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RESEARCH

Exosomal PD-L1 derived from hypoxia nasopharyngeal carcinoma cell exacerbates CD8$^+$ T cell suppression by promoting PD-L1 upregulation in macrophages

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Abstract

Immunotherapy targeting the programmed death ligand-1/programmed cell death protein-1 (PD-L1/PD-1) pathway exhibits limited effectiveness in individuals with recurrent and metastatic nasopharyngeal carcinoma (NPC). Recent studies have noted that hypoxia within the tumor microenvironment (TME) triggers intricate interplay, termed "hypoxia-induced exosome-mediated communication", between cancer cells and various immune cells. However, the role of hypoxia in modulating the immunosuppressive environment and its implications on the efficacy of immunotherapy in NPC remains poorly understood. In this study, we found hypoxia inducible factor-1 (HIF-1α) was positively associated with increased PD-L1 levels and decreased CD8$^+$ T cell infiltration, and correlated with a poor prognosis. Mechanistically, we demonstrated that hypoxia regulated the expression of PD-L1 in NPC cells and their exosomes by activating the binding of HIF-1α to the PD-L1 promoter. Meanwhile, using in vitro approaches, we found that macrophages could upregulate their PD-L1 expression through the phagocytosis of exosomal PD-L1 derived from NPC cells. Furthermore, we confirmed that PD-L1$^+$ macrophages could induce CD8$^+$ T cell exhaustion and reduce their proliferation. In conclusion, our study revealed that hypoxia (via HIF-1α) upregulated the expression of PD-L1 in exosomes derived from NPC cells, while macrophages induce the suppression of CD8$^+$ T cells by phagocytosis of exosomal PD-L1. Targeting the PD-L1$^+$ macrophages could potentially serve as a promising approach to augment the effectiveness of immune checkpoint blockade in NPC.

Keywords Nasopharyngeal carcinoma · Hypoxia · PD-L1 · Macrophage · Immune escape

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Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial squamous cell carcinoma originating from the nasopharyngeal mucosa, characterized by a significant infiltration of immune cells in the tumor microenvironment (TME) [1]. Metastasis and recurrence are the predominant contributors to mortality in NPC [2]. In the past decade, immune checkpoint blockade (ICBs), such as blocking programmed death ligand-1/programmed cell death protein-1 (PD-L1/PD-1) immune checkpoints, have achieved great success in many solid tumors [3]. PD-1/PD-L1 blockade therapy has been approved for the treatment of metastasis and recurrence NPC [4]. However, the therapeutic efficacy of PD-1/PD-L1 blockade therapy is limited by the TME, especially the tumor immune microenvironment (TIME), with only 20–30% objective response rates (ORR) among metastasis and recurrence NPC [5, 6]. Therefore, investigating the immune microenvironment of NPC can provide insights

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into immune evasion mechanisms and an effective strategy for the immunotherapy of NPC.

Hypoxia is prevalent in a majority of TMEs due to the uncontrolled growth and rapid proliferation of cancer cells [7]. Among the microenvironmental factors influencing neoplasia, hypoxia is considered one of the most significant in affecting the neoplastic response of tumor cells [8]. Several studies have implicated hypoxia-inducible factor 1α (HIF-1α) can upregulate PD-L1 expression in tumor cells [9–11]. However, the potential for hypoxia to induce the upregulation of PD-L1 in NPC has not yet been reported. Therefore, investigating anti-tumor immunity under hypoxia may help to develop novel approaches for NPC immunotherapeutic strategy.

Currently, clinical observations have revealed inconsistencies between the expression levels of PD-L1 in tumor cells and the treatment outcomes [12]. This discrepancy may result from various types of immune cells and tumor cells in the TME cooperatively inhibit the immune response, rather than the immunosuppressive effect of individual cells. Macrophages are the most abundantly infiltrating immune cells within the TME [13]. Instead of triggering antitumor immune responses, they can also undergo training within the TME to drive disease progression through varied mechanisms, which encompass tumor angiogenesis, tumor cell invasion, and metastasis [13]. Moreover, macrophages exhibit an immunosuppressive function by facilitating the recruitment of other immunosuppressive cells (such as regulatory T cells and myeloid-derived suppressor cells) [14]. In recent years, PD-L1$^+$ macrophages for immune escape were investigated in various cancers [15, 16]. In our previous study [17], we also found that a substantial infiltration of PD-L1$^+$ macrophages in NPC. However, the immunosuppressive effect of PD-L1$^+$ macrophages in NPC remains unclear. Additionally, it is yet to be determined whether signaling interactions between NPC cells and macrophages within the TME contribute to the upregulation of PD-L1 on macrophages.

Exosomes, which are small vesicles ranging in size from 30 to 150 nm, have recently been identified as crucial mediators of intercellular communication [18]. They play an important role in facilitating hypoxia-induced communication within the TME by transferring miRNAs, proteins, and mRNAs [19]. Recent studies have revealed that exosomal miRNAs secreted by tumor cells can induce the upregulation of PD-L1 in macrophages [15, 16]. Additionally, existing literature indicates that exosomes in NPC can carry PD-L1 protein on their surface, which can bind to PD-1 on T cells, thereby promoting immune evasion [20]. Whether macrophages phagocytose exosomal PD-L1 secreted by NPC cells to upregulate their own PD-L1 expression deserves further study.

Therefore, we conducted this study to verify the impact of hypoxia (via HIF-1α) on PD-L1 expression in the TME of NPC and to elucidate the specific mechanisms underlying the generation of PD-L1$^+$ macrophages. In addition, we further explored the effect of PD-L1$^+$ macrophages on CD8$^+$ T cells. This study aims to identify new therapeutic targets and provide a theoretical foundation for improving the response rate to immunotherapy in NPC.

Materials and methods

Clinical samples

NPC tissue specimens (n = 32) for multiplex immunohistochemistry (mIHC) were collected from biopsies of patients diagnosed with NPC in 2021 at Nanfang Hospital of Southern Medical University (Guangzhou, China). These specimens were obtained through biopsies from patients diagnosed with NPC between 2005 and 2015, and all cases were pathologically confirmed as NPC. Detailed pathological assessments, clinical data, and survival information were comprehensively collected through outpatient consultations and telephone follow-ups. The clinical characteristics of these patients with NPC are shown in Supplementary Table 1.

The NPC tissue specimens (n = 32) used for multiplex immunohistochemistry (mIHC) were collected from the Nanfang Hospital of Southern Medical University (GuangZhou, China). These specimens were obtained from biopsy samples collected in 2021 from patients diagnosed with NPC. The eighth edition of the American Joint Committee on Cancer (AJCC) staging system was used for stage classification. This study was approved by the Ethics Committee of Nanfang Hospital of Southern Medical University (Approval no.: NFEC-2017-165), and written informed consent was obtained from all participants prior to sample collection and data use.

Cell culture

The human NPC cell lines HK-1 and HNE-1, as well as the human monocytic leukemia cell line THP-1, were obtained from the Cancer Research Center of Southern Medical University. Macrophages were differentiated from THP-1 cells. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy donors. NPC cells and PBMCs were cultured in RPMI-1640 medium (11,875,176, Gibco, USA) supplemented with 10% fetal bovine serum (FSP500, ExCell Bio, China), 100 mg/mL streptomycin, and 100 U/mL penicillin (C125C5, NCM Bio, China) at 37 °C. Under normoxic conditions, the gas mixture consisted of 74% N_2, 21% O_2 and 5% CO_2, while

under hypoxic conditions, the composition was adjusted to 94.9% N_2, 0.1% O_2, and 5% CO_2. For exosome extraction, cells were cultured in medium containing exosome-depleted FBS. The culture conditions for THP-1 cells were the same as for NPC cells, except for the addition of β-mercaptoethanol (M917637, Macklin, China). THP-1 cells were differentiated into macrophages by incubation with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (M4647, Abmole, USA) for 48 h.

T cell activation

T cells were activated following a standardized protocol to ensure reproducibility. Briefly, a 6-well flat-bottom plate was first coated with anti-human CD3 monoclonal antibody (clone OKT3, 16-0037-81, eBioscience, USA) at 1 μg/mL diluted in sterile PBS, and incubated either overnight at 4 °C or for 2 h at 37 °C. After incubation, wells were washed twice with sterile PBS to remove unbound antibody. Subsequently, Anti-human CD28 monoclonal antibody (16-0289-81, eBioscience, USA) was added at 3 μg/mL in complete RPMI-1640 medium supplemented with 10% FBS. Freshly isolated T cells were resuspended in pre-warmed complete RPMI-1640 medium at a density of 1×10^6 cells/mL, and 2–3 mL of the cell suspension was seeded into each well. The T cells were then incubated at 37 °C in a humidified 5% CO_2 incubator for 72 h to ensure full activation. After activation, T cells were harvested for downstream applications such as flow cytometry or co-culture experiments.

Co-culture experiment

In the co-culture experiments, macrophages were seeded in the lower chamber of a 6-well plate, while nasopharyngeal carcinoma (NPC) cells (HK-1 or HNE-1) were seeded in the upper chamber of a Transwell insert with a 0.4-μm pore size. This setup allowed the diffusion of soluble factors while preventing direct cell–cell contact. Additionally, exosomes (10 μg/10^5 cells) isolated from NPC cells under different treatment conditions were added to macrophages in a separate co-culture system to investigate the role of exosomes in macrophage regulation. In subsequent co-culture experiments, macrophages pretreated either with NPC cells (via Transwell) or NPC cell-derived exosomes were co-incubated with PBMCs freshly isolated from healthy donors. Furthermore, $CD8^+$ T cells isolated from PBMCs using magnetic bead separation were co-cultured with the pretreated macrophages to assess their cytokine production (e.g., IL-2, IFN-γ, and Granzyme B) and activation status (e.g., PD-1 expression). All co-culture experiments were conducted for 48 h.

RNA extraction, reverse transcription and qRT-PCR

The cellular RNA was extracted from HK-1, HNE-1 NPC cells, and THP-1-derived macrophages using an RNA isolation kit (RC112-01, Vazyme, China) and subsequently reverse transcribed into cDNA utilizing a reverse transcription kit (R323-01, Vazyme, China). ChamQ SYBR RT-qPCR Master Mix (Low ROX Premixed) (Q331-02, Vazyme, China) was employed to prepare a 20 μL reaction amplification system for conducting reverse transcription quantitative polymerase chain reaction (RT-qPCR) on an ABI QuantStudio 6 System. GAPDH were used as the internal control for normalization, and the relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method. The primer sequences for RT-qPCR are shown in Supplementary Table 2a.

Western blot

The proteins were extracted from NPC cell lines (HK-1 and HNE-1), THP-1-derived macrophages, and exosomes derived from NPC cells using the radioimmunoprecipitation assay (RIPA) lysis buffer (P0013B, Beyotime, China), supplemented with a protease-inhibitor cocktail (HY-K0010, MCE, USA). The protein samples were mixed with loading buffer (LT101S, Epizyme, China), separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore, USA). The membranes were incubated with primary antibodies against HIF-1α (20960-1-AP, Proteintech, USA), PD-L1 (13,684, Cell Signaling Technology, USA), and GAPDH (60004-1-Ig, Proteintech, USA), followed by HRP-conjugated secondary antibodies. Signals were detected using enhanced chemiluminescence (ECL) reagents (SQ101, Epizyme, China).

Isolation and characterization of exosomes

Exosomes were isolated from NPC cell culture supernatants by differential centrifugation. Briefly, collected culture supernatants were differentially centrifuged at 300 × g for 10 min, 2000 × g for 20 min, and 10,000 × g for 45 min, and the supernatant was filtered and ultracentrifuged at 110,000 × g for 75 min (all steps were per formed at 4 °C). The exosomes were collected and resuspended in phosphate-buffered saline (PBS). The size distribution and concentration of exosomes were measured by nanoparticle tracking analysis (NTA) by using the Nanosight NS300 (Malvern, England) or Zeta View nanoparticle tracking

analyzer (Particle Metrix, Germany). And the exosome morphology was observed under the transmission electron microscope (HT-7700, Hitachi, Japan). The characteristics of exosome marker proteins TSG101 (28283-1-AP, Proteintech, USA), CD9 (20597-1-AP, Proteintech, USA), and Calnexin (2679 T, Cell Signaling Technology, USA) were analyzed by Western blot.

Macrophage phagocytosis experiment

To investigate macrophage phagocytosis of exosomal PD-L1, NPC cells were transfected to overexpress mCherry-tagged PD-L1. After 48-h, the culture supernatant was collected and exosomes were isolated by ultracentrifugation. The purified exosomes were stained with the green fluorescent dye PKH67 (MIDI67-1KT, Sigma, USA) according to the manufacturer's instructions. The labeled exosomes were incubated with macrophages at 37 °C for 6 h. Following incubation, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, and stained with DAPI to visualize nuclei. Internalization of mCherry-PD-L1-containing exosomes by macrophages was observed using confocal microscopy (LSM980, Zeiss, Germany).

Immunofluorescence staining (IF)

The pretreated HK-1 and HNE-1 cells and THP-1-derived macrophages were seeded onto sterilized glass coverslips placed in 24-well plates and allowed to adhere. After appropriate treatment, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Following fixation, cells were washed three times with PBS and then blocked with 5% goat serum for 1 h at room temperature to reduce nonspecific binding. Subsequently, cells were incubated overnight at 4 °C with primary antibody against HIF-1α (20960-1-AP, Proteintech, USA) diluted in 1% BSA/PBS. The next day, cells were washed three times with PBS and incubated with Alexa Fluor 488-conjugated secondary antibody (A0423, Beyotime, China) for 2 h at room temperature in the dark. After additional PBS washes, cells were counterstained with DAPI-containing mounting solution (S2110, Solarbio, China) to visualize nuclei. Coverslips were mounted onto slides, and fluorescence images were captured using a fluorescence scanning microscope (BX63, Olympus, Japan).

ChIP-PCR

The EZ-Magna ChIP™ A/G kit (17-10086, Millipore, USA) was used to conduct perform chromatin immunoprecipitation (ChIP) assays according to the manufacturer's instructions HK-1 and HNE-1 nasopharyngeal carcinoma cells were cultured under hypoxic conditions (0.1% O_2) for 24 h prior to ChIP. Cells were crosslinked with 1% formaldehyde at room temperature for 10 min to preserve protein-DNA interactions, and the reaction was quenched with 125 mM glycine. Nuclei were isolated and chromatin was sheared to an average length of 200–500 bp using sonication. Immunoprecipitation was carried out using anti-HIF-1α antibody (36,169, Cell Signaling Technology, USA) overnight at 4 °C with rotation. Normal IgG served as the negative control. After extensive washing, the protein-DNA complexes were eluted and reverse crosslinked at 65 °C. DNA was purified using spin columns provided in the kit. Enrichment of HIF-1α binding to the PD-L1 promoter region was assessed by qPCR using three primer sets (Supplementary Table 2b) targeting distinct sites within the human PD-L1 promoter.

Dual-luciferase assay

293 T cells were seeded in 24-well plates at a density of 1×10^5 cells per well and cultured for 24 h until reaching appropriate confluency. For transfection, Lipofectamine 2000 reagent (116,680,119, Invitrogen, USA) was used following the manufacturer's protocol. Cells were co-transfected with 4.0 μg/ml of either the control pcDNA3.1 plasmid or the HIF-1α expression plasmid, together with 4.0 μg/ml of either the pGL3-basic control vector or the pGL3-PD-L1 promoter luciferase reporter plasmid. After 36 h of transfection, the cells were lysates, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (E1910, Promega, USA) according to the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla luciferase activity to control for transfection efficiency. The specific primer sequences used for cloning the PD-L1 promoter constructs are provided in Supplementary Table 2c.

Immunohistochemistry (IHC) and multiplex immunohistochemistry (mIHC)

IHC was performed on 4-μm sections of paraffin-embedded tissue samples, including 152 NPC tissue samples collected from patients diagnosed with nasopharyngeal carcinoma (2005–2015). Primarily, sections were deparaffinized, rehydrated and immersed in 10 mM citrate buffer for heat-induced antigen recovery. Antigen blocking was carried out using 10% goat serum (SP-9000, Zsbio, China). Overnight at 4 °C, the sections were subjected to probing with primary antibodies against HIF-1α (20,960–1-AP, Proteintech, USA), PD-L1 (ET1701-41, Huabio, China), and CD8 (AF5126, Affinity, USA). Then, staining was performed using the DAB color reagent kit (ZLI-9018, Zsbio, China). For mIHC assays, a multiple fluorescent immunohistochemical staining kit (abs50028, Absin, China) was used for

immunofluorescence staining on 32 NPC tissue samples collected in 2021, also from nasopharyngeal carcinoma patients. Primary antibodies against CD68 (ER1901-32, Huabio, China), PD-L1 (13,684, Cell Signaling Technology, USA), and CD8 (AF5126, Affinity, USA) were applied. The evaluation criteria of IHC and mIHC were shown in Supplementary Materials and Methods.

Flow cytometry (FC)

Macrophages were labeled with fluorescently tagged monoclonal antibodies targeting HLA-DR (555,811, BD Pharmingen, USA) and CD163 (333,606, Biolegend, USA) following the guidance provided by the manufacturer. PBMCs were extracted from the peripheral blood of healthy donors with a mononuclear cell isolation reagent (abs9645, Absin, China). PBMCs were stained with fluorochromeconjugated monoclonal antibodies against CD8a (300,912, Biolegend, USA) and PD-1 (329,906, Biolegend, USA). Staining of cells with IgG isotype were considered as negative control. The cells were then subjected to flow cytometric analyses on a BD LSRFortessa cytometer and analyzed using FlowJo software (10.9.0).

Enzyme linked immunosorbent assay (ELISA)

Cytokine levels of human IFN-γ, IL-2, and Granzyme B in the culture supernatants of $CD8^+$ T cells co-cultured with macrophages under different conditions were quantified using ELISA DuoSet kits (Multi Sciences, China), following the manufacturer's protocol. Briefly, 96-well plates were pre-coated with capture antibodies and incubated overnight at 4 °C. After blocking, culture supernatants were added to the wells and incubated at room temperature. Biotinylated detection antibodies and streptavidin-HRP were then added sequentially. After substrate development, absorbance was measured at 450 nm using a microplate reader, with 570 nm used as the reference wavelength for background correction. All samples were analyzed in duplicate or triplicate to ensure reliability.

Statistical analysis

Statistical analyses were performed using SPSS version 23.0. All data were obtained from at least three independent experiments. Experimental results are presented as mean ± SEM. A two-tailed Student's t test or the Wilcoxon-Mann–Whitney test was used to evaluate differences between two groups, and one-way analysis of variance (ANOVA) was used for comparisons among three or more groups. Overall survival (OS) and progression-free survival (PFS) were analyzed using the Kaplan–Meier method, and differences were assessed by the log-rank test. Correlation analyses were performed using the Pearson's correlation method. All data statistical tests were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Result 1 The expressions of HIF-1α, CD8, and PD-L1 are interrelated in NPC, among which the expressions of HIF-1α and CD8 are associated with patient prognosis

Immunohistochemistry (IHC) analysis of 152 NPC tissues revealed significant correlations among HIF-1α, CD8, and PD-L1 expression. Specifically, HIF-1α expression was positively correlated with PD-L1 expression ($r = 0.6799$, $p < 0.001$) and negatively correlated with CD8 expression ($r = -0.4143$, $p < 0.001$) (Fig. 1a–c). PD-L1 expression CD8 expression also showed a negative correlation ($r = -0.5105$, $p < 0.001$) (Fig. 1d). In addition, PD-L1 expression was significantly upregulated in patients with advanced-stage NPC (clinical stage III-VI) compared with those in early-stage (clinical stage I-II) (Fig. S1).

Prognostic analysis revealed that higher expression of HIF-1α or CD8 was associated with worse overall survival (OS) and progression-free survival (PFS), whereas PD-L1 expression was not associated with prognosis (Fig. 1e, f). Together, these findings suggest that HIF-1α is positively correlated with increased PD-L1 expression and decreased CD8 expression in NPC tissues, with both HIF-1α and CD8 serving as potential prognostic markers in NPC.

Result 2 Hypoxia up-regulates expression of PD-L1 on NPC cell lines via transcriptional regulatory factor HIF-1α

To investigate the impact of hypoxia on PD-L1 expression in NPC cells, HK-1 and HNE-1 cells were cultured under hypoxic (0.1% O_2) and normoxic (21% O_2) conditions. Western blot analysis revealed significantly higher levels of HIF-1α and PD-L1 under hypoxia at both 24 h and 48 h compared with normoxia (Fig. 2a, b). Based on these findings, 0.1% O_2 for 48 h was selected for subsequent experiments.

Immunofluorescence analysis showed that HIF-1α was primarily localized in the cytoplasm under normoxic conditions but translocated to the nucleus under hypoxia, suggesting its role in transcriptional regulation (Fig. 2c). Using UCSC and JASPAR databases, three putative HIF-1α binding sites were identified within the PD-L1 promoter

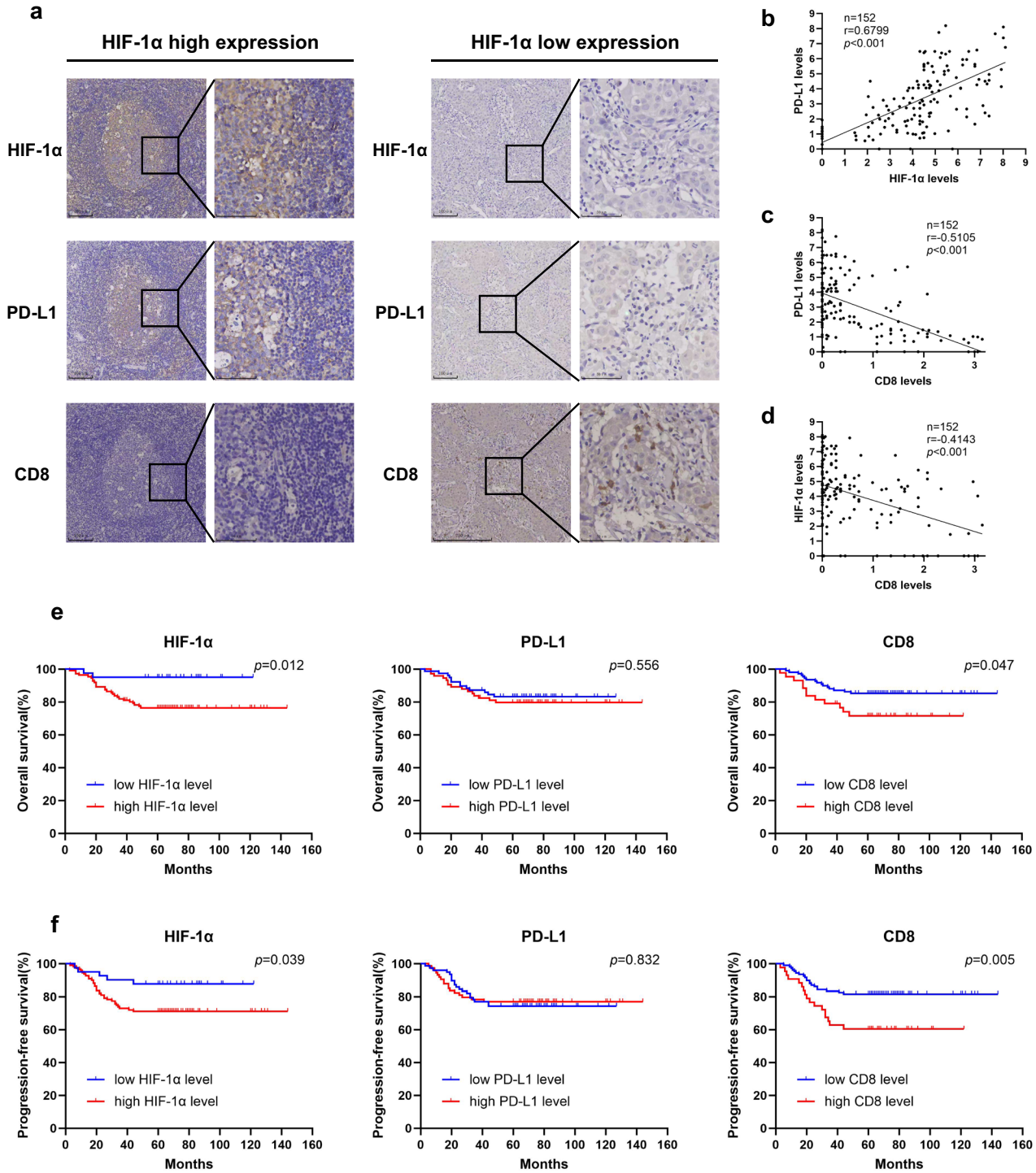


Fig. 1 HIF-1α expression was positively correlated with elevated PD-L1 expression and reduced CD8 expression, and high HIF-1α or CD8 expression was associated with a negative prognosis. **a** Representative pictures of PD-L1 and CD8 expression in NPC patients in HIF-1α-low and high expression groups. Scale bars represented 50 or 100um. **b** Pearson correlation analysis of the association between HIF-1α and PD-L1 (r = 0.6799, $p < 0.001$). **c** Pearson correlation analysis of the association between HIF-1α and CD8 (r = −0.4143, $p < 0.001$). **d** Pearson correlation analysis of the association between PD-L1 and CD8 (r = −0.5105, $p < 0.001$). **e** Kaplan–Meier curves for OS between high HIF-1α, PD-L1 or CD8 groups and low HIF-1α, PD-L1 or CD8 groups. **f** Kaplan–Meier curves for PFS between high HIF-1α, PD-L1 or CD8 groups and low HIF-1α, PD-L1 or CD8 groups

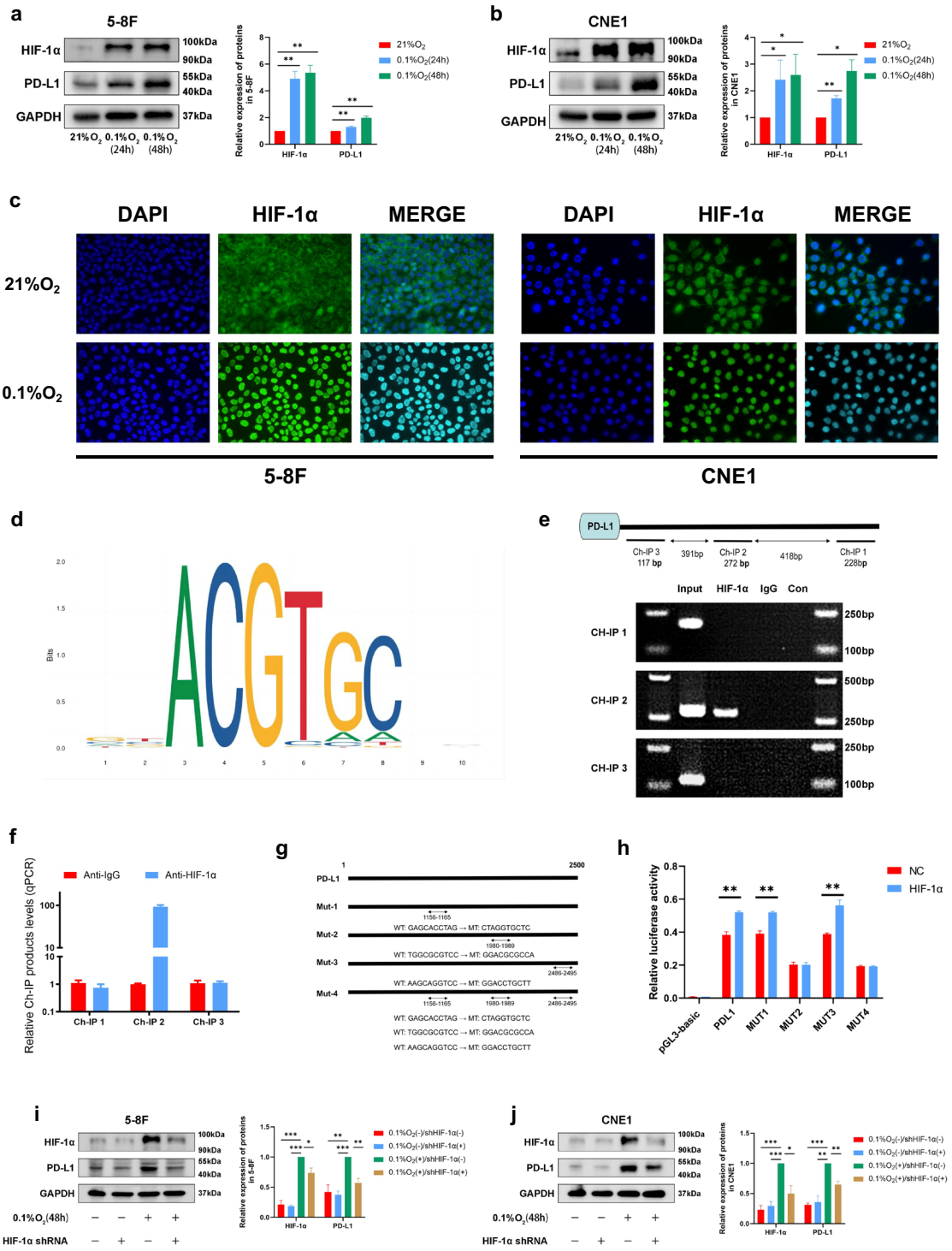


Fig. 2 Hypoxia upregulated PD-L1 expression in NPC cells through HIF-1α. **a**, **b** Western blot analysis of HIF-1α and PD-L1 within normoxia condition or different hypoxia conditions in HK-1 and HNE-1 cells. **c** IF staining localization of HIF-1α expression in within normoxia condition or hypoxia condition in HK-1 and HNE-1 cells. **d** The binding motif of HIF-1α from JASPAR database. **e** The CHIP-PCR assay was used to assess the binding of PD-L1 promoter region. **f** Anti-HIF-1α-pulled down chromatins were analyzed by qRT-PCR. **g** A diagram showing the relationship of full-length and mutant PD-L1 promoters. **h** Dual-luciferase reporter gene assay was performed to indicate the interaction between HIF-1α and PD-L1. **i**, **j** Western blot analysis of HIF-1α and PD-L1 within normoxia condition or hypoxia condition after silencing HIF-1α in HK-1 and HNE-1 cells. *Notes*: $*p<0.05$, $**p<0.01$, $***p<0.001$; ns, not significant

region. Chromatin immunoprecipitation (ChIP) assays confirmed direct binding at the second site (Fig. 2d–f). Luciferase reporter assays further demonstrated that HIF-1α significantly enhanced the transcriptional activity of PD-L1 through this binding site, while mutation at this site (Mut-2) led to reduced activity (Fig. 2g, h).

Finally, shRNA-mediated knockdown of HIF-1α in NPC cells resulted in a marked decrease in PD-L1 expression under hypoxia conditions (Fig. 2i, j). These findings demonstrate that hypoxia promotes PD-L1 expression in NPC cells through HIF-1α–mediated transcriptional regulation.

Result 3 Hypoxia NPC cells up-regulate the expression of PD-L1 in macrophages

Immunohistochemistry (IHC) revealed a noteworthy phenomenon: PD-L1 was highly expressed not only in NPC cells but also in immune cells infiltrating the vicinity of PD-L1-positive NPC cells (Fig. 3a). As macrophages are the most abundant immune cells in the tumor microenvironment (TME), we hypothesized that PD-L1$^+$ NPC cells could induce the upregulation of PD-L1 expression in macrophages. To test this hypothesis, THP-1-derived macrophages were co-cultured with NPC cells pretreated under different conditions. The results showed that macrophages incubated with NPC cells under hypoxic conditions (0.1% O_2) exhibited significantly higher PD-L1 expression compared with those incubated with NPC cells under normoxic conditions (21% O_2) or untreated macrophages (Fig. 3b).

Furthermore, multiplex immunofluorescence (mIF) staining revealed an inverse correlation between the percentages of PD-L1$^+$ macrophages and CD8$^+$ T cells in NPC tissues, suggesting that PD-L1$^+$ macrophages suppress CD8$^+$ T cell infiltration in the TME (Fig. 3c, d). To confirm the role of HIF-1α in this process, macrophages were co-cultured with NPC cells pretreated under various conditions (shCTRL/21% O_2, shHIF-1α/21% O_2, shCTRL/0.1% O_2, shHIF-1α/0.1% O_2). Macrophages co-cultured with hypoxic NPC cells (shCTRL/0.1% O_2) exhibited the highest PD-L1 levels, whereas macrophages co-cultured with NPC cells in other conditions showed significantly lower PD-L1 expression (Fig. 3e, f). Interestingly, macrophages incubated with hypoxia-pretreated NPC cells displayed a shift toward the HLA-DR^low/CD163^high phenotype compared to untreated macrophages. However, there were no significant differences in HLA-DR and CD163 expression among the different treatment groups (Fig. 3g, h).

Collectively, these findings indicate that hypoxic NPC cells enhance PD-L1 expression in macrophages without significantly affecting their HLA-DR or CD163 expression. These results highlight the interaction between hypoxic NPC cells and macrophages in the TME, where in hypoxia-induced mechanisms contribute to the immunosuppressive phenotype of macrophages.

Result 4 PD-L1$^+$ macrophages induced by hypoxic NPC cells can inhibit CD8$^+$ T cells

To investigate whether PD-L1$^+$ macrophages induced by NPC cells exert immunosuppressive effects in the tumor microenvironment (TME) by regulating CD8$^+$ T cells, untreated macrophages and macrophages preconditioned with NPC cells under different conditions were individually co-cultured with freshly isolated human PBMCs. The results showed that macrophages co-cultured with NPC cells under hypoxic conditions (shCTRL/0.1% O_2) significantly reduced the proportion of CD8 + T cells while increasing the proportion of PD-1$^+$ CD8$^+$ T cells compared to untreated macrophages or macrophages co-cultured with NPC cells under normoxic conditions (shCTRL/21% O_2) or with HIF-1α knockdown (shHIF-1α/21% O_2, shHIF-1α/0.1% O_2). Importantly, these immunosuppressive effects were almost completely abrogated by the addition of anti-PD-L1 antibodies (Fig. 4a, c).

To further evaluate the effects of PD-L1$^+$ macrophages on CD8$^+$ T cell effector functions, untreated macrophages and macrophages precultured with NPC cells under various conditions were separately co-cultured with CD8$^+$ T cells. The results demonstrated that macrophages co-cultured with NPC cells under hypoxic conditions (shCTRL/0.1% O_2) significantly reduced the production of IL-2, IFN-γ, and Granzyme B by CD8$^+$ T cells compared to untreated macrophages or macrophages co-cultured with NPC cells under normoxic or HIF-1α knockdown conditions. Similarly, these inhibitory effects were nearly completely reversed by the addition of anti-PD-L1 antibodies (Fig. 4b, d).

Together, these findings demonstrate that PD-L1$^+$ macrophages, induced by hypoxic NPC cells, suppress the activity and effector functions of CD8$^+$ T cells in the NPC TME through PD-L1-mediated mechanisms.

Result 5 Hypoxia increases exosomal PD-L1 levels in NPC cells, macrophages increase PD-L1 expression by phagocytizing PD-L1 in exosomes

To investigate the mechanism underlying the upregulation of PD-L1 in macrophages induced by NPC cells, we first measured PD-L1 mRNA levels in macrophages co-cultured with NPC cells using qRT-PCR. The results showed a significant reduction in PD-L1 mRNA expression in macrophages co-cultured with NPC cells compared with untreated macrophages, with the lowest expression observed in macrophages co-cultured with hypoxic NPC cells (Fig. 5a). These findings indicates that the upregulation of PD-L1 protein in macrophages is not mediated by

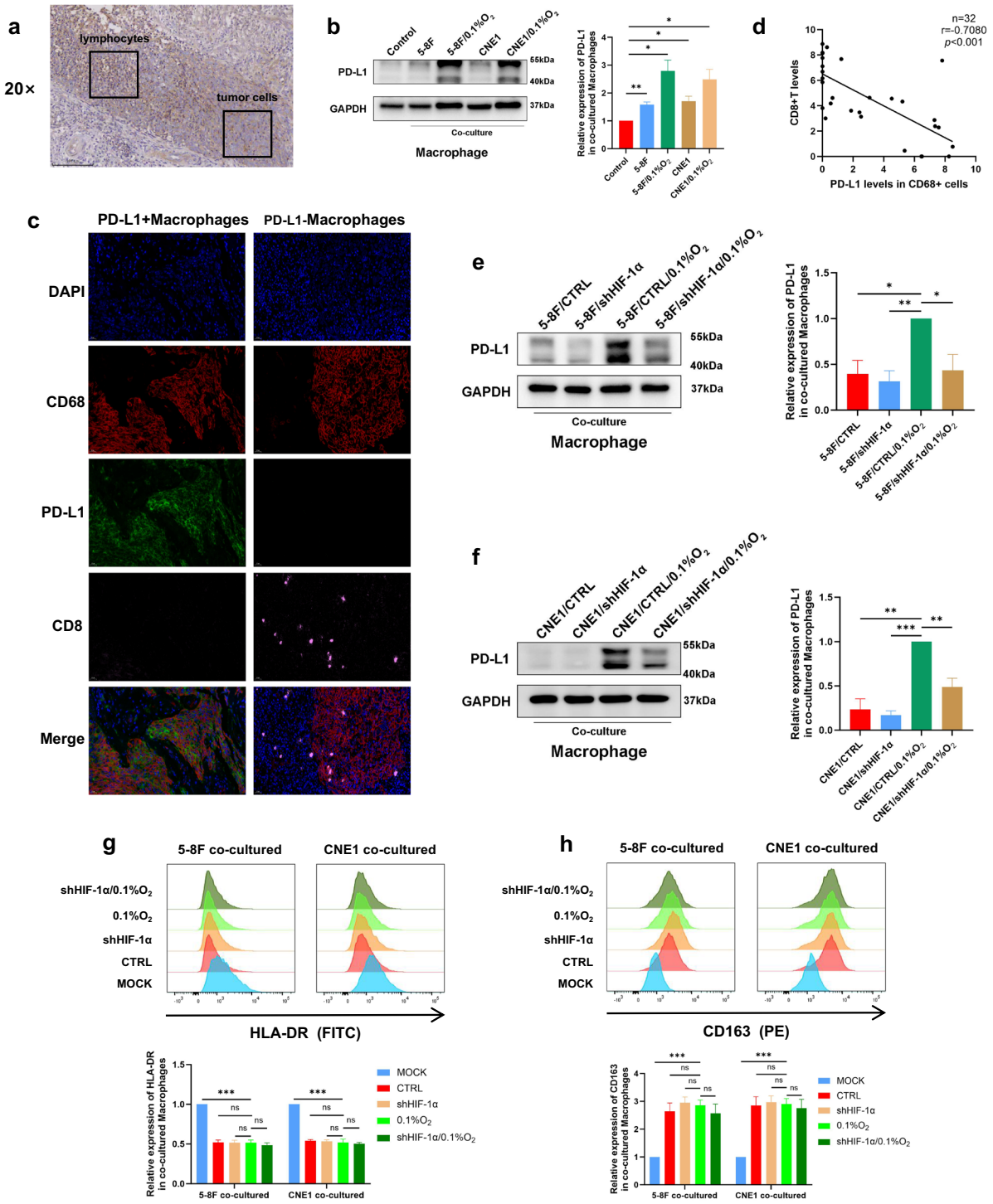


Fig. 3 Hypoxia NPC cells upregulate the expression of PD-L1 in macrophages. **a** IHC staining of PD-L1$^+$ NPC cells and PD-L1$^+$ immune cells. **b** Western blot analysis of PD-L1 in macrophages co-cultured with differently pretreated HK-1 and HNE-1 cells (normoxia and hypoxia) for 48 h. **c** The mIF staining of PD-L1$^+$ macrophages and CD8$^+$ T cells was performed in NPC tissue samples. **d** Pearson correlation analysis of the association between the levels of PD-L1$^+$ macrophages and CD8$^+$ T cells (r = −0.7080, $p < 0.001$). **e**, **f** Western blot analysis of PD-L1 in macrophages co-cultured with differently pretreated HK-1 and HNE-1 cells (normoxia, normoxia/shHIF-1α, hypoxia and hypoxia/shHIF-1α) for 48 h. **g**, **h** Flow cytometry was performed to detect the expression of HLA-DR and CD163 in in macrophages co-cultured with differently pretreated HK-1 and HNE-1 cells (normoxia, normoxia/shHIF-1α, hypoxia and hypoxia/shHIF-1α). *Notes*: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, not significant

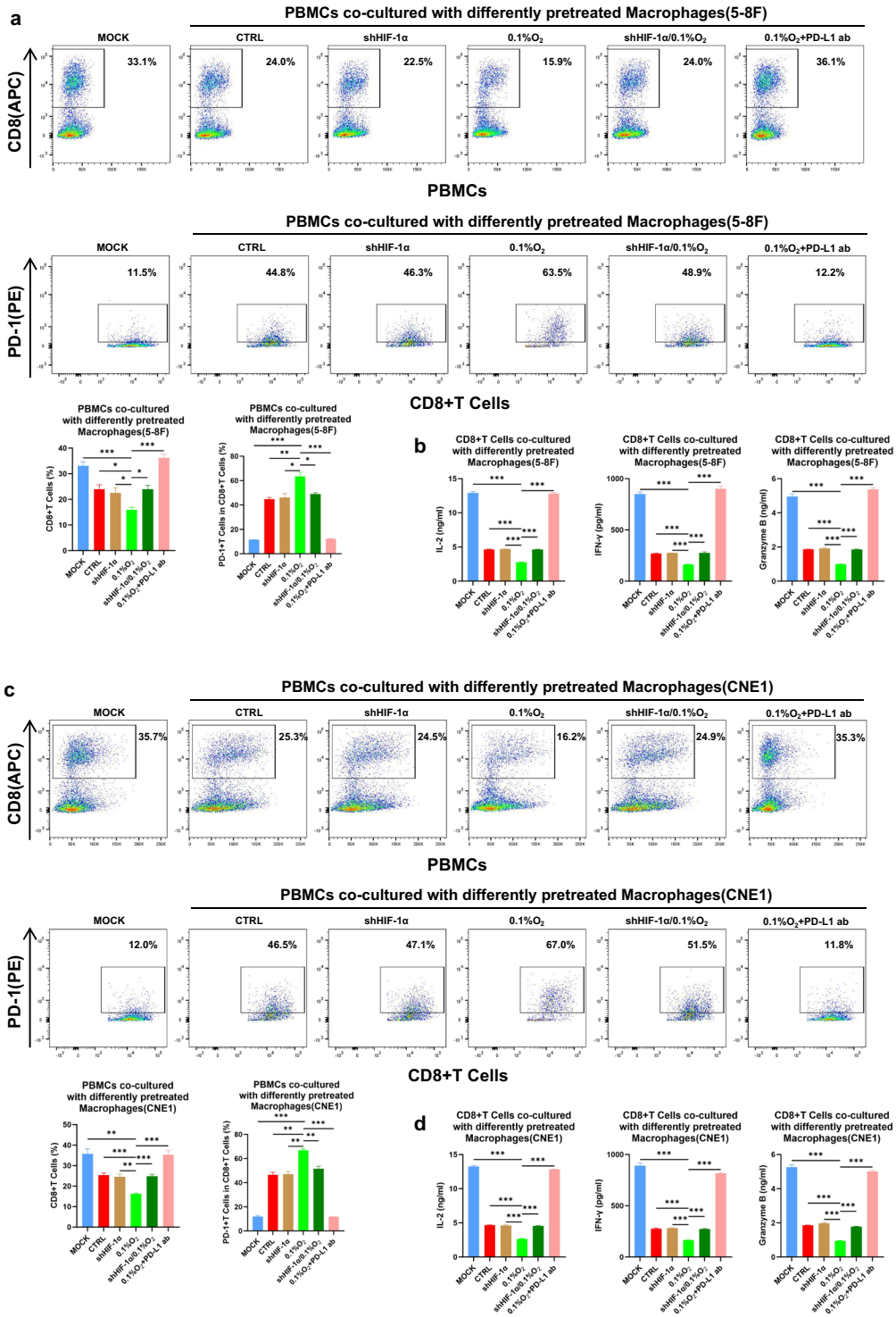


Fig. 4 PD-L1$^+$ macrophages induced by hypoxic NPC cells could inhibit CD8$^+$ T cells. **a** Flow cytometry was performed to detect the levels of CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells in PBMCs co-cultured with differently pretreated macrophages (macrophages were co-cultured with differently pretreated HK-1 cells). **b** IL-2, IFN-γ and Granzyme B levels were detected in the culture supernatants of human peripheral CD8$^+$ T cells co-cultured with macrophages that underwent different pretreatments (macrophages were co-cultured with differently pretreated HK-1 cells) by ELISA. **c** Flow cytometry was performed to detect the levels of CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells in PBMCs co-cultured with differently pretreated macrophages (macrophages were co-cultured with differently pretreated HNE-1 cells). **d** IL-2, IFN-γ and Granzyme B levels were detected in the culture supernatants of human peripheral CD8$^+$ T cells co-cultured with macrophages that underwent different pretreatments (macrophages were co-cultured with differently pretreated HNE-1 cells) by ELISA. *Notes*: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, not significant

transcriptional activation. We therefore hypothesized that the increased PD-L1 expression in macrophages may result from the phagocytic uptake of exogenous PD-L1.

To test this hypothesis, exosomes secreted by NPC cells (HK-1 and HNE-1) were characterized using transmission electron microscopy, nanoparticle tracking analysis (NTA), ZetaView, and western blotting (Fig. 5b–d). PD-L1 was overexpressed in NPC cells with a red fluorescent protein tag. After incubating macrophages with PKH67-labeled exosomes derived from PD-L1-overexpressing NPC cells (HK-1/OE-PD-L1 and HNE-1/OE-PD-L1), colocalization of PD-L1 (red) and exosome (green) signals was observed within macrophages, confirming the delivery of PD-L1 via exosome uptake (Fig. 5e).

We next evaluated whether hypoxia affects PD-L1 levels in NPC cell-derived exosomes. Western blot analysis revealed significantly higher PD-L1 levels in exosomes from hypoxic NPC cells (shCTRL/0.1% O_2) compared with exosomes from normoxic cells (shCTRL/21% O_2) or from cells with HIF-1α knockdown under either normoxic or hypoxic conditions (Fig. 5f–g). Furthermore, macrophages co-cultured with exosomes derived from hypoxic NPC cells (shCTRL/0.1% O_2) exhibited higher PD-L1 expression than those exposed to exosomes from normoxic NPC cells (shCTRL/21% O_2) or from HIF-1α knockdown NPC cells (Fig. 5h, i).

Interestingly, macrophages incubated with exosomes from hypoxic NPC cells exhibited an HLA-DR^low/CD163^high phenotype compared with untreated macrophages. However, no significant differences in HLA-DR or CD163 expression were observed between macrophages treated with exosomes from differently preconditioned NPC cells (Fig. 5j, k).

Altogether, these findings demonstrate that NPC cells enhance PD-L1 expression in macrophages through the uptake of PD-L1-containing exosomes, revealing a novel mechanism by which hypoxic NPC cells modulate the tumor microenvironment in vitro.

Result 6 PD-L1$^+$ macrophages induced by hypoxic NPC cell exosomes can inhibit CD8$^+$ T cells

To investigate the immunosuppressive role of PD-L1$^+$ macrophages induced by exosomes from hypoxic NPC cells in the tumor microenvironment (TME), particularly their regulatory effects on CD8$^+$ T cells, untreated macrophages and macrophages preconditioned with exosomes from NPC cells under different conditions were co-cultured with freshly isolated human PBMCs. The results showed that macrophages co-cultured with exosomes from hypoxic NPC cells (shCTRL/0.1% O_2) significantly decreased the proportion of CD8$^+$ T cells and increased the proportion of PD-1$^+$ CD8$^+$ T cells compared to untreated macrophages or macrophages co-cultured with exosomes from normoxic NPC cells (shCTRL/21% O_2) or HIF-1α knockdown NPC cells (shHIF-1α/21% O_2, shHIF-1α/0.1% O_2). Notably, these immunosuppressive effects were almost completely abolished by the addition of anti-PD-L1 antibodies (Fig. 6a, c).

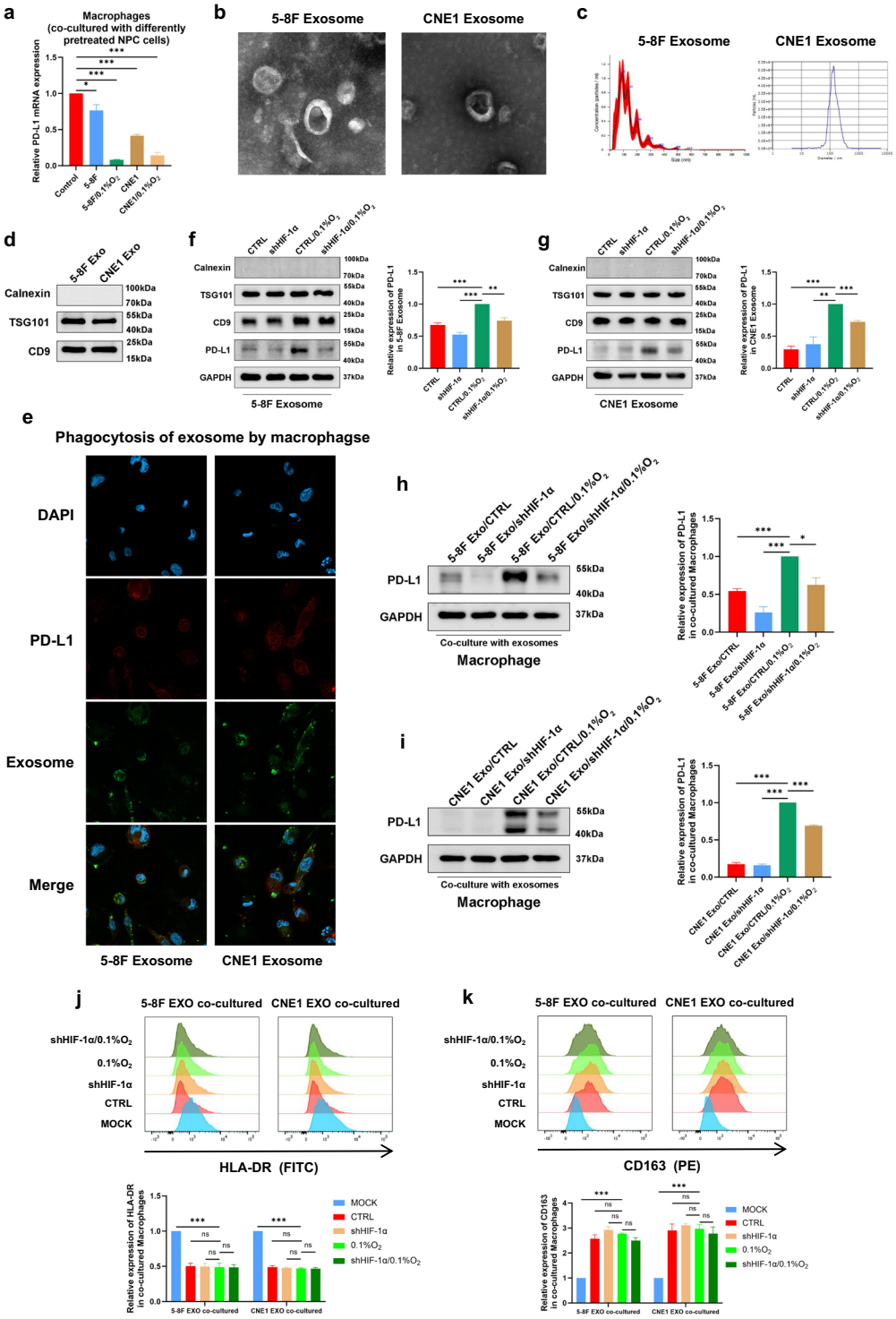
To further examined whether PD-L1$^+$ macrophages affect the effector functions of CD8$^+$ T cells, untreated macrophages and macrophages precultured with exosomes from NPC cells under various conditions were individually co-cultured with CD8$^+$ T cells. The results revealed that macrophages co-cultured with exosomes from hypoxic NPC cells (shCTRL/0.1% O_2) significantly reduced the production of IL-2, IFN-γ, and Granzyme B by CD8$^+$ T cells compared with untreated macrophages or macrophages co-cultured with exosomes from normoxic or HIF-1α knockdown NPC cells. These inhibitory effects were also almost completely reversed by the addition of anti-PD-L1 antibodies (Fig. 6b, d).

Together, these findings indicate that exosomes derived from hypoxic NPC cells, with elevated PD-L1 levels, induce PD-L1 expression in macrophages, which subsequently suppresses CD8$^+$ T cell activity and effector functions. This highlights a critical mechanism by which hypoxic NPC cells contribute to immunosuppression in the TME.

Discussion

The immune evasion of malignant tumors is a complex process involving multiple factors, steps, and stages, characterized by the dynamic interaction between tumor cells and the TME across both temporal and spatial dimensions [21]. Numerous factors contribute to immune escape in NPC at various stages of the immune response. Therefore, this study investigates the interactions among NPC tumor cells, macrophages, and CD8$^+$ T cells within a hypoxic microenvironment, aiming to elucidate the specific mechanisms underlying immune escape during the immune response.

Hypoxia is a common feature of solid tumors. Increased expression of HIF-1α is a key marker of tumor cell adaptation to hypoxic stress, promoting tumor growth, invasion, and metastasis, and contributing to resistance to immunotherapy and radiotherapy, failure of immunotherapy, and poor prognosis [22]. Over the past decade, research on hypoxia and HIF-1α has predominantly focused on the regulation of genes involved in angiogenesis, glucose and energy metabolism, and tumor cell growth and survival, such as vascular endothelial growth factor (VEGF), glucose transporter (GLUT), lactate dehydrogenase A (LDHA), and epithelial-mesenchymal transition (EMT) genes [23]. However, increasing evidence suggests that hypoxia facilitates immune evasion in malignant tumors



[24, 25]. HIF-1α signaling enables cancer cells to escape immune surveillance in the hypoxic TME by upregulating immunosuppressive molecules, impairing tumor antigen presentation, and disrupting immune cell activity [7]. Despite these advances, the role of the hypoxic TME in immune escape in NPC remains largely unclear.

In the present study, we observed that advanced-stage NPC patients exhibited significantly higher PD-L1 expression than early-stage patients. Additionally, higher HIF-1α expression was positively correlated with increased PD-L1 and negatively correlated with CD8 expression in NPC tissues. Although HIF-1α is expressed in both cancer cells and stromal immune cells within the TME,

◂**Fig. 5** Hypoxia increases exosomal PD-L1 levels in NPC cells, macrophages increase their PD-L1 expression by phagocytizing PD-L1 in exosomes. **a** The PD-L1 mRNA expression of macrophages co-cultured with differently pretreated HK-1 and HNE-1 cells (normoxia and hypoxia) for 48 h by qRT-PCR. **b** Transmission electron micrograph of HK-1 or HNE-1 cells-derived exosomes. **c** Exosomes released by HK-1 cells or HNE-1 cells were detected by nanosight particle tracking analysis or ZETA view analysis. **d** Exosome markers CD9, TSG101 and Calnexin proteins were detected by western blot. **e** IF detection of macrophages phagocytosing exosomal PD-L1(exosomes: green fluorescent; PD-L1: red fluorescent). **f**, **g** Western blot analysis of PD-L1 in exosomes derived from differently pretreated HK-1 and HNE-1 cells (normoxia, normoxia/shHIF-1α, hypoxia and hypoxia/shHIF-1α). **h**, **i** Western blot analysis of PD-L1 in macrophages co-cultured with exosomes derived from differently pretreated HK-1 and HNE-1 cells (normoxia, normoxia/shHIF-1α, hypoxia and hypoxia/shHIF-1α) for 48 h. **j**, **k** Flow cytometry was performed to detect the expression of HLA-DR and CD163 in in macrophages co-cultured with exosomes derived from differently pretreated HK-1 and HNE-1 cells (normoxia, normoxia/shHIF-1α, hypoxia and hypoxia/shHIF-1α). *Notes*: $*p<0.05$, $**p<0.01$, $***p<0.001$; ns, not significant

our in vitro experiments focused on hypoxia-exposed NPC cells, supported by IHC results showing predominant HIF-1α expression in NPC cells rather than in infiltrating immune cells. Further mechanistic investigation revealed that hypoxia upregulated PD-L1 expression via the HIF-1α signaling pathway. Although the ability of HIF-1α to induce PD-L1 has been demonstrated in other solid tumors, its role in NPC had not been well characterized [10, 11]. In the hypoxic TME, stabilized HIF-1α drives PD-L1 expression on tumor and regulatory immune cells (e.g., macrophages and MDSCs), thereby suppressing T cell activity and facilitating immune escape. As highlighted by Shurin and Umansky (2022) [26], inhibition of HIF-1α can downregulate PD-L1 expression, positioning HIF-1α as a promising target for enhancing the efficacy of anti-PD-1/PD-L1 immunotherapies. Targeting the HIF-1α/PD-L1 axis may therefore improve anti-tumor immune responses, overcome therapy resistance, and provide novel therapeutic strategies for NPC, ultimately supporting the development of more personalized treatment approaches.

Despite NPC tumor cells exhibiting high PD-L1 expression rates of 89% to 100%, only 20% to 30% of NPC patients respond favorably to anti-PD-L1/PD-1 therapy [12, 27]. recognition of antigenic peptides presented by antigen-presenting cells (APCs) via the T cell receptor (TCR), as well as binding of costimulatory molecules (such as B7) on APCs by costimulatory receptors (such as CD28) on T cells [28]. If T cell activation is impaired during interactions with APCs, then regardless of the level of PD-L1 expression by tumor cells, the response rate to PD-1/PD-L1 blockade will remain low. Macrophages, a key type of APC, can be categorized into M1 and M2 subtypes based on their distinct functional phenotypes [29]. In solid tumors, macrophages constitute over 50% of immune-related stromal cells within the TME. These macrophages are predominantly of the M2 subtype and are often referred to as tumor-associated macrophages (TAMs) [30]. TAMs generally possess anti-inflammatory, tissue repairing, pro-tumorigenic, and immunosuppressive properties [30]. With the advancement of single-cell technologies, an increasing number of studies have moved beyond the simplistic M1/M2 dichotomy, adopting surface markers or functional characteristics for more precise categorization of macrophage subsets [13]. In recent years, PD-L1$^+$ macrophages have been identified in various solid malignancies and are closely associated with suppression of the TIME [15, 16, 31, 32]. However, the generation and function in NPC remain poorly understood. In the current study, we demonstrated that hypoxic NPC cells can upregulate PD-L1 expression in macrophages, which in turn induces CD8$^+$ T cell exhaustion and suppresses their proliferation. These findings suggest that PD-L1$^+$ macrophages play a critical role in mediating immune escape within the NPC TME. It is well known that macrophages, particularly those with an M2 phenotype, are capable of expressing PD-L1. However, our study does not focus solely on this intrinsic property. Instead, we aimed to investigate whether hypoxic NPC cells could further enhance macrophage PD-L1 expression through the delivery of exosomal PD-L1, thereby amplifying their immunosuppressive capacity. Our findings revealed that PD-L1 mRNA levels in macrophages were downregulated despite increased protein expression, suggesting a post-transcriptional mechanism involving exosomal transfer. Similar phenomena have been reported in other malignancies. In colorectal cancer, tumor-derived exosomes upregulate PD-L1 expression in macrophages, resulting in the formation of a PD-L1$^+$CD206$^+$ subset associated with poor prognosis, and these macrophages inhibit CD8$^+$ T cell activity [15]. In ICC, tumor-derived exosomes also promote PD-L1 expression in macrophages, contributing to an immunosuppressive environment [16]. These findings suggest that hypoxic NPC cells may drive PD-L1 expression in macrophages, thereby collectively support the hypothesis that hypoxic NPC cells may drive PD-L1 expression in macrophages, thereby facilitating immune evasion. Targeting these PD-L1$^+$macrophages may represent a promising therapeutic strategy to enhance anti-tumor immunity in NPC.

Increasing evidence indicates that tumor-derived exosomes play a crucial role in the upregulation of PD-L1 expression in macrophages across various cancers, such as colorectal cancer [15], intrahepatic cholangiocarcinoma [16], and ovarian cancer [31]. These studies have demonstrated that tumor-derived exosomal miRNAs can upregulate PD-L1 mRNA expression in macrophages, leading to immune evasion. In our study, we also investigated the mRNA expression of PD-L1 in macrophages co-cultured with NPC cells. However, unexpectedly, PD-L1 mRNA expression in these macrophages was found to be

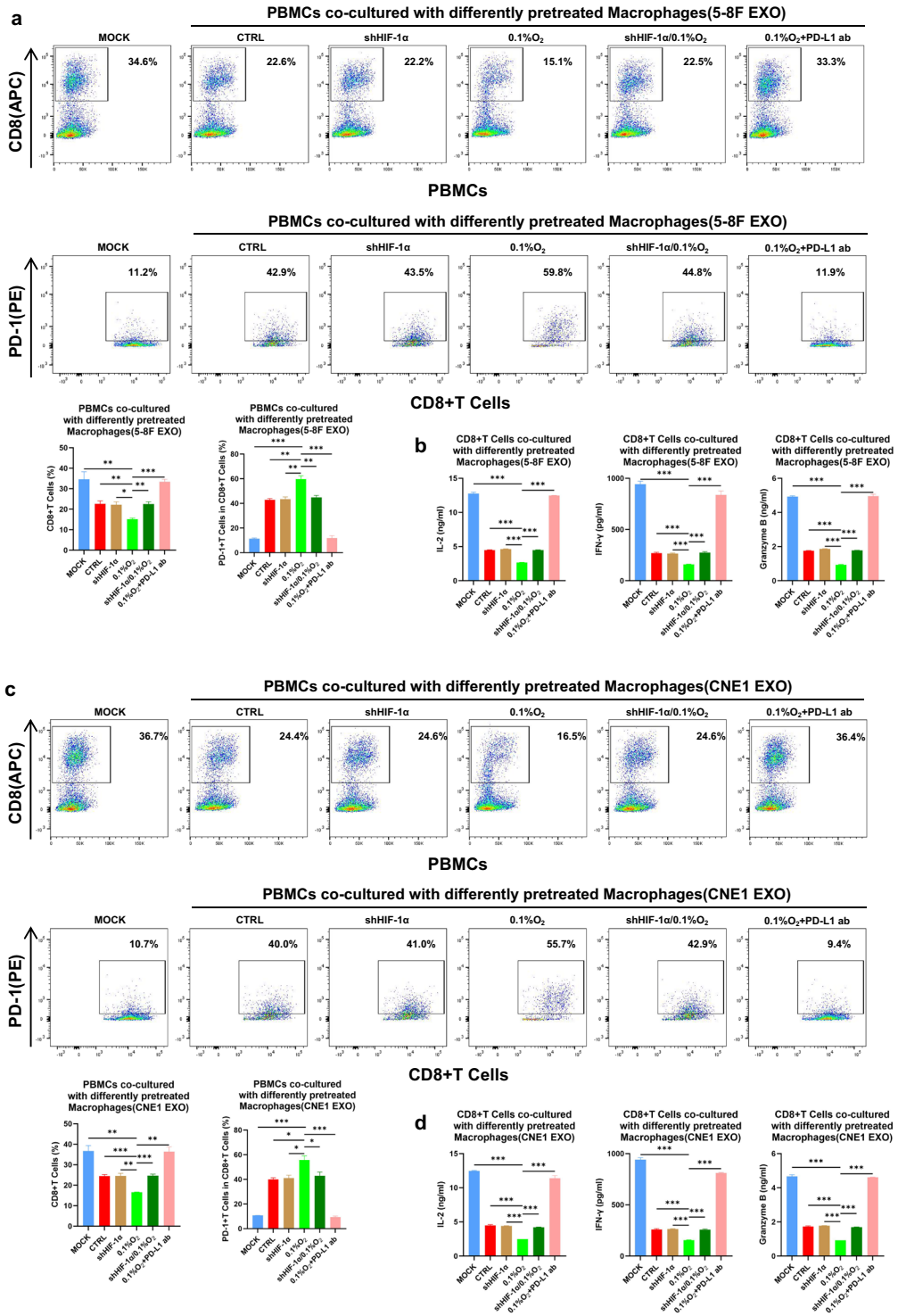


Fig. 6 PD-L1$^+$ macrophages induced by exosomal PD-L1 derived from hypoxic NPC cells could inhibit CD8$^+$ T cells. **a** Flow cytometry was performed to detect the levels of CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells in PBMCs co-cultured with differently pretreated macrophages (macrophages were co-cultured with exosomes derived from differently pretreated HK-1 cells). **b** IL-2, IFN-γ and Granzyme B levels were detected in the culture supernatants of human peripheral CD8$^+$ T cells co-cultured with macrophages that underwent different pretreatments (macrophages were co-cultured with exosomes derived from differently pretreated HK-1 cells) by ELISA. **c** Flow cytometry was performed to detect the levels of CD8$^+$ T cells and PD-1$^+$ CD8$^+$ T cells in PBMCs co-cultured with differently pretreated macrophages (macrophages were co-cultured with exosomes derived from differently pretreated HNE-1 cells). **d** IL-2, IFN-γ and Granzyme B levels were detected in the culture supernatants of human peripheral CD8$^+$ T cells co-cultured with macrophages that underwent different pretreatments (macrophages were co-cultured with exosomes derived from differently pretreated HNE-1 cells) by ELISA. *Notes*: $*p<0.05$, $**p<0.01$, $***p<0.001$; ns, not significant

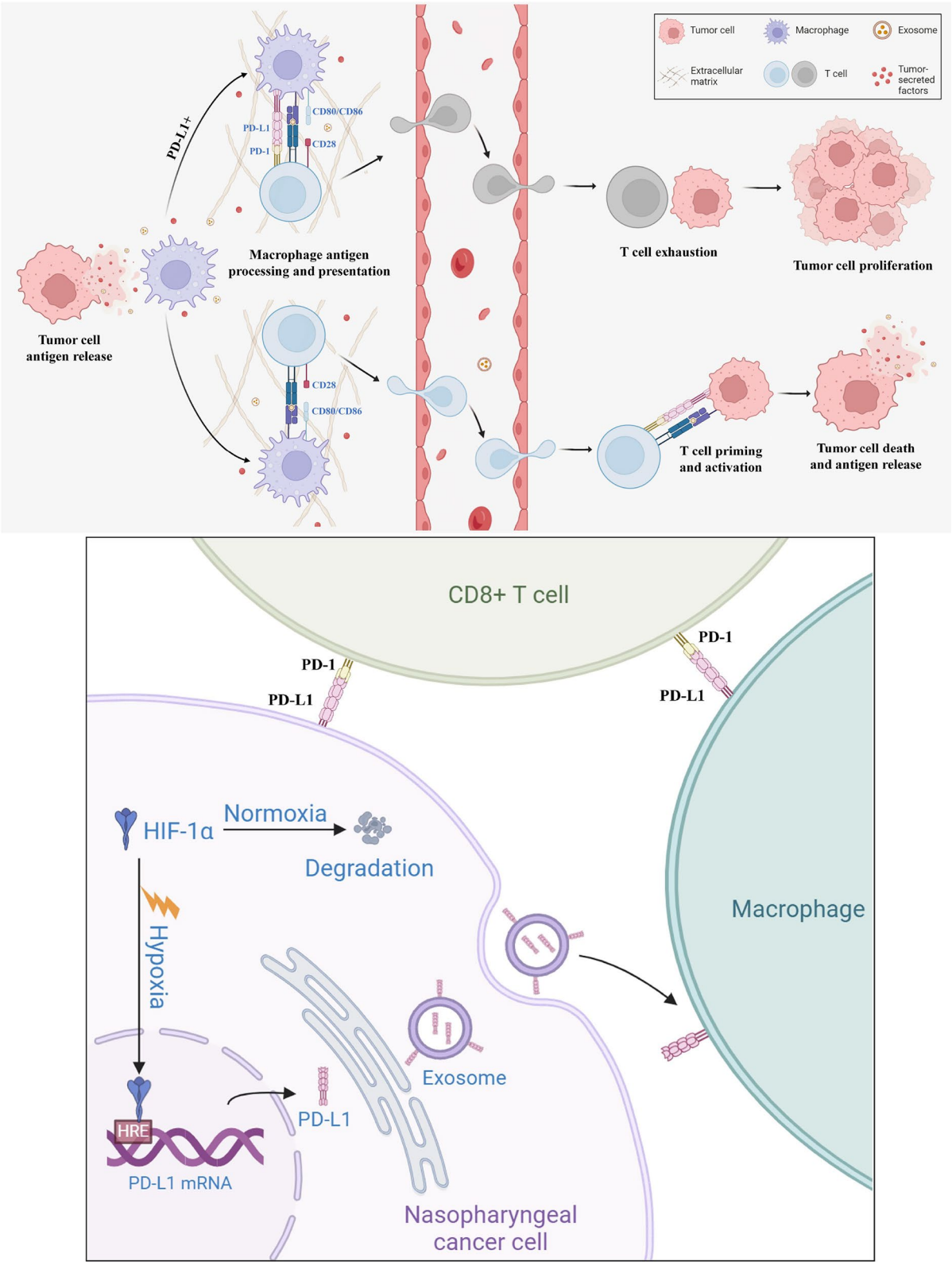


Fig. 7 The schematic diagram of this article. **a** Immune response process of NPC. **b** Hypoxia (via HIF-1α) upregulated the expression of PD-L1 in exosomes derived from NPC cells, while macrophages induce the suppression of $CD8^+$ T cells by phagocytosis of exosomal PD-L1

downregulated. This discrepancy may be due to alternative mechanisms, such as protein-mediated regulation via exosomes. In addition to miRNAs, exosomes also carry proteins, including immune-related molecules, which could influence the immune response [33]. One possibility is that exosomal proteins, such as interferon-stimulated genes (ISGs), may be involved in the regulation of PD-L1 expression [34]. The activation of ISGs, particularly in the context of innate immunity and interferon signaling, may modulate the immune functions of macrophages, including their ability to upregulate PD-L1 expression in response to tumor-derived signals. These interactions highlight the complexity of immune modulation within the tumor microenvironment and emphasize the need to explore both the miRNA and protein contents of exosomes in regulating PD-L1 expression. A recent study by Yang and colleagues showed that abundant PD-L1-enriched exosomes could derive from both NPC cells and the plasma of NPC patients [20]. Therefore, we hypothesized that macrophages might upregulate their PD-L1 expression through the phagocytosis of exosomal PD-L1 secreted by NPC cells. In our study, we found that hypoxia enhanced the expression of exosomal PD-L1 secreted by NPC cells. We further confirmed that macrophages could upregulate their PD-L1 expression through internalization of exosomal PD-L1. In addition, macrophages that had phagocytosed exosomal PD-L1 could induce $CD8^+$ T cell exhaustion and reduce their proliferation. Our study elucidated the mechanisms underlying PD-$L1^+$ macrophage formation and their immunosuppressive role in NPC, providing insight for the development of rationally designed therapies to counteract the immunosuppressive tumor microenvironment.

Furthermore, we observed interesting results indicating that NPC patients who exhibited higher HIF-1α expression or higher CD8 expression were closely associated with worse 5-year PFS and OS. This finding reveals that the HIF-1α and CD8 expression display opposite trends in terms of their correlations and prognostic significance. The relationship between $CD8^+$ T cells infiltration and cancer prognosis has long been controversial. In the study by Blessin et al. [35], high $CD8^+$ T cells infiltration was associated with a better prognosis in colorectal carcinoma, a worse prognosis in renal cell carcinoma, and showed no significant association with prognosis in breast carcinoma, ovarian carcinoma, pancreatic carcinoma, or gastric carcinoma. Therefore, further in-depth research is warranted to elucidate the interactions and effects of $CD8^+$ T cells on tuomor cells (including NPC) and within the TME.

In conclusion, considering the immune response process associated with NPC (Fig. 7a), we propose a novel model that incorporates progressive immunosuppression in NPC (Fig. 7b). Our study revealed that hypoxia, mediated by HIF-1α, induces upregulation of PD-L1 expression both in NPC cells and tumor-derived exosomes. Macrophages subsequently upregulate PD-L1 expression through phagocytosis of exosomal PD-L1. In addition, PD-$L1^+$ macrophages promote immune escape by inducing $CD8^+$ T cell exhaustion and reducing their proliferation. These findings provide new insights into the mechanisms of resistance to anti-PD-1/PD-L1 therapy in NPC, and suggest that targeting PD-$L1^+$ macrophages may represent a promising strategy to enhance the efficacy of immune checkpoint blockade in NPC. Recent study suggests that antihistamines, such as cetirizine, may enhance the efficacy of immune checkpoint inhibitors by promoting M1 macrophage polarization in advanced cancers [36]. Although we did not assess antihistamine use or allergy status in our NPC cohort, this emerging evidence warrants future investigation into how concomitant medications might influence immunotherapy outcomes in NPC. However, it is important to note that our current study is limited to in vitro experiments and clinical sample analysis. The use of in vitro models may not fully recapitulate the complexity of the tumor microenvironment in vivo, including immune system interactions and drug responses. While the findings are promising, future studies involving in vivo animal models will be essential to validate our results and better understand the therapeutic potential of targeting the HIF-1α pathway or PD-$L1^+$ macrophages in NPC. In particular, combining HIF-1α inhibition or depletion of PD-$L1^+$ macrophages with existing immune checkpoint blockade therapy may enhance treatment efficacy. However, the challenges of such combination approaches include potential off-target effects, toxicity, and the need to optimize dosing and delivery methods. These animal studies will help confirm the role of these mechanisms in immune escape and the overall efficacy of immune checkpoint blockade therapies.

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Author contributions X.Y., X.L. and D.J. contributed equally to this work. X.Y., X.L. and D.J.: Conceptualization; Methodology; Data curation; Formal analysis; Writing—Original Draft; Writing—Review & Editing. Z.Z.: Conceptualization; Methodology; Writing—Review & Editing. X.M. and S. W.: Resources: Statistic analysis; Visualization; Writing—Review & Editing. X.L., J.L.and M. F.: Conceptualization; Methodology; Supervision; Funding acquisition; Project administration; Writing—Review & Editing. All authors read and approved the final version of the manuscript.

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Data availability The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethical approval This study was approved by the Ethics Committee of Nanfang Hospital of Southern Medical University (Approval no.: NFEC-2017-165). All participants provided written informed consent for the use of their samples and data in research. The study adhered to the Declaration of Helsinki and ensured participant confidentiality.

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