1:1000 (Santa Cruz, USA) overnight at 4 \( ^\circ \) C. Membranes were subsequently washed in TBST.
and incubated with appropriate secondary antibodies (Anti-rabbit/mouse/goat, 1:2000, Cell Signalling) in TBST for 1 h at room temperature. The membranes were developed using the Amersham ECL Prime detection kit, which was prepared as per the manufacturer’s instructions and membranes were imaged using Image Studio Images were analysed using ImageJ software to determine the optical density of the bands.

2.9. ELISA

ABTS ELISA kit (PeproTech) was purchased together with an ABTS ELISA buffer kit (PeproTech). ELISA for the detection of IFN-γ and IL-10 was performed on immune cell-conditioned media. An ELISA was performed following the manufacturer’s protocols.

2.10. In vivo model

Six to eight weeks old female C57BL/6 mice were used for the studies. The animal room maintained a 12 h light–dark cycle (lights on at 6 am). Mice were housed in a noise-free environment and allowed to acclimatise for 1 week after transport. Mice were handled daily (for approximately 5 min/mouse) for 2 weeks before the studies. Food and tap water were provided ad libitum. All mouse experiments comply with the ARRIVE guidelines and were carried out by the U.K. Animals (Scientific Procedures) Act, 1986 (Workman, 2010).

C57BL/6 mice were injected with either 4x10^6 (for the syngeneic model validation) IP or 7 × 10^6 ID8 cells (for the therapy studies to account for the shortened time for the Alzet pump use) IP. An osmotic pump (Alzet) was surgically inserted in the back of animals to provide a constant release of PRO (Sigma Aldrich) 2 mg/Kg/d (Thaker, 2006) or vehicle (PBS). Animals underwent a restraint-stress procedure for 2 h per day (Flint, 2005; Flint, 2011; Hermann, 1993). PD-L1 inhibitor (200 μg/mouse) (Cambridge Bioscience) was administered with IP injections 3 times a week for 2 weeks (Bucsek, 2017). PD-L1 inhibitor was solubilized in DMSO and diluted in sterile PBS with DMSO not exceeding 5 ml/Kg or 10 % of the injected volume (Workman, 2010).

2.11. Real-time qPCR

RNA was extracted from cells using the RNeasy Mini kit (QIAGEN) following the manufacturer’s protocol and diluted in RNase-free water. Extracted RNA was quantified using a NanoDrop spectrophotometer set to read absorbance at 260 nm and 280 nm. RNase-free water was used as a reference for zero absorbance and 1 μL of RNA dilution was loaded. Purity was assessed with a 260/280 ratio; RNA was considered pure with a ratio ≈ 2. Tissues were harvested, stored in a freezing vial containing RNA later and flash frozen by submersion in liquid nitrogen. Each tissue was homogenized using pipette tips and syringe needles. RNA was extracted from 30 mg of tissue using the RNeasy Mini kit (QIAGEN) according to the manufacturer’s instructions and diluted in RNase-free water. cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer’s instructions. For real-time PCR assay, the Rotor-Gene SYBR green PCR kit (Qiagen) was used with bioinformatically validated primers (QuantiTect Primer Assay, Qiagen). The ΔΔC_T method was used to analyse the C_T values collected for the gene of interest and a housekeeping gene (Actin β) in each sample. The biological sample used for normalization is specified in each experiment.

2.12. Flow cytometry

Flow cytometry was performed on a single-cell suspension of splenocytes. 1 × 10^6 cells were re-suspended in PBS buffer 1 % BSA and the fluorescent labelled specific antibody in the appropriate dilution. Our antibody panel includes a cell viability dye 7-AAD-(PE)-Cy5 (Miltenyi Fig. 2. IFN-γ induced EVs carry PD-L1 and show decreased immune cell viability compared to PRO. ID8 cancer cells were treated with NA (1 μM), PRO (1 μM), PRO/NA (1 μM) and IFN-γ (20 ng/ml) alone or in combination. (A) Total EVs from ID8 cells were isolated and examined by NTA. (B) Cancer EVs were examined from each treatment group and compared to the control. (C) Proteins were extracted and PD-L1 and CD81, HSP70, CD9 and GAPDH were examined by western blot. (D) Co-incubation of EVs and splenocytes. EVs from ID8 cells treated with NA, PRO, PRO/NA or IFN-γ were cultured with splenocytes (20 μg/mL) in triplicate for 72 h. The immune cells were then analysed using flow cytometry. Data are shown as Mean ± SEM, and statistical significance was determined with a one-way ANOVA test (Post-test: Sidak). * = p < 0.05.
Biotech), anti-CD4-APC (Miltenyi Biotech) and anti-CD8-PE (Miltenyi Biotech). Cells were incubated with fluorescent antibodies at 4 °C for 20 min, then washed and re-suspended in 1 ml of buffer. Data were acquired from a cytoflex flow cytometer and analysed using FlowJo v10.

3. Results

3.1. PD-L1 mRNA expression increased following IFN-γ treatment in ovarian cancer cell lines

To explore the regulation of PD-L1, PD-L1 mRNA expression was assessed in different ovarian cancer cell lines by qPCR. Cells were treated with NA (1 μM), PRO (1 μM) alone or 30 min before NA (PRO/NA 1 μM) and recombinant IFN-γ (20 ng/ml) for 2, 6 or 24 h. In mouse ID8 cells, PD-L1 mRNA was not detected at 2 h in any group but was significantly increased in cells treated with IFN-γ compared to cells treated with NA or PRO/NA at 6 and 24 h (Fig. 1A). Furthermore, cells treated with NA or PRO in the presence of IFN-γ did not differ significantly from cells treated with IFN-γ alone. Neither NA nor NA/PRO significantly affected PD-L1 expression suggesting that they do not directly regulate PD-L1. In human SKOV3ip1 and OVCAR8 ovarian cancer cells, similar patterns of expression were observed with IFN-γ significantly inducing PD-L1 expression compared to NA or PRO/NA alone (p < 0.001 and p < 0.01, respectively Fig. 1B).
3.2. IFN-γ induced EVs carry PD-L1 and decreased immune cell viability compared to PRO.

We next determined if NA, PRO, PRO/NA or IFN-γ could: a) regulate the release of EVs from ID8 cells and b) if these EVs could carry PD-L1. Cancer EVs were isolated from ID8 cells treated with NA (1 μM), PRO (1 μM), NA/PRO (1 μM) and IFN-γ (20 ng/ml). NTA analysis confirmed that the isolated EVs were within the expected size range (~30 to 150 nm) (Supplementary Fig. 1 and Fig. 2 A/B). Although we observed that PRO slightly reduced the number of EVs produced, this result was not statistically significant (Fig. 2B). We also showed that these EVs carry the usual EV proteins ALIX but not the cellular marker calnexin (Supplementary Fig. 2). We extracted proteins from EVs concentrated fraction and further probed for positive exosome markers, CD81, HSP70 and CD9. We found that cell lysates expressed CD81, HSP70 and CD9 but they were enriched in the EVs compared to the lysate (Fig. 2C). We next examined PD-L1 and we found that PD-L1 was expressed in the cell lysates of all treatment groups but it was highly expressed in the IFN-γ group. Moreover, we found that only IFN-γ induced EVs carried significant amounts of PD-L1 (Fig. 2C). This suggests that, upon IFN-γ treatment, PD-L1 increased both in the cell lysate and in released EVs. To determine whether EVs carrying PD-L1 affected immune cell viability of different immune subsets; splenocytes were activated and co-incubated with EVs isolated from cancer cells treated with NA, PRO, PRO/NA or IFN-γ. EVs from cancer cells stimulated by IFN-γ induced a significant decrease in the viability of CD8+ cells (Fig. 2E) compared to PRO indicating increased immunosuppression. Moreover, NA alone also showed a significant decrease in the viability of CD8+ cells compared to PRO.

3.3. PRO reduced IFN-γ levels in PMA/IONO and NA-activated immune cells

To determine if NA or PRO/NA could regulate IFN-γ, we examined the effect of NA or PRO/NA on the release of IFN-γ by splenocytes. We first determined that the beta-2 adrenergic receptor (β2 adrenoceptor) was expressed on both ID8 cells and splenocytes (Supplementary Fig. 3). We examined the effect of NA and PRO/NA on the release of IFN-γ and IL-10 from control and splenocytes activated ex vivo with PMA and Ionomycin (Fig. 3). In immune cells activated for 3 h, the addition of NA to PMA and Ionomycin significantly increased levels of IFN-γ (p < 0.05) as expected, however, these levels were significantly reduced by PRO (Fig. 3A). At 24 h, activated splenocytes in the presence or absence of NA showed similar levels of IFN-γ (Fig. 3B, p < 0.001). The increase in IFN-γ was again reverted by the addition of PRO during immune cells’ activation (p < 0.001). Furthermore, low levels of the immunosuppressive cytokine IL-10 were detectable in the media, and levels did not change when the splenocytes were activated or treated with either NA or PRO/NA at 24 h (Fig. 3C).

3.4. PRO reduced levels of IL-10 and the expression of PD-L1 in cancer cells in a cancer-splenocyte co-culture

We next examined the release of IFN-γ in an immune-cancer cell co-culture. C57BL/6-derived ID8 cells only were used for these experiments because we could combine them with C57BL/6 mouse splenocytes without concerns of immune rejection. Mouse ID8 cells were co-cultured with splenocytes activated ex vivo with PMA and Ionomycin in the presence or absence of NA or NA/PRO for 24 h. IFN-γ levels were lower than activated splenocytes alone (below 50 pg/mL) and levels did not change significantly regardless of the treatments (Fig. 3D). However, in the cancer-splenocyte co-culture, IL-10 levels increased when immune cells were activated with PMA/IONO alone and in the presence of NA (p < 0.01, Fig. 3E) enabling a more immunosuppressive tumour environment. This upregulation of IL-10 was significantly reversed by PRO (p < 0.001). We also examined PD-L1 expression on ID8 cells in the co-culture model. There was a significant increase in PD-L1 expression on ID8 cells co-cultured with activated splenocytes and activated splenocytes + NA (p < 0.05); whereas PRO significantly reduced this increase (p < 0.05; Fig. 3F).

3.5. Syngeneic mouse model of ovarian cancer undergoing restraint stress

We next wanted to ascertain if PRO combined with a PD-(L)1 inhibitor decreases tumour burden and metastatic nodules. To achieve this, we elected to use a syngeneic mouse model of ovarian cancer using ID8 cells. We first assessed if 2 h daily restraint stress (RS) could increase tumour burden and metastasis in our syngeneic mouse model of ovarian cancer. C57BL/6 mice were injected with 4 × 10^6 ID8 cells. One week later, one group of mice (n = 10) was restrained daily for six weeks. Non-restrained mice (n = 10) remained in their home cages in a noise-free environment. After the RS protocol, mice were sacrificed to assess tumour burden and tumour weight by gross necropsy. No significant change in overall mouse weight was shown throughout the duration of the study (Fig. 4A). The restrained mice had significantly more metastatic tumours (liver, stomach, and peritoneum)/mouse than the non-stressed mice (p = 0.04, Fig. 4B) and a higher number of the stressed mice had metastatic tumours compared to non-stressed mice (p < 0.05, Fig. 4C). Whereas 40 % of non-stressed mice developed ascites, 50 % of the stressed mice did so (data not shown). These findings substantiate the use of syngeneic mice as a suitable model for studying in vivo stress-
Fig. 5. Combined therapy with PRO + PD-L1i decrease tumour burden and metastatic nodules. Mice (n = 8) were injected with 7x10^6 ID8 cells and randomised into one of the following groups: 1) Control; 2) Restraint stress (RS), 3) Restraint stress and PRO (RS + PRO); 4) Restraint stress and PD-L1 inhibitor (RS + PD-L1i) and 5) Restraint stress, PRO, and PD-L1 inhibitor (Combo). After 4 weeks mice were sacrificed. (A) Schematic view of treatments for each group. (B) Mouse weight was measured before surgery and every week after ID8 cell injection. (C) Primary tumours were weighed at the end of the study. (D) Total number of organs per mouse showing metastatic nodules were reported by gross necropsy. Data are shown as Mean ± SEM, and statistical significance was determined with a one-way ANOVA test (Post-test: Bonferroni). * = p < 0.05, ** = p < 0.01, *** = p < 0.001. Data are representative of 2 different in vivo experiments.
induced immune regulation in ovarian cancer.

3.6. Propranolol combined with PD-(L)1 inhibitor decreases tumour burden and metastatic nodules in a syngeneic mouse model of ovarian cancer

We next assessed if PRO combined with a PD-(L)1 inhibitor decreases tumour burden and metastatic nodules. Mice were injected with $7 \times 10^6$ ID8 cells (a higher number to account for the shortened time allowed for cancer progression due to the Azelut pump use) and randomised into one of the following groups: 1) Control; 2) Restraint stress (RS), 3) Restraint stress and PRO (RS + PRO); 4) Restraint stress and PD-L1 inhibitor (RS + PDL1i) and 5) Restraint stress, PRO, and PD-L1 inhibitor (Combo). PRO was administered via an Azelut pump 2 weeks before ID8 injections and restraint stress started a week before the tumour injection and lasted for 5 weeks (Fig. 5A). Mice initially demonstrated a weight gain, possibly due to the insertion of the Azelut pump ($p < 0.001$). At the end of the study, mice treated with the combined therapy of PRO and PDL1i had significantly lower weight compared to the control mice ($p < 0.05$, Fig. 5B). The combined treatment with PRO and PDL1i significantly reduced primary tumour weight in mice ($p < 0.001$, Fig. 5C). Mice subjected to restraint stress showed increased metastatic nodules ($p < 0.05$) correlating to what we had observed in Fig. 4B, and this was significantly decreased in groups treated with PRO alone ($p < 0.001$) or the combined therapy ($p < 0.001$, Fig. 5D Table 1).

3.7. Propranolol combined with PD-L1 inhibitor improves the anti-tumour immune signature in an orthotopic mouse model of ovarian cancer

Due to the small tumour size observed in our model, we used PCR to examine tumour immune cells rather than IHC. The immune cell subsets in the spleen of mice injected with ID8 cells showed that the combined therapy with PRO + PDL1i significantly decreased CD8+ cells resident in the spleen (Supplementary Fig. 4). Tumour tissues of mice injected with ID8 cells were examined by qPCR to show the mRNA expression of different immune markers. We made two comparisons; one to look at the effects of treatments compared to the cancer control, and another compared to restraint stress. We found that CD3 and CD4 were elevated in mice when subjected to restraint stress compared to controls ($p < 0.05$ and $p < 0.0001$) and in all treatment groups compared to controls and restraint stress (Fig. 6A & B). However, only mice treated with PD-L1 inhibitor ($p < 0.001$) and PRO and PD-L1 inhibitor ($p < 0.05$) showed significantly elevated CD4 compared to restraint stress only. Furthermore, only the combined therapy with PRO and PD-L1 inhibitor showed a significant increase in CD8 compared with controls ($p < 0.05$ Fig. 6C). Interestingly PD-L1 was higher in the PD-L1i groups compared to the restraint stress groups suggesting compensation for the inhibitor ($p < 0.01$, Fig. 6D). We also examined several immune-related markers such as VEGF, Granzyme B, CCL10 and IDO1 based on their previously reported roles in the TME (Supplementary Fig. 5). Although in our model, VEGF was elevated in both the PRO alone and PD-L1i groups ($p < 0.001$), VEGF was not significantly different when compared to restraint stress or controls (Supplementary Fig. 5A). Granzyme B was downregulated in the group treated with PRO alone compared to the control group ($p < 0.05$, Supplementary Fig. 5B). CCL10 and IDO1 were not significantly changed by restraint stress or with any treatments (Supplementary Fig. 5C and 5D).

4. Discussion

The data presented here suggest that Propranolol combined with PD-(L)1 inhibitor decreased tumour burden and metastatic nodules in a syngeneic mouse model of ovarian cancer. To begin to understand the mechanism, we first determined that NA does not directly regulate PD-L1. Furthermore, evidence in the literature showing that IFN-γ induces PD-L1 (Abiko, 2015; Bellucci, 2015; Mimura, 2018) and that immune cells are affected by adrenergic signalling (Chen, 2018) led us to hypothesise that NA has an indirect role in PD-L1 expression via the regulation of IFN-γ. Firstly, NA increases IFN-γ release, which may contribute to PD-L1 overexpression in cancer cells (Fig. 7A). This evidence might explain the inefficacy of PD-L1 inhibitor-based immunotherapy. Cancer cell-derived EVs contain PD-L1, which acts as an immunosuppressive switch on immune cells, as evidenced by an increase in IL-10 and a decrease in IFN-γ (Fig. 7B). This lends support to the idea that PRO can reduce PD-L1 overexpression by preventing immune cells from producing IFN-γ (Fig. 7C). We propose that PRO, by blocking the adrenergic mechanism, may prevent PD-L1 overexpression in ovarian cancer and PD-L1-mediated immunosuppression. Since PD-L1 has been shown to be down-regulated by PRO the use of a PRO/PD-L1 inhibitor combination may appear counterintuitive. However, PD-L1 may be intrinsically expressed in cancer cells and not just induced by IFN-γ leading to a more specific response to PD-L1 inhibitors (Shi, 2018). In our study, we did not assess the localization of PD-L1, which is known to be expressed on the surface of T and B cells, dendritic cells, and macrophages. As most immune cells can be regulated by adrenergic signalling and PRO can decrease PD-L1 on macrophages (Kokolus, 2018), further work may be warranted to understand where PD-L1 is regulated. Work in our laboratory will involve the use of patient-derived xenografts, whereby we can further characterise immune cells in ascites as described in (Gandhi, 2021). Although further investigation is needed, this study suggests that PRO might improve PD-L1 inhibitors’ effectiveness in ovarian cancer, as demonstrated by a reduced tumour and metastatic burden.

Although IFN-γ has been shown to have both tumour-promoting and inhibiting properties in cancer, the molecular relevance of the decrease in IFN-γ levels in the immune-tumour cell co-culture may be explained by the physical/EV contact between cancer and immune cells. It has been shown that PD-L1 can be released in EVs that increase in number after IFN-γ stimulation (Chen, 2018). Our data also suggest that IFN-γ stimulates ovarian ID8 cell-derived EVs to deliver PD-L1 which regulates CD8+ T cells, suppressing cytotoxic CD8+ T cell function and allowing for immune escape. Interestingly NA derived EVs were also immunosuppressive which could be attributed to other mechanisms.

The decrease in IFN-γ levels in activated immune cells treated with PRO is an important finding that supports the hypothesis of immune regulation of the beta-blocker. This is consistent with the fact that the synergistic effect of PRO with PD-L1 inhibition was previously shown in melanoma (Kokolus, 2018) and tested in patients with metastatic melanoma (Gandhi, 2021). PRO reduces PD-L1 on cancer cells that is expressed in response to the IFN-γ. This influences the regulation of the immune response to cancer and the cancer-immune escaping mediated by PD-L1.

Previous studies have shown that restraint stress increases tumour burden and metastasis in in vivo cancer models and this can be reduced by PRO (Thaker, 2006; Avraham, 2010). Our in vivo data suggests that a combined therapy using PRO and a PD-L1 inhibitor reduces tumour burden and metastatic spread. The reason behind a combined therapy resides in the assumption that PD-L1 can either be induced by IFN-γ or intrinsically expressed by cancer cells. With the combined therapy, PRO reduces IFN-γ dependent PD-L1 while PD-L1 inhibitors block the
Fig. 6. Combined therapy with Propranolol and PD-L1 inhibitor improved an anti-tumour immune signature in mouse primary tumour tissues. C57BL/6 mice were injected with $7 \times 10^6$ ID8 cells and randomised into one of the following groups: 1) Control; 2) Restraint stress (RS), 3) Restraint stress and PRO (RS + PRO); 4) Restraint stress and PD-L1 inhibitor (RS + PD-L1i) and 5) Restraint stress, PRO, and PD-L1 inhibitor (Combo). After 4 weeks mice were sacrificed. Tumours were homogenized and total RNA was extracted. Expressions of immune markers were assessed by qPCR. (A) Expression of CD3, (B) CD4, (C) CD8, (D) PD-L1. β-Actin was used as an endogenous control. Results are presented as relative quantification calculated using the $\Delta\Delta$Ct method normalised to control. Mean ± SEM expressed. Data were checked for normal distribution by the Shapiro-Wilk test. A one-way ANOVA or nonparametric test followed by a multiple comparison (Dunnett’s) was used to compare all columns. * Represents significant difference $^* = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$, $^{****} = p < 0.0001$. Data are representative of 2 in-vivo experiments; replicates are representative of different mice.
intrinsic PD-L1 mediated immunosuppression. Interestingly, although restraint stress alone did not increase VEGF, a key player in ovarian cancer metastasis, the combined therapy significantly reduced VEGF. It is now understood that VEGF also has a role in tumour immune infiltration. Pharmacologic inhibition of VEGF has been shown to diminish ovarian tumour endothelial Fast expression, causing a significant increase in the influx of CD8+ T cells, leading to a CD8-dependent tumour growth suppression (Motz, 2014). Previous findings showed that beta-adrenergic activation of the cAMP-PKA pathway is a major mechanism by which restraint stress can enhance tumour angiogenesis and promote malignant cell growth (Thaker, 2006). The combined therapy also affected the expression of specific immune markers in the tumour tissues suggesting modulation of the tumour signature towards an anti-tumour immune response. PRO shows promising potential as an anti-metastatic agent by reducing the number of metastatic nodules.

The adrenergic signalling pathway also influences the peripheral distribution of immune cells (Rogausch, 1999). For the complexity of multi-factorial effects, the role of restraint stress regulating tumour immunity cannot be easily interpreted. However, the analysis of the tumour immune milieu is the basis of diagnostic strategies used to determine the prognosis and predict the response to immunotherapy (Angell and Galon, 2013). One of the most important parameters used to assess the cancer immune signature is an assessment of the TILs populations. The ratio of CD4+/CD8+ cells in the tumour has a crucial prognostic value, especially in ovarian cancer (Pinto, 2018). Although we didn’t assess TILs by IHC here, due to the small size of the tumours, future work in our laboratory will involve the use of patient-derived xenografts whereby we can further characterise immune cells in ascites as described in (Almeida-Nunes, 2022). Nonetheless, our data show that CD8 expression was up-regulated when mice were treated with a combined therapy with PRO and PD-L1 inhibitor suggesting an increase in the CD8+ T cell infiltration in tumours of mice receiving the combined therapy. The immune cell subsets in the spleen of mice injected with ID8 cells showed that the combined therapy with PRO + PD-L1i significantly decreased CD8+ cells resident in the spleen. This suggests a mobilisation of CD8+ T cells from the spleen, presumably because of an altered chemokine expression. Indeed, the decreased abundance of T cell populations has also recently been suggested in cancer (Hiam-Galvez et al., 2021). Systemic changes and plasticity of the immune microenvironment in cancer have been described in breast cancer, and splenic remodelling was specifically characterized by increases in frequencies of neutrophils, eosinophils, and monocytes and decreased abundance of B and T cells (Allen, 2020). This supports the hypothesis of increased T-cell infiltration and a shift towards a CD8+ cytotoxic phenotype (CTLs). Interestingly, we did not see a correlation between the expression pattern of the gene encoding for granzyme B, a serine protease produced...
specifically by CTLs. In tumours of mice receiving the PRO monotherapy, the expression of granzyme B was significantly lower compared to the control indicating that PRO is not sufficient to restore the effector functions of the immune cells; hence, the addition of the immune checkpoint blockade is essential to restore the T cell cytotoxic function.

In summary, our data suggest that PRO decreases PD-L1 mediated immunosuppression and improves anti-tumour immune signature in ovarian cancer biology, paving the way for dual therapies in EOC patients.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability
Data will be made available on request.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.02.011.

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