Unveiling the Mechanisms of Immune Suppression by Quiescent Cancer Cells in Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) poses a significant clinical challenge due to its aggressive nature and limited targeted treatment options. New treatments are emerging with greater outcomes such as immune checkpoint blockade (ICB) which has recently been approved by the FDA (Shah et al., 2022). Clinical trials have proven that ICB can improve outcomes in TNBC patients, but the response rate is only 20%. This highlights the need to understand the mechanisms that lead to resistance to ICB in these types of cancers. Recent data (Baldominos et al., 2022) reveals that in the tumor microenvironment (TME), a subset of quiescent cancer cells are immune-invasive, resistant to immune cell killing, and possess stem cell-like properties.

This thesis tries to investigate 1) the induction of these quiescent cancer cells (QCCs) in TNBC, and 2) the possible mechanism by which QCCs alter dendritic cell (DC) function.

In the first part of the study, we studied the induction and characteristics of QCCs within the TNBC TME. Previous work in the lab had shown that QCCs show a hypoxia-induced gene expression signature. To address the role of hypoxia in the induction of QCCs, I utilized experimental approaches to mimic hypoxia and hypothesized that TNBC cells induce quiescence through the upregulation of HIF1a.

Furthermore, we described that QCCs are highly glycolytic related to hypoxia-induced transcriptional signatures. Thus, in the second part of the study, we examined the role of cancer-immune metabolism, such as lactate, in modulating DC activation and function. Through in vitro experiments using TNBC cell lines and primary DC cultures, we hypothesized and demonstrated that lactate in the environment impairs DC activation. These findings provide valuable insights into the interplay between cancer cell metabolism and immune cell function in TNBC, with implications for immune evasion and tumor progression.

This thesis aims to unveil the mechanism of QCC induction and how it shapes the immune suppressive environment through lactate-impairing DC activation and function. This study sheds light on the interactions between cancer cell metabolism, immune responses, and tumor dormancy in TNBC. These findings may help the development of novel therapeutic approaches aimed at targeting metabolic vulnerabilities and restoring immune surveillance in this aggressive subtype of breast cancer.

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Chapter 1

1. Introduction

1.1. Cancer Immunotherapy

Cancer immune surveillance is the front line of our immune system to recognize and eliminate tumor cells. However, tumor cells are able to evade the immune system through a variety of mechanisms: 1) down-regulate their major histocompatibility complex class I (MHC-I) to decrease immunogenicity; 2) up-regulate immune checkpoint ligands to prevent immune cell killing; 3) secrete metabolites to suppress immune cell function or recruit suppressor immune cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg) that will suppress T-cell killing. The tumor microenvironment (TME) is immune-suppressed, hypoxic, and nutrient-deficient for immune cells to execute their functions—resulting in tumorigenesis (Kim & Cho, 2022).

Immunotherapy has been widely used to utilize our immune system to fight against diseases such as cancer. One of which, targets immune checkpoints upregulated by tumor cells, is called immune checkpoint blockade (ICB). Immune checkpoints are part of the immune system to prevent over-reactive immune response damaging normal tissue. Cytotoxic T lymphocyte–associated protein 4 (CTLA-4) is a checkpoint protein that plays a crucial role in regulating the activation of T cells, which are a key component of the immune system's response against cancer cells. After the T cell is activated by antigen-presenting cells (APC) through major histocompatibility complex (MHC) class II (MHC II) and T cell receptor (TCR), CTLA-4 is upregulated as a brake of T cell immunity, binding to its ligand B7 on APCs will dampen their response (Fig.1a). This is detrimental in cancer patients as T cell antitumor immunity is dysfunctioned (He & Xu, 2020).

While tumor cells themselves do not typically express B7 ligands as APCs, they can indirectly influence the immune response by modulating the expression of molecules involved in immune regulation, such as ligand-programmed death ligand-1 (PD-L1). Tumor cells utilize and upregulate PD-L1 on their surface interacting with programmed cell death protein (PD-1) on T cells, inhibiting their cytotoxic function and leading to immune tolerance (Ramos-Casals et al., 2020). These two immune checkpoints are the major focus of ICB design to facilitate antitumor immunity by preventing inhibitory signaling on T cell killing (Fig. 1b).

ICB blocks this checkpoint protein from binding to its partner protein allowing the T cell to elicit cytotoxic function; This goal can be accomplished through the inhibition of CTLA-4 or PD-1/PD-L1 pathways by antibodies, either individually or in combination. Blocking immune checkpoints using antibodies that specifically target CTLA-4 and the PD-1/PD-L1 has shown potential in treating various types of cancers. Nonetheless, cancers respond to anti-PD-1/PD-L1 and CTLA-4 monoclonal antibodies differently and predictions are still quite limited due to the lack of biomarkers or systemic immune activation (Fitzsimmons et al., 2023).

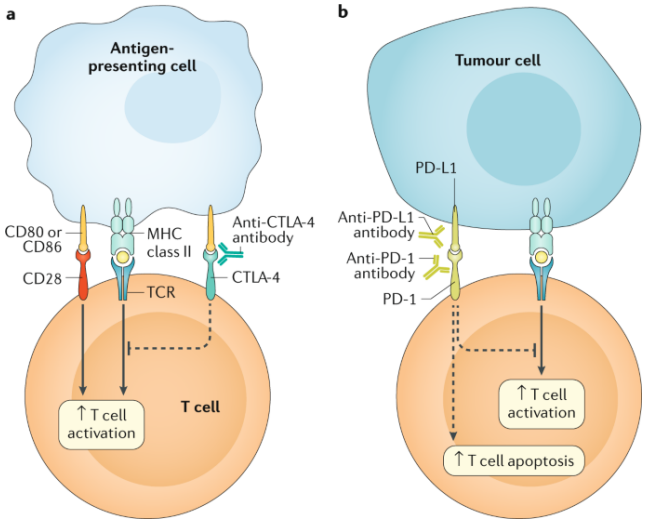


Figure 1. Immune checkpoint blockade (ICB) schematic a) T cell activation requires two signals: one is antigen presented by the antigen-presenting cells such as dendritic cells through major histocompatibility complex (MHC) class II and TCR, costimulatory molecules CD80/86 and CD28. CTLA-4 binding will impede T cell activation and function. b) PD-L1 expressed by tumor cells binding to PD-1 on T cells can impair T cell activation leading to T cell exhaustion and apoptosis. PD-1 and PD-L1 inhibitors block the PD-1–PD-L1 interaction, facilitating T cell activation and survival. Image modified from https://www.nature.com/articles/s41572-020-0160-6/figures/1.

1.2. Triple Negative Breast Cancer (TNBC)

Triple-negative breast cancer (TNBC) is one of the most aggressive forms of breast cancer that lacks estrogen receptor (ER) progesterone receptor (PR), and HER2 gene amplification expression. Similar to other types of breast cancer, TNBC exhibits biological heterogeneity, resulting in varied clinical and epidemiological characteristics. However, unlike other clinical subtypes, TNBC currently lacks targeted therapy tailored specifically to the tumor. This leads to poor prognostic outcomes and the use of targeted treatment of the disease. TNBC also exhibits higher levels of tumor mutation burden, increased expression of PD-L1, and greater infiltration of tumor-infiltrating lymphocytes (TILs) into the tumor microenvironment. This characteristic makes ICB a potentially ideal treatment benefiting TNBC patients (Zhu et al., 2023). New therapies are emerging with greater outcomes such as the ICB drug Pembrolizumab targeting PD-L1 which has recently been approved by the FDA. However, TNBC's resistance to treatment due to its lack of markers and tumor heterogeneity remains a significant issue (Cortes et al., 2022). The current clinical primarily focuses on primary tumor surgical resection, chemotherapy, or radiation therapy. However, these efforts with limited target on TNBC tumor heterogeneity to preventing tumor relapse, which remains a critical concern following tumor metastasis.

1.2.1 Quiescent Cancer Cell (QCC)

With advanced clinical treatment like chemotherapy and published immunotherapy drugs, the overall TNBC patients' survival rates increase. However, not all patients respond to treatment with subsequent tumor relapse, which may be due to tumor heterogeneity, so some immune-evasive tumor cells are not targeted. In 2022, Baldominos et al. discovered a subset of tumor cells, called quiescent cancer cells (QCC), forms clusters with reduced immune cell infiltration in TNBC

tumors. They also processed great tumor-initiating potentials with immune evasion, chemotherapy resistance, and stem-cell-like genes. This cell population plays a major role in subsequent tumor relapse. Precise spatial analysis was used to profile TILs and tumor cells in and outside the QCC niche. Single-cell RNA sequencing and transcriptomics revealed a hypoxia-induced program. In those clusters, a larger proportion of exhausted T-cells were identified. QCC are nonproliferating cancer cells that can arrest their cell cycle in the G0 phase, identified by $Ki67^{low}$ and $p27^{high}$ (Baldominos et al., 2022). As most of the current therapy targets rapidly proliferating cancer cells, QCC that stays 'dormant' evades therapies and repopulates tumors after therapies. Thus, it is likely that by resisting therapies, QCCs contribute to the persistence of residual tumor cells and eventual recurrence.

1.2.2 Quiescent cancer cell evasive properties

Kabraji et al. conducted a study to analyze the percentage of QCCs in primary TNBC tumor samples both before and after multidrug, multicycle neoadjuvant chemotherapy. They found that the proportion of QCCs was higher in mastectomy specimens obtained after treatment compared to the biopsies taken before treatment (Kabraji et al., 2017). Baldominos et al. utilized a mVenus-$p27K^{-}$, a cell cycle fluorescent reporter to visualize QCCs in conjunction with CD3 staining to observe the QCC niche (Oki et al., 2014). They discovered that the area of QCCs, had minimal infiltration of CD3-positive T-cells. Subsequent profiling of QCCs showed a hypoxic signature and Hyproxyprobe (Pimonidazole) labeling confirmed that QCCs reside in hypoxic areas. This indicates a potential involvement of hypoxia-inducible factor 1-alpha (HIF1a) in regulating cell cycle arrest. They also revealed that QCC residing in hypoxic TME displayed enhanced glycolytic phenotypes than the heterogenous tumor cell population, resulting in a nutrient-deficient

environment (Baldominos et al., 2022). By integrating findings from single-cell RNA sequencing, which reveal elevated expression of genes associated with resistance to chemotherapy, initiation of tumors, glycolysis, and hypoxia, Quiescent Cell Clusters (QCC) orchestrate immune evasion by inhibiting T cell activity within a hypoxic, immune-suppressive tumor microenvironment (TME). Apart from excluding immune cells within the TME, QCCs that arrest their cell cycle are more susceptible to immune editing, such as diminishing tumor antigen presentation by downregulating MHC-II and increasing engagement of PD-1/PD-L1 in primary mammary carcinoma (Payne et al., 2016).

1.3. Hypoxia-inducible factor 1

Hypoxia-inducible factor 1 (HIF-1), a dimeric protein complex discovered in 1993 by Gregg L. Semenza, has emerged as an important transcriptional activator and serves as a central player in the body's response to low oxygen levels (hypoxia). It has garnered significant attention in oncology research as a master regulator of cancer progression. HIF-1 orchestrates the expression of genes involved in angiogenesis, maintenance of cancer stem cell phenotypes, extracellular matrix remodeling, invasion, metastasis, and metabolic reprogramming. HIF-1's role in cancer progression raises therapeutic prospects. Conversely, inhibiting HIF-1 in QCC could prevent tumorigenesis by thwarting angiogenesis, inhibiting metastasis, and metabolic reprogramming. The potential for manipulating HIF-1a transcriptional activity through small molecules offers promising avenues for therapeutic intervention. Although gene therapy has shown promise in preclinical studies for simultaneously promoting vessel proliferation and inducing tumor regression, further refinement is needed before clinical application (Ziello et al., 2007).

1.3.1 HIF1a signaling and mechanism

HIF1 complex consists of a HIF1b subunit and one of three subunits: HIF1a, HIF2a, or HIF3a. HIF1a contains PAS A/B domains and one bHLH domain that mediates the HIF1a-HIF1b heterodimer and is also responsible for DNA binding. It also has an oxygen-dependent degradation domain (ODDD) with two prolyl residues: Pro402 and Pro564; these are susceptible to hydroxylation by von Hippel-Lindau tumor suppressor gene product (pVHL) under normoxic conditions. Under hypoxic conditions, HIF1a translocates to the nucleus and dimerizes with HIF1b, and functions as a transcriptional activator. Various post-translational modifications, such as hydroxylation, acetylation, and phosphorylation, play crucial roles in modulating the stability and function of HIF-1a.

The HIF1a mRNA is constantly produced and translated into the cytoplasm, under normoxia with oxygen, HIF1a is subjected to be hydroxylated by prolyl hydroxylase (PHD)—addition of -OH group on two prolyl residues. pVHL has conserved hydroxyproline-binding pockets that recognize the hydroxylated residues. This engagement elicits a VCB E3 ubiquitin-protease pathway that rapidly degrades HIF1a. Under hypoxia where there is limited oxygen, the prolyl subunits on HIF1a won't get hydroxylated. The active HIF1a-HIF1b heterodimeric transcription factor complex is stabilized, enters the nucleus binds to hypoxia-responsive elements (HRE), and accumulates (Fig.2) (Weidemann & Johnson, 2008). Its stabilization will induce HIF-1 target genes that regulate cell proliferation and survival such as insulin-like growth factor-2 (IGF2*)* and

transforming growth factor-a (TGF-a); it also regulates the expression of the glycolytic pathway as well as glucose transporters such as GLUT1 and GLUT3 (Lee et al., 2004).

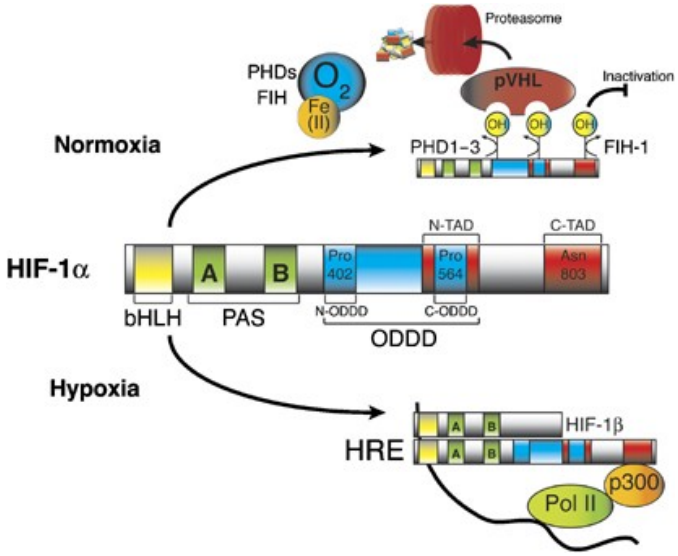


Figure 2 Biology of HIF1a Under normoxic conditions, HIF-α subunits have a very short half-life. HIF1a will be constantly degraded by the ubiquitin-proteasome pathway in normoxia. Under hypoxia, prolyl hydroxylation is suppressed, HIF-α protein escapes proteasomal destruction and can stabilize and accumulate. Image modified from: https://www.nature.com/articles/cdd200812/figures/1

1.3.2 Hypoxia and HIF1a in tumor microenvironment

Tumor hypoxia, a hallmark of the TME, profoundly influences cancer progression and treatment outcomes. Among the key regulators of cellular responses to hypoxia is HIF1a which orchestrates adaptive mechanisms to sustain tumor growth and survival. Under hypoxic conditions, HIF-1α stabilization and nuclear translocation occur, leading to the activation of genes involved in immune modulation. HIF-1α-mediated upregulation of immunosuppressive factors, such as PD-L1, creates an immunosuppressive milieu within the tumor microenvironment (Noman et al., 2014). Major components of tumor-infiltrating myeloid cells such as MDSC promote tumor progression by inhibiting the anti-tumor function of T cells; hypoxic stress induces PD-L1 upregulation resulting in an MDSC-mediated immune suppression. Consequently, T cell activation and proliferation are impaired, inhibiting effective cellular immune response and eventually leading to immune escape.

Moreover, HIF-1α promotes the recruitment of regulatory T cells (Tregs), further dampening immune surveillance and fostering immune evasion by tumor cells (Wei et al., 2015). Tregs express co-inhibitory molecule CTLA-4, cytokines production such as TGF-b and IL-10, and competitive binding of interleukin-2 (IL-2) directly contributes to impaired T cell functions. It is also reported that hypoxia impairs antigen presentation, the fundamental key of the immune response, through upregulating immunosuppressor indoleamine 2,3-dioxygenase (IDO) production in dendritic cells. IDO inhibition in hypoxia-environment DC reversed MHC-II and CD86 upregulation(Song et al., 2018). Thus, implicating HIF1a as a hypoxic molecular target could reveal the mechanism of immune escape and provide insights for tumor immunotherapies.

1.4. Dendritic cell

Dendritic cells (DC) are professional antigen-presenting cells and are central for immune recognition and initiation of antigen-specific immunity and tolerance. They have the ability to integrate environmental cues and transfer captured antigens to other immune cells, thereby molding both adaptive and innate immune responses. As the regulator of upstream cytotoxic cell functions, they possess the capacity to coordinate adaptive immune reactions by activating naive T cells and subsequent differentiation of the effector T cell. Through immunomodulatory signals and cytokine, DCs initiate immune response or tolerance. Crucially, DCs also play an essential role in directing the immune system to react to foreign antigens while preventing the development of autoimmune reactions against the healthy, self-tissues.

1.4.1 Dendritic cell maturation and activation

DCs typically reside in peripheral tissues such as the skin, mucosal surfaces (e.g., respiratory, and gastrointestinal tracts), and lymphoid organs (e.g., spleen and lymph nodes), where they are constantly surveilling for foreign pathogens and antigens. DC expresses pattern recognition receptors (PRRs) that enable them to detect specific molecular patterns associated with pathogens (PAMPs) or cellular damage (DAMPs) in the microenvironment. In their resting state, DC receives tonic signals such as Flt3L or type 1 interferon to maintain their survival. Immature DC express chemokine receptors CCR1, CCR2, CCR5, and CXCR1. Upon utilizing a combination of phagocytosis, receptor-mediated endocytosis, and macropinocytosis to internalize foreign antigens, DC undergoes maturation and migration to lymphoid organs; this process is characterized by upregulation of co-stimulatory molecules CD40, B7-1/CD80, and B7-2/CD86. Meanwhile, DC will secrete pro-inflammatory cytokines such as interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-a), and interferon-alpha (IFN-a). Matured DC also modulates their chemokines receptor expression levels of CCR6 and CCR7, upon maturation guiding their migration from peripheral tissue to secondary lymphoid organs; there, they present captured antigen T cells and initiate the adaptive immune response (Dalod et al., 2014).

Common DC precursors derived from hematopoietic stem cells in bone marrow can give rise to plasmacytoid DCs (pDCs) and will migrate into lymphoid and non-lymphoid tissue where they differentiate into conventional DCs (cDCs), that express high-level of MHC molecules and CD11c in both lymphoid and peripheral organs. There are two subsets of cDC classified as type 1 cDC which specializes in the cross-presentation of antigens to $CD8^+$ T cells in promoting Th1 immune response; type 2 cDC involved in presenting antigens to CD4 T cells and promoting Th2 immune response (Eisenbarth, 2019). Such asymmetric antigen-presentation of DC positioning T cell

differentiation influences the characteristics of effector T cells, including Th1, Th2, Th17 cells, and Tregs.

1.4.2 Dendritic cell cross-presentation

DC cross-presentation, particularly by type 1 cDC (cDC1), is a crucial process by which DCs can present exogenous antigens on major histocompatibility complex class I (MHC-I) molecules to activate $CD8^+$ T cells. This allows for a more diverse antigen recognition priming $CD8^+$ T cells, initiating cytotoxic T lymphocyte (CTL) responses against intracellular pathogens and tumor antigens. cDC1 employs endosomal proteases to degrade and subsequently load captured antigens onto MHC class I molecules within endosomal compartments (Nierkens et al., 2013). Hildner et al. adapted *Batf3*$^{-/-}$ mice lacking expression of Batf3 protein, ablating $CD11c^{hi}$ $CD8a^+$ DCs, were defective in cross-presentation with restricted $CD8^+$ T cell response. Moreover, *Batf3*$^{-/-}$ mice were unable to reject highly immunogenic tumors due to failed DC functions priming $CD8^+$ T cells (Hildner et al., 2008). These findings indicate the significance of cDC1 in vivo cross-presentation in eliciting CTL responses.

1.4.3 Dendritic cell metabolism

As DCs undergo maturation and activation, it is conceivable that they adjust their stimulatory capacities following metabolic reprogramming. In both normal and pathological conditions, dendritic cells exhibit dynamic metabolic adaptations to meet their functional demands. One of which is glycolysis, a process by glucose metabolization to produce energy, fuels DC proliferation and activation.

DC activation induces metabolic reprogramming by switching from oxidative phosphorylation (OXPHOS) to glycolysis. DC increases their glycolysis need through increased expression of glycolytic enzymes and glucose transporters, facilitating ATP production and biosynthetic intermediates. Early glycolysis is found to support DC survival and is responsible for co-stimulatory molecule upregulation, while late glycolysis is not essential for DC maturation (Møller et al., 2022). DC activation involves a transition from mitochondrial metabolism to glycolysis under both strong and weak stimuli, supporting the metabolic demands of DC function. Inhibition of glycolysis impairs CCR7 oligomerization, affecting DC motility, and migration to draining lymph nodes (Guak et al., 2018). Meanwhile, the DC antigen presentation process is facilitated by antigen proteolysis and trafficking of MHC to the plasma membrane. This process requires NADPH oxidase 2 (NOX2) as the major cellular ROS energy source, reducing antigen degradation and retaining MHC-loading antigen-peptide. In LPS-activated DCs, mTOR, an upstream regulator of glycolysis, can be activated by PRR signaling through the PI3K/Akt signaling pathway. Inhibiting mTORC, through ablating its mechanistic target tuberous sclerosis complex subunit 1 (TSC1), preserved DC development but resulted in decreased T cell proliferation and reduced cohort of memory-phenotype of $CD8^+$ T cells. This illuminates the metabolic activity of mTOR and contributes to DC function on T cell priming (Shi et al., 2019).

1.4.4 Dendritic cell in antitumor immunity

While most therapy targeting cancer focuses on cytotoxic T cells, there has been shifted attention toward DC function, which set the basis for cancer immunotherapeutic strategies approaches such as neoantigen-pulsed DC vaccine that promote tumor recognition leading to significant tumor regression (Ding et al., 2021).

Being the most upstream mediator in initiating and regulating antitumor immunity, DC is critical for capturing, process, and presenting tumor antigens to T cells, in activating specific immune responses against cancer cells. They patrol the TME and peripheral tissues, constantly uptaking debris from abnormal cells; upon encountering tumor-derived antigens or danger signals, DC initiates an adaptive immune response by presenting tumor antigens to T cells and recruiting TILs to the TME. Meanwhile, different DC subtypes are subjected to build tolerance that supports tumorigenesis. pDC has lower antigen-presentation efficiency compared to cDC; they are more prone to evoke tolerance and induce T cell anergy through secreting tolerogenic factors such as PD-L1, IL-10, and TGF-b (Matta et al., 2010). Interfering such tolerogenic mechanisms through ICB targeting PD-L1 on DC has proven effective in patients with metastatic melanoma, renal cell carcinoma, and non-small lung cancer (Sánchez-Paulete et al., 2016).

Within the TME, characterized by the intricate metabolic alterations driven by cancer cells, it is suggested that nutrient deprivation and metabolic dysregulation hinder DC's conventional functions in antigen presentation and immune activation. Hypoxic or aerobic glycolysis in TME, known as the Warburg effect, indicates that tumor cells competitively uptake high glucose levels. Instead of converting glucose to pyruvate entering the tricarboxylic acid (TCA) cycle, cancer cells secrete lactate as their metabolic product (Warburg et al., 1927). Lactate accumulation in the environment will acidify the TME which favors tumorigenesis processes such as metastasis, angiogenesis, and most importantly, immune suppression (de la Cruz-López et al., 2019). Lactate-mediated signaling will damage DC function including their activation, and expression of pro-inflammatory factors, and facilitate antigen degradation by downregulating their membrane trafficking proteins (Manoharan et al., 2021). Such twisted metabolism will not only restrict DC

differentiation and maturation but also generate an acidic and hypoxic TME limiting DC infiltration.

DCs are believed to play an important role in the initiation and programming of the antitumor response. In the antitumor immune response, the pathway of priming and activating tumor-specific CD8+ T cells focuses mainly on DC cross-presentation. DCs residing in tumor-draining lymph nodes (TDLN) have been observed to engage in the cross-presentation of tumor antigens to naive CTLs. Pilato et al. also revealed how $CCR7^{+}$ DCs expressing CXCL16 in the tumor microenvironment create a specialized niche where CTLs are positioned. This facilitates the local expansion and survival of CTLs through the presentation of interleukin-15 (IL-15), a vital cytokine for CTL persistence and anti-tumor activity (Pilato et al., 2021).

In cancer patients, DC detected in tumor tissue or TDLN displayed low expression of CD80, CD86, or CD86 indicating altered DC functions (McDonnell et al., 2010). Thus, given the central importance of DCs, particularly cDC1, in orchestrating antitumor immune responses, any activation or maturation dysfunction, as well as in their abilities to provide co-stimulatory signals and present antigens, have the potential to hinder the development of effective adaptive immune responses against tumors.

Chapter 2

2. Materials and Methods

2.1 Mice

Animals were housed at the Dana-Farber Cancer Institute animal facility and all procedures performed were approved by the DFCI-IACUC under protocol number #17-017. The study is compliant with all relevant ethical regulations regarding animal research.

2.2 Cell lines

4T07 TNBC cell line was available in the laboratory. All cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM), 10% heat-inactivated FBS (Gibco), 1% Glutamax (Gibco) and 1% Penicillin-Streptomycin (Life Technologies).

2.3 Generation of HIF1a knock-out cell

The HIF1a KO was generated from 4T07 p27-mVenus mCherry cells. Amaxa 4D-Nucleofector X Unit device (Lonza) was used to generate knockout cell lines through CRISPR/Cas9-mediated genome editing. Briefly, 2.5μL 200μM trRNA, 2.5μL 200μM gRNA, and 5μL 20μM recombinant CAS9 (Integrated DNA Technologies) were mixed to make Cas9/gRNA ribonucleoprotein (RNP). The RNP was delivered into 4T07 TNBC cell lines using program A549 by SF Cell Line 4D-NucleofectorTM X Kit S (Lonza). 200,000 cells were used per reaction and recovered in a 6-well plate for 4 days after nucleofection, and then subjected to a 2nd round of the same process. HIF1a$^{-/-}$ cells were validated by flow cytometry using the HIF1a antibody from Biolegend. The guide RNA sequence is HIF1a (AGTGCACCCTAACAAGCCGG)

2.4 Flow cytometry

All antibodies were purchased from BioLegend (San Diego, CA). 4T07 TNBC cells were stained with Alexa Fluor® 647 anti-human HIF1α antibody. Dendritic cells were stained with anti-mouse CD86 APC, CD11c PE, and MHC-II (I-A/I-E) PB antibodies. Antibodies were diluted 1:100 in FACS buffer (PBS, 5% BSA, 2 mM EDTA). Cells were resuspended in FACS buffer with 1:10,000 DAPI to discriminate the dead and live cells. All flow cytometry experiments were performed using a CytoFLEX flow cytometer instrument (Beckman Coulter, Brea, CA). Analysis of flow cytometry data was done with Flowjo software for Mac (FlowJo LLC, Ashland, OG).

2.5 HIF1a Trans-Nuclear staining

For intracellular HIF1a protein staining, cells were fixed and permeabilized with True-Nuclear Transcription Factor kit (BioLegend). Transcription Factor Fix working solution was prepared by diluting the 4X Fix Concentrate (1 part) with the Fix Diluent (3 parts). Perm Buffer was prepared by diluting the 10X Perm Buffer with distilled water. Following the manufacture protocol, 4T07 cells were stained with HIF1a AF647 with 1:100 dilution with True-Nuclear 1X Perm Buffer (1:1000). After 30 min staining incubation, antibody was washed off by flow buffer (2mM EDTA 0.1% BSA PBS).

2.6 Splenic cDC extraction and isolation

cDC were purified from spleens and lymph nodes (LNs) from mice, extracted, and processed with flow buffer. After grinding, the spleen and LNs were transferred to a 6-well plate tube with 2mL digestion solution (HBSS+10% FBS, 1:100 Col IV, and 1:1000 DNase) and incubated at 37 °C for

20 minutes. Digested samples were then filtered through a 100μm cell strainer along washing with flow buffer, spun down, and resuspended with 1ml of flow buffer. cDC cells were selected with the mouse EasySep™ Mouse Pan-DC Enrichment Kit (STEMCELL) following the manufacturer's instructions. This kit follows immuno-magnetic negative selection to isolate the cDC from the rest of the splenocytes and lymphocytes. They were finally resuspended in an adequate volume of PBS for seeding, approximately 300,000 cells/well in 96 well plates.

2.6.1 In vitro stimulation of splenic cDCs

After seeding, cDC was cultured in corresponding media as negative control; experimental groups and positive control were stimulated with 50μg/mL of Polyinosinic: polycytidylic acid (Poly I: C).

2.7 DC lactate experiment media

Dendritic cells were cultured in 0mM, 20mM, 40mM lactate in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher), 10% heat-inactivated FBS (Gibco), 1% Glutamax (Gibco), and 1% Penicillin-Streptomycin (Life Technologies) with varied glucose.

2.8 DC conditioned media

Seed 4T07 p27-mVenus mCherry WT cells from 100000, 500000, and 1 million in 6-well plates. Use a Hypoxia Incubator Chamber (STEMCELL) and 0.1% oxygen to generate hypoxic and normoxic conditioned media for 48h in a tissue culture incubator. Collect media from the plate and use it to culture DC.

2.8.1 JEDI-T Extraction and Isolation

T cells were purified from spleens and lymph nodes (LNs) from JEDI mice, extracted, and processed with flow buffer. Ground samples were then filtered through a 100μm cell strainer along washing with flow buffer, spun down, and resuspended with 1ml of flow buffer. $CD8^+$ T cells were selected with the mouse EasySep™ Mouse $CD8^+$ T Cell Isolation Kit (STEMCELL) following the manufacturer's instructions. This kit follows immuno-magnetic negative selection to isolate the $CD8^+$ T Cell from the rest of the splenocytes and lymphocytes. They were finally resuspended in an adequate volume of PBS for seeding.

2.8.2 JEDI-T cell culture media

JEDI T cells were cultured in RPMI 1640 Medium(Thermo Fisher), 10% heat-inactivated FBS (Gibco), 1% Glutamax (Gibco), 1% Penicillin-Streptomycin (Life Technologies), and 55uM B-mercaptoethanol.

2.8.3 DC – JEDI-T cell coculture

DCs were isolated as indicated in section 2.6, cultured in 0mM and 40mM lactate and Poly I: C in a 24-well plate for 6 hours. Treat with 100ng/mL GFP peptide for 30 mins. Spin down and resuspend in T cell media. The cell seeding ratio of DC and $CD8^+$ T cells was 1:5 (DC 20,000 cells/well and $CD8^+$ T cell 100,000 cells/well). The coculture period was overnight 16 hours.

3. Result

3.1 Hypoxia upregulates HIF1a and induces quiescence in breast cancer cells

Baldominos et al. discovered a subset of tumor cells that were highly quiescent in the TME, hence, termed quiescent cancer cells (QCC). They cluster together forming an hypoxic and immune-invasive niche that exclude immune cells. These cells possess stem cell-like characteristics and exhibit the remarkable ability to resist direct T cell killing, drive $CD8^+$ T cell exhaustion, and impair Dendritic cell activation and function. These characteristics supports them in cancer initiation, metastasis, and tumor recurrence. Understanding how QCCs are induced and how they drive immune dysfunction is key for the immunotherapy efficacy. First, we aimed to elucidate how QCCs arise in TNBC. We found that hypoxic QCCs displayed an increase of HIF1a-induced genes compared to the rest of tumor cells. Thus, we hypothesized that activation of HIF1a may lead to induction of quiescence, the reciprocal relationship between HIF1a activation and quiescence remains elusive (Fu et al., 2021).

In order to understand the role of HIF1a in QCC induction, we first generated 4T07 cells carrying the mVenus-$p27K^-$ reporter that was HIF1a knockout (KO) cells using the CRISPR-Cas9 system. We used a scramble gRNA to generate a negative control paired cell line. To enhance knockout efficiency, we conducted CRISPR KO twice. Verification of knockout efficiency was carried out by chemically inducing HIF1a stabilization with $CoCl_2$ (cobalt chloride), a chemical stabilizer of HIF1a (Rana et al., 2019). HIF1a protein was quantified by flow cytometry post-treatment through trans-nuclear staining. While $CoCl_2$ increased HIF1a levels in control cells, treatment with CoCl2 yielded no statistical increase in HIF1a expression in HIF1a KO cells compared to DMSO-treated

cells, confirming efficient HIF1a KO (Fig.3). This provided us with a suitable cell line to study the role of HIF1a in the induction of the quiescent phenotype.

To enhance the specificity of the population I tried to investigate, rather than gating the $p27^+$ population, I decided to gate the $p27^+$ high population. This allows for a more precise identification of cells with elevated levels of the p27 marker. By focusing on cells with high p27 expression, I focus on the subset of cells that are most likely to be quiescent. This enhances the specificity of the analysis and reduces the inclusion of heterogeneous cancer cells, including non-quiescent cells that exhibit low to moderate levels of p27 expression.

Then, to test quiescence induction by HIF1a stabilization, I treated both 4T07 WT and HIF1a KO with CoCl2 to measure the p27+ high% cell population using flow cytometry. In WT cells, there was a 5-fold increase in p27 high% following cobalt treatment. Conversely, the HIF1a KO p27 high% increment was not significant (Fig. 4A). Trans-nuclear HIF1a staining shows an increase in WT HIF1a expression after treatment (Fig. 4B). This result aligns with accessing HIF1a KO efficiency, suggesting that CoCl2-induced HIF1a stabilization can promote quiescence. It suggests that CoCl2-induced HIF1a triggers a cellular response characterized by an increase in quiescence, possibly as a survival mechanism in response to oxygen deprivation. The upregulation of HIF1a expression leads to the activation of hypoxia-responsive pathways, which may promote the adaptation of cancer cells to the hypoxic microenvironment. In summary, our data suggest that cancer cells upregulate HIF1a to induce QCC.

Subsequently, we aimed to replicate these results under physiologically relevant hypoxic conditions using a hypoxia incubator chamber. To mimic the high cell density and low oxygen

conditions of the TME, cells were seeded in two 6-well plates at increasing cell densities (100,000 cells/well, 500,000 cells/well, and 1 million cells/well) under normoxic and hypoxic conditions for 48 hours. The results showed a generally higher percentage of $p27^+$ high cells under hypoxia compared to normoxia. In normoxia, the baseline quiescent population was observed which was likely to represent the natural state of cells in the absence of hypoxic stress (Fig. 5A). While in hypoxic condition, cells were exposed to 0.1% oxygen level resembling hypoxic niche within tumors. There was a significant fold change in $p27^+$ high cells in hypoxia compared to normoxia, indicating that hypoxic conditions, combined with increased cell density, promote cell quiescence within the TME. Moreover, with increasing cell densities, $p27^+$ high% had a much higher fold-increase in hypoxia (Fig. 5B). While in both conditions, WT cells processed a much higher $p27^+$ quiescence population compared to HIF1a KO cells; This indicates that wildtype cells respond to hypoxic conditions by entering a quiescent state, potentially mediated by HIF1a signaling pathways. In the absence of HIF1a, cancer cell attenuates the cellular response to hypoxia and mitigate the induction of quiescence but obtain a higher proliferation rate than WT. Thus, the result indicates a correlation between cell density and the induction of QCC under hypoxic conditions (Fig. 5C).

Collectively, these *in vitro* findings suggest that stabilization of HIF1a induces quiescence, shedding light on the mechanisms underlying tumor cell dormancy within the hypoxic TME. The comparisons highlight the role of hypoxia and HIF1a in regulating cell quiescence within the TME and underscore the importance of HIF1a signaling pathways in mediating the cellular response to hypoxic stress.

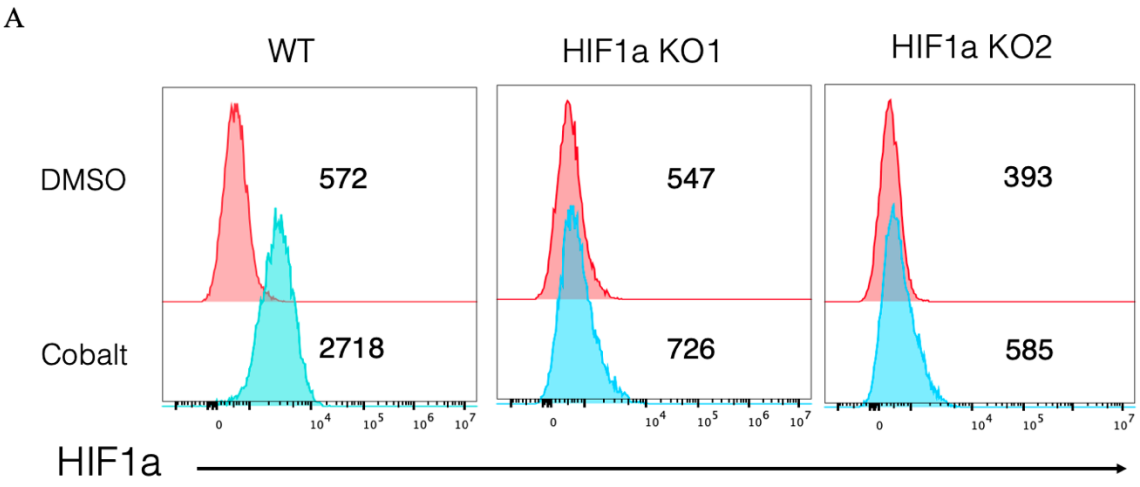


Figure 3. Cobalt treatment to test HIF1a knock-out efficiency. Trans-nuclear staining of HIF1a expression, WT (Red), HIF1a KO (blue).

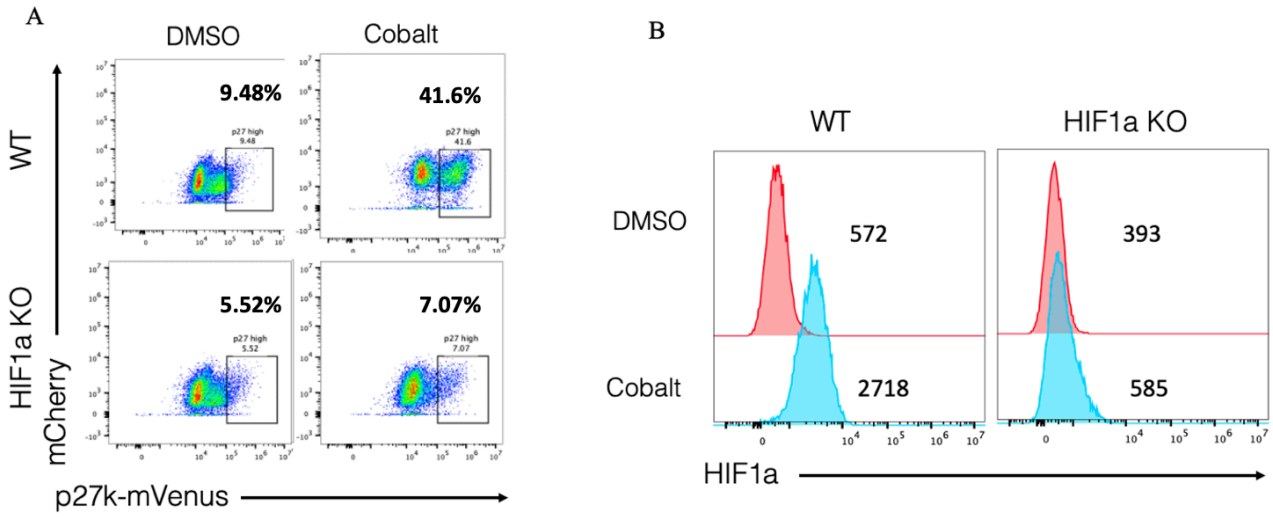


Figure 4. Cobalt Treatment to 4T07 WT cells under normoxia induce quiescence. A) 4T07 tumor cell p27 high% gating. B) Trans-nuclear staining of HIF1a expression in WT (Red) and in HIF1a KO (blue).

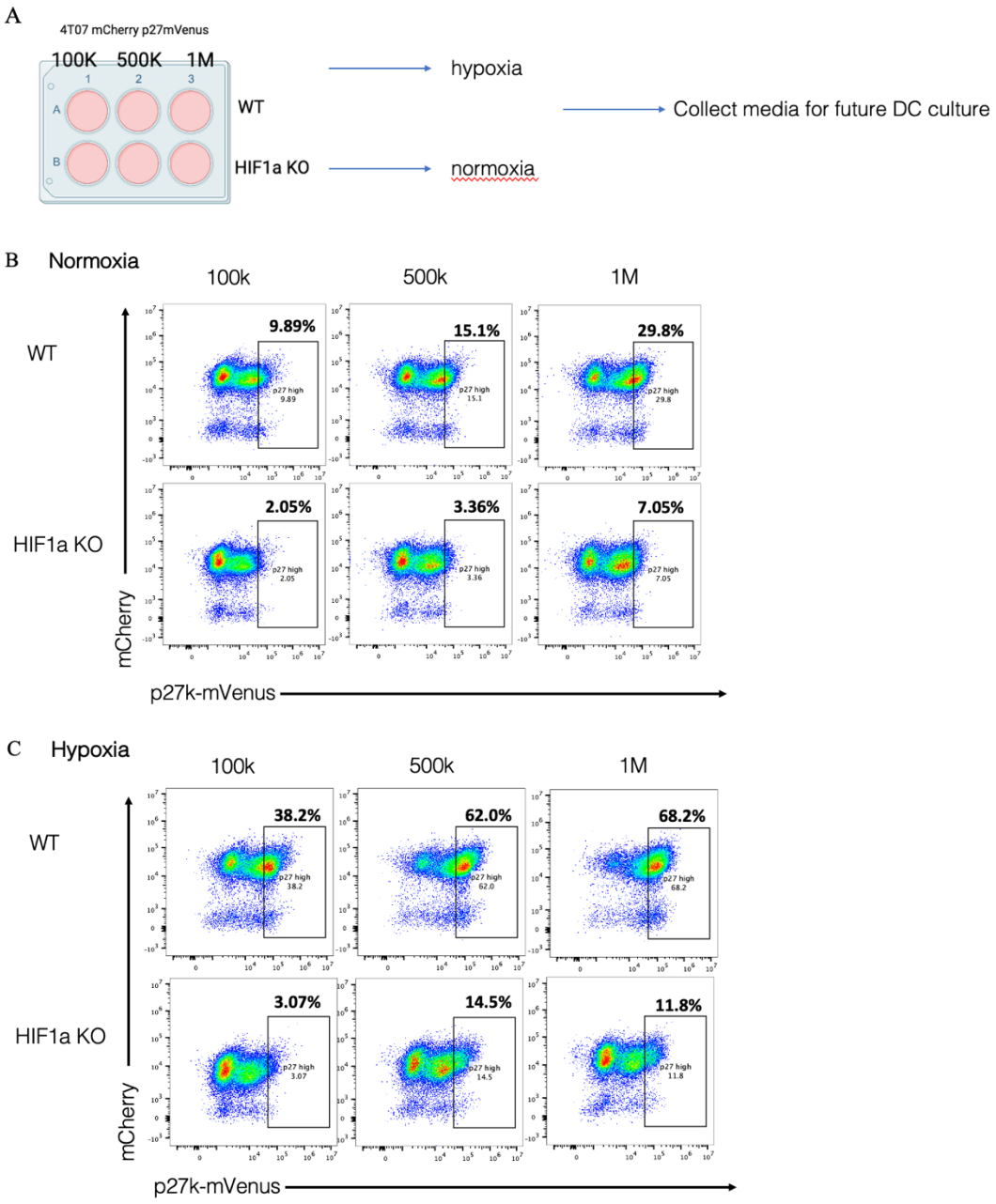


Figure 5. Hypoxia drives quiescence. A) Experiment schematic. B) 4T07 tumor cell p27 high% gate under normoxia with WT and HIF1a KO cells. C) 4T07 tumor cell p27 high% gate under hypoxia.

3.2 Unveiling the Metabolic Crosstalk: Cancer Cells' Impact on Dendritic Cell Function in Breast Cancer

Given the growing attention to the dynamic interactions within the TME, particular attention has been directed towards understanding the interplay between cancer cells and immune cells. DC, as the major antigen-presenting cells, orchestrate the anti-tumor T cell response.

Many studies indicate that cancer cell metabolism facilitates a pro-tumorigenesis environment that is hypoxic, immune-excluded, and evasive (Martínez-Reyes & Chandel, 2021). Cancer cells undergo nutrient scavenging mechanisms, to sustain proliferation and secrete metabolites like lactate that are unfavored by immune cells. Therefore, to elucidate the impact of cancer cell metabolic activity, we designed a nutrient-deficient culture along with lactate to mimic the metabolic properties of the TME. By varying glucose and lactate concentrations, we investigate how metabolite availability influences DC functions, starting with their activation.

3.3 Validation of Dendritic Cell Extraction Efficiency

Splenic dendritic cells (DCs) were isolated using an immuno-magnetic negative selection kit (STEMCELL), yielding approximately 3 - 4x10^6 DCs per extraction. To assess and validate the efficiency of the extraction kit, an experimental setup comprising three groups was devised: 1) Spleens extracted from wild-type mice without DC isolation, 2) Spleens extracted from wild-type mice with DC isolation, and 3) Spleens extracted from macrophage-depleted mice with DC isolation(Fig. 6A). Flow cytometry analysis was conducted using MHC-II and CD11c antibodies to identify and gate dendritic cells, with CD86 antibody staining employed as a marker for dendritic cell activation. We expected to observe an increase in the proportion of DCs identified by CD11c and MHC-II markers. Additionally, we used CD64 staining to exclude macrophages from our analysis (Fig. 6B). Our findings showed that unprocessed spleen samples from WT mice contained only 2.87% DCs, whereas processed samples post-extraction contained 32.8% DCs,

indicating a substantial increase in DC yield. Furthermore, the successful extraction of DCs from macrophage-depleted samples (41.5%) confirms the effectiveness of the extraction kit in isolating dendritic cells (Fig. 6C).

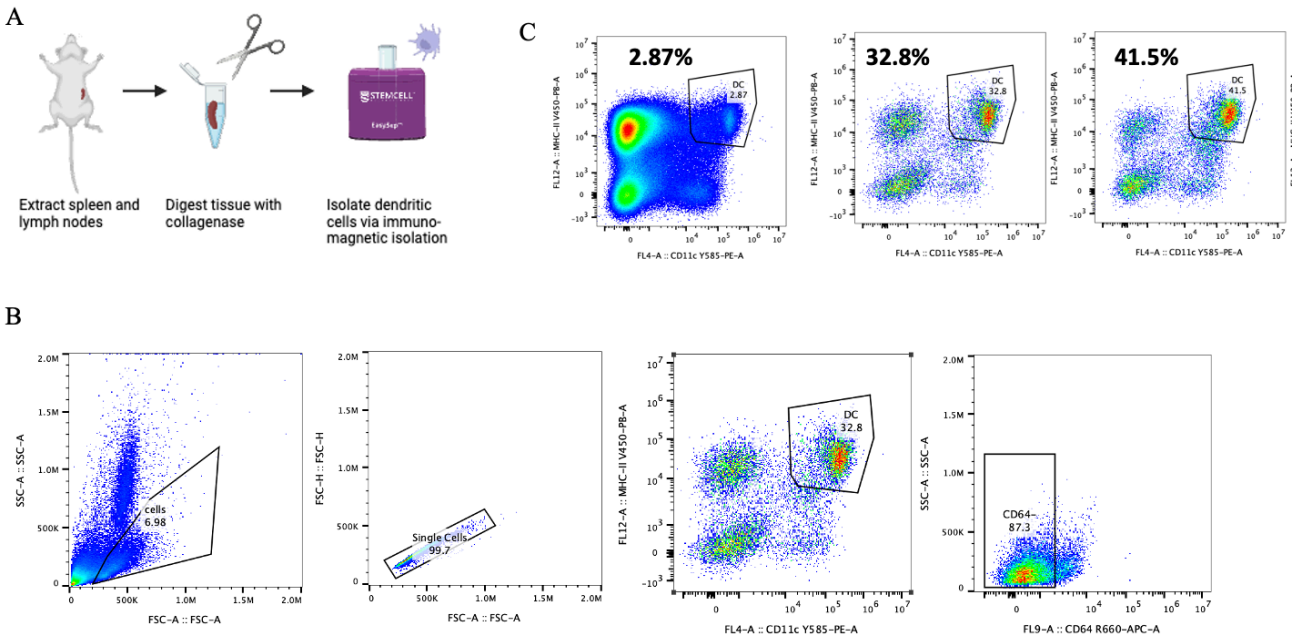


Figure 6. Validation of Dendritic Cells Extraction Efficiency. A) Dendritic cell isolation schematic, B) Gating strategy in splenic DC, stained with CD11c, MHC-II, and CD64. C) DC gating WT unprocessed, WT processed, and macrophage-depleted groups.

3.4 High Cancer Cell Densities Acidify the Media, Impaired Dendritic Cell Activation

We sought to investigate the impact of cancer cell metabolism on DC activation, considering both the creation of a nutrient-deficient TME and the influence of metabolic byproducts on immune cells in the tumor mass. After investigating QCC and gaining insights into their quiescence regulation dynamics, I have redirected my focus toward understanding the role of TNBC QCC metabolism altering DC function. Recognizing the importance of immune cell interactions in shaping the tumor niche, particularly in the context of QCC, I aim to elucidate the impact on DC function through cancer cell metabolite secretion under hypoxic and normoxic conditions.

To address this question effectively, I designed experiments to culture cancer cells under both normoxic and hypoxic conditions, modeling the diverse oxygen gradients present within tumors. Within the culture media, tumor cells secrete tumor-associated antigens, exosomes, and extracellular particles that may affect DC activation. Additionally, I hypothesized that increased cell density within the culture will lead to heightened secretion of metabolites, potentially influencing the behavior of both cancer cells and DCs within the QCC niche.

In order to include variations in cell density and oxygen availability, I cultured 4T07 breast cancer cells under normoxic and hypoxic conditions at different confluences for 48 hours (Fig. 5). Under increasing confluence and oxygen availability, the metabolic profile of the cancer cells would vary. We used the 4T07 conditioned media to add to DCs in vitro. To test the functionality of DCs with the various conditioned media, we used the TLR3 agonist polyI:C, to activate them and quantified their activation by measuring CD86 levels by flow cytometry (Fig. 7A).

Flow cytometry analysis of CD86 levels in DCs revealed that DCs cultured with conditioned media from the lowest cancer cell densities (100,000 cells/well) had a lower activation compared to DCs with conditioned media from high cancer density (500,000 cells/well) (Fig. 7B). Interestingly, DC activation appeared similar regardless of oxygen availability but was dependent on cell density, suggesting that it may be the presence of QCC or their metabolites that alter DC activation. The observed increase in CD86 expression suggests heightened activation of DC in response to higher cell density. This could indicate that the presence of a greater number of cells, regardless of oxygen

levels, triggers an immune response, potentially due to the increased release of metabolites, danger signals, or immunomodulatory factors from the populated cell culture.

Upon extracting media from both hypoxic and normoxic plates, we observed a gradient yellow coloration corresponding to increasing cell densities (100,000, 500,000, and 1,000,000 cells/well), indicating a proportionally lower pH in the media. The change in media color and CD86 mean fluorescence intensity (MFI) decreased between 500,000 and 1M cells/well raising the interest in investigating cancer cells affecting media pH. To minimize the potential influence of pH variation on DC activation, we replaced the culturing media (IMDM) with DMEM supplemented with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). HEPES is known for maintaining physiological pH despite changes in carbon dioxide concentration under high cell density.

There was no clear variation observed between CD86 levels in hypoxic and normoxic media, we proceeded with normoxic conditioned media only, adjusting cell densities to 100,000, 1 million, and 5 million cells/well. Consistently, we observed a trend where conditioned media from higher cell density cultures induced higher CD86 expression, indicative of enhanced DC activation (Fig. 7C) However, a decrease in CD86 (MFI) was again noted between 1 million and 5 million cells/well, which the cultured media was again yellow and acidified. The CD86 MFI drop due to increased acidity elucidates that HEPES may not neutralize pH fluctuations effectively. The pH shift impaired DC activation compared to the previous two density conditions. Moreover, CD86 MFI at 1M cells/well increased unlike the previous result. This elucidates that after adding HEPES, media acidification was countered at 1M confirming that pH, rather than metabolite secretion, is the bigger player (Fig 7B and 7C).

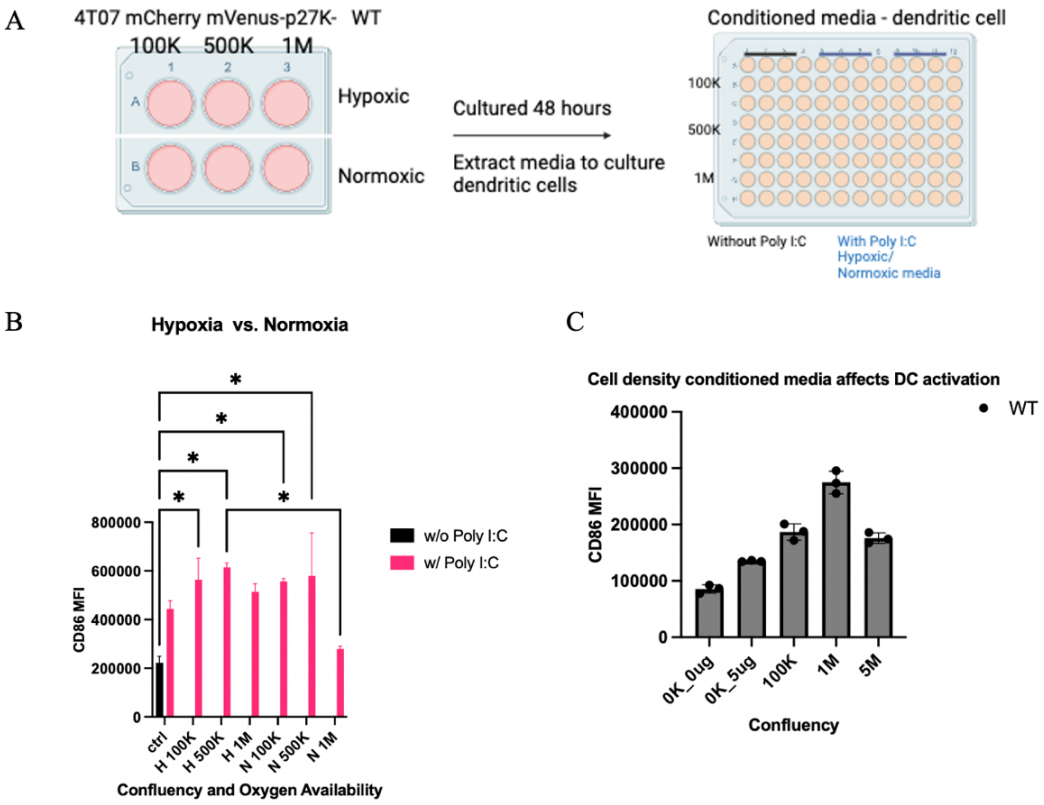


Figure 7. Cancer cell-conditioned media affects DC activation. A) Experiment schematic B) CD86 MFI of Dendritic cells under different cell densities in both normoxia (N) and hypoxia (H). C) CD86 MFI of Dendritic cells under different cell densities in normoxia, with HEPES added to the culture media.

3.5 **Lactate in TME Impairs Dendritic Cell Function**

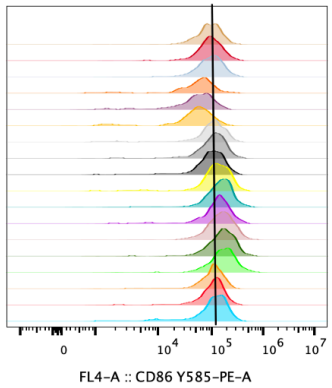
After discovering how cancer cell shapes their immune suppressive environment, as we found out that pH is one of the factors contributing to CD86 downregulation, combining the studies that revealed lactate accumulation drives the environment acidic, I want to delve into the effect of cancer cell metabolism, that is the role of lactate.

In TME, metabolite secreted by cancer cells can influence and impair immune cells in or surrounding the niche, one of which is lactate(de la Cruz-López et al., 2019). Higher confluency and abundance of cancer cells lead to higher lactate in the media. This correlates with the higher density of cancer cells in the QCC niche. To investigate the effect of lactate on DCs we developed

an *in vitro* approach to expose DCs to controlled concentrations of lactate independent of pH and oxygen.

DCs were cultured in different concentrations of lactate in the media (0mM, 10mM, 20mM, and 40mM). Our findings revealed that treatment of DCs with lactate *in vitro* culture led to a dose-dependent reduction in DC activation. Specifically, we observed a decrease in DC activation, as indicated by reduced CD86 expression, in response to increasing lactate concentrations, irrespective of whether the DCs were treated with poly I:C (Fig. 8). The decrease in CD86 expression observed in DC cultured in environments with increasing lactate concentrations suggests potential immunosuppressive effects associated with elevated lactate levels. At concentrations of 20mM and 40mM lactate treatment, CD86 MFI is at a similar level as un-activated DC, negative control 0mM without Poly I:C treatment. Alternatively, lactate could directly interfere with DC maturation processes or intracellular signaling pathways, impairing their ability to activate and express co-stimulatory molecules like CD86 properly. These findings suggest that lactate secreted by cancer cell metabolism in the TME may impair DC activation, thereby potentially compromising immune surveillance and antitumor responses.

A



	Sample Name	Subset Name	Count	Median : FL4-A
	40mM 5ug 3.fcs	DC	327	88915
	40mM 5ug 2.fcs	DC	381	92675
	40mM 5ug 1.fcs	DC	313	94289
	40mM 0ug 3.fcs	DC	162	57052
	40mM 0ug 2.fcs	DC	128	52371
	40mM 0ug 1.fcs	DC	152	53641
	20mM 5ug 3.fcs	DC	500	102441
	20mM 5ug 2.fcs	DC	799	109397
	20mM 5ug 1.fcs	DC	637	96931
	10mM 5ug 3.fcs	DC	596	125643
	10mM 5ug 2.fcs	DC	966	144828
	10mM 5ug 1.fcs	DC	712	131887
	0mM 5ug 3.fcs	DC	693	151508
	0mM 5ug 2.fcs	DC	1043	159050
	0mM 5ug 1.fcs	DC	718	164668
	0mM 0ug 3.fcs	DC	1386	104227
	0mM 0ug 2.fcs	DC	673	108268
	0mM 0ug 1.fcs	DC	699	109019

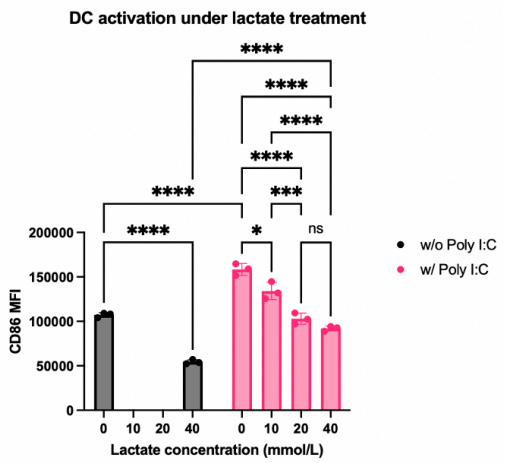


Figure 8. Lactate in the Media Drives Dendritic Cell Activation Dysfunction. (A) Histogram of CD86 MFI in increasing lactate concentration. B) Summary graph of CD86 MFI in increasing lactate concentration.

Along with impaired DC activation by the accumulation of lactate, we wonder if the presence of lactate will influence DC-T cell crosstalk. One of DC's main functions is presenting antigens to and activating T cells, thereby initiating immune responses. Thus, I designed a DC-T cell co-culture to unveil the DC-T crosstalk. Here, I used JEDI (Just EGFP Death-Inducing) $CD8^+$ T cells extracted from JEDI mice, which have an engineered T cell receptor that recognizes green fluorescent protein (GFP) as its T cell antigen. The JEDI model allows us to interrogate the immune interactions by incorporating GFP in the cells of interest (Agudo et al., 2015).

As we understood that increasing lactate concentration can impair DC activation, here, DCs were cultured in specific concentrations of lactate for 8 hours (0mM and 40mM); and their ability to activate $CD8^+$ T cells was studied. Then, I introduced 100ng/mL of GFP peptide onto the MHC-I on DCs. This process over-loads DC's MHC-I with GFP peptide. This can provide a robust system for studying antigen presentation and DC-T cell interactions. Antigen presentation is a complex process involving antigen recognition, co-stimulation, cytokine production, and multiple

checkpoints. Thus, this *in vitro* culture focused on the crosstalk between DC and T cells by the read-out of the T cell activation marker (CD69 expression) without assessing antigen presentation fully (Fig 9A).

After 6 hours of lactate treatment, aliquots of DCs were extracted to check their live/dead and cell loss. The result showed a consistent survival (~95%) and cell population percentage (~30%) of dendritic DCs across lactate concentration groups, as determined by DAPI staining and DC gating, suggesting that lactate does not directly induce cytotoxic effects or alter the overall viability of DCs under the lactate treatment (Fig 9B). Furthermore, manual DC counting shows a similar number of cells before and after lactate treatment indicating it is not affecting DC survival and population—the 30% is consistent to result from DC isolation validation. After confirming this, we shift focus to how DC functions are affected by examining DC-T cell crosstalk in overnight coculture.

The T cell-only condition, which serves as a negative control, did not express CD69 confirming unactivated T cells. Culturing DCs in the presence of 40mM lactate resulted in a significant reduction in $CD69^{+}$% on T cells compared to DCs cultured in lactate-free (0mM) conditions (Fig. 9C). This observation suggests that higher lactate concentrations impair the capacity of DCs to effectively stimulate T-cell activation. Given that CD69 serves as an early activation marker on T cells, its diminished expression indicates a potential suppression of T cell activation.

This finding indicates that the observed differences in T cell activation, specifically the reduced $CD69^{+}$% expression, are unlikely to be attributed to variations in survival or population size of DCs. Instead, it strengthens the hypothesis that the observed impairment in DC-mediated T-cell activation is primarily due to functional changes induced by lactate exposure. Therefore, while

lactate did not appear to affect DC viability in this study, its influence on DC function, particularly in modulating T-cell activation, remains a critical area for further investigation.

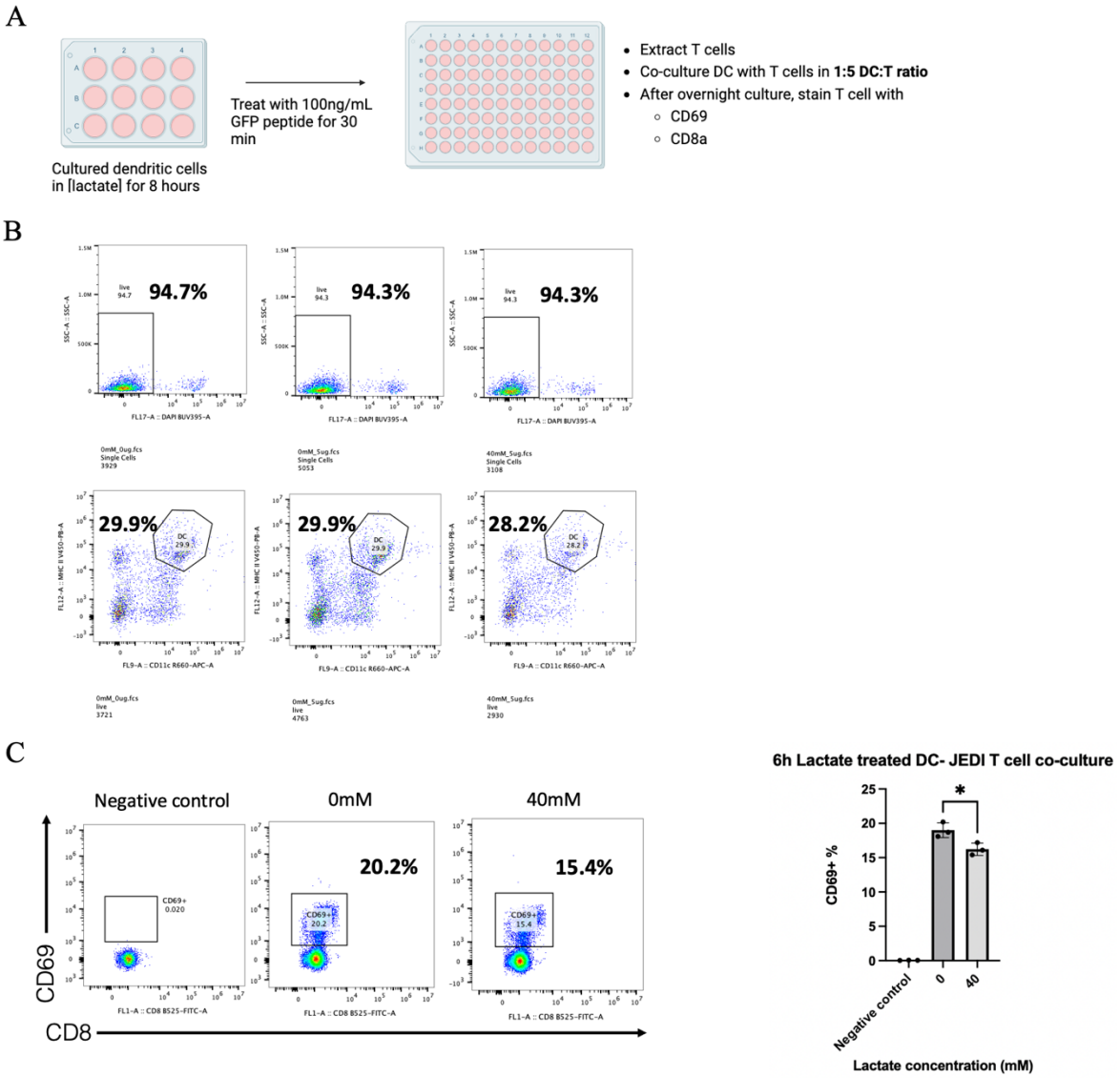


Figure 9. Lactate impairs DC-T cell crosstalk A) Experiment Schematic. B) Flow cytometry result of DC survival and gated cell percentage, cells were stained with DAPI, CD11c, and MHC-II. C) Flow cytometry result of T cell $CD69^{+}$% after overnight DC-T cell coculture

Chapter 3

4. Discussion

4.1 Quiescent Cancer Cell Induction and Mechanism:

The induction of QCC within the TME in TNBC represents a critical aspect of immune recognition and immune-killing resistance. Our findings reveal that, in the hypoxic tumor niche, TNBC cells upregulate HIF1a and enter a quiescent state, a mechanism that provides these cells with a survival advantage and contributes to resistance to therapy. These results underline the importance of illustrating the molecular mechanisms underlying QCC induction and dormancy in TNBC. Future studies investigating the signaling pathways involved in HIF1a-mediated quiescence induction in TNBC cells are necessary.

According to immunofluorescent imaging (IF) of TNBC tumors, the $p27^{+}$ QCC clusters are compacted with limited immune cell infiltration (Baldominos et al., 2022). Thus, it would be necessary to consider that cell density may also play a role in inducing quiescence and forming an immune cell-unfavored niche. Investigating the heterogeneity of TNBC tumors and their interactions with the immune microenvironment (immune cell) could offer further insights into their functional significance. Utilizing single-cell sequencing and imaging techniques to characterize QCC niche and their spatial distribution within the TME can help analyze the correlation between QCC and high-density areas.

Additionally, exploring the crosstalk between hypoxia-induced signaling pathways and other key regulators of cell cycle progression and dormancy may uncover therapeutic targets for preventing tumor relapse and metastasis in TNBC.

4.2 Metabolism Effect on Dendritic Cell Function:

Our study elucidates the impact of nutrient availability and cancer cell metabolism on DC maturation, particularly lactate and glucose. From the conditioned media experiment, we found that cancer cells secrete metabolites in the media that will lead to DC dysfunction. However, the reason for the decreasing trend between cell densities is unclear. When more cancer cells are taking up nutrients, the carbon dioxide will acidify the media. We did observe that the media shows a yellow color indicating acidification. In the TME, the accumulation of extracellular lactate results in an acidic niche via hypoxia affecting immune cells in the environment. Low pH would cause mannose receptor (MR), a DAMP receptor on DC, to deform, leading to a decreased capacity for antigen uptake and decreased DC-T cell cross-presentation activity (Burgdorf et al., 2020). Thus, the factor of media pH needs to be considered. Thus, in future experiments, the use of a buffer to prevent acidification will be necessary. Moreover, we can incorporate a pH meter to keep track of culture media.

Along with conditioned media, we will proceed with cancer cell and DC co-culture to measure a more direct effect on both glucose availability and lactate concentration. There are lactate assay measurement kits available from Sigma-Aldrich for detecting lactate acid concentration in the cell culture media.

To further elucidate the underlying mechanisms of cancer cell metabolism on DC function in TNBC, future studies could focus on exploring the metabolic pathways involved in mediating these effects. To understand the underlying mechanism of lactate affecting DC, we are interested in lactate dehydrogenase (LDH), which is the primary metabolic enzyme that converts pyruvate to lactate and vice versa (de la Cruz-López et al., 2019). LDHA and LDHB are two isoenzymes of

LDH; we generated LDHA/B KO in 4T07 TNBC cell lines. In our in vitro findings, lactate will impair DC activation dose-dependently. Thus, after the removal of the metabolic enzyme, the lactate produced and extruded into the TME by the cancer cell will be eliminated. We can also utilize LDH inhibitors or metabolic modulators to directly disable lactate production by cancer cells. With these, investigate DC activation.

Past work also underscored the broader impact of lactate metabolism reprogramming the immune landscape within the TME. Notably, lactate has been shown to exert inhibitory effects on tumor-associated macrophages (TAMs), a key component of the tumor microenvironment. While DCs play a critical role in antigen presentation and immune priming, TAMs represent another key population of immune cells that shape the TME. Lactate-mediated metabolic reprogramming drives TAMs toward a pro-tumor and immunosuppressive phenotype (M2), fostering collaboration with tumor cells to enhance angiogenesis (Tao et al., 2023). Concurrently, elevated lactate levels in the TME foster the upregulation of PD-1 expression in Treg cells, thereby sustaining their immunosuppressive function and dampening effector T cell response (Kumagai et al., 2022). In contrast, Feng et al. highlighted that subcutaneous administration of sodium lactate to mice bearing MC38 (murine colon adenocarcinoma) results in $CD8^+$ T cell-dependent antitumor response, improving the efficacy of anti-PD-1 therapy (Feng et al., 2022). Hence, the mechanism by which lactate reprograms different immune cell populations within the TME remains controversial and worth further exploration.

In conclusion, our findings shed light on the complex interplay between cancer cell metabolism, immune responses, and tumor dormancy in TNBC. By unraveling the mechanisms driving QCC induction and metabolic effects on DC function, we lay the groundwork for further exploration of

fundamental biological processes underlying QCC immune evasion properties and TNBC progression.

4.3 Future perspectives

In this study, we elucidated the induction of QCC in the hypoxic tumor microenvironment of TNBC, driven by elevated HIF1a expression, which confers a survival advantage and contributes to metastasis and tumor relapse. Concurrently, we uncovered the detrimental impact of cancer cell-secreted metabolites, particularly lactate and glucose, on dendritic cell (DC) function, potentially compromising immune surveillance. Future investigations will delve into the signaling pathways mediated by HIF1a in QCC induction. Additionally, we will investigate altered metabolism in DC by studying lactate dehydrogenase isoforms and assess the efficacy of LDH inhibitors or metabolic modulators in restoring DC function. We will also move to in vivo experiments generating universal DC lactate transporter knockout better to understand the influence of the DC metabolic mechanism. These efforts will deepen our understanding of tumor-immune interactions and inform the development of immunotherapeutic strategies for TNBC. Hence, the mechanism by which lactate reprograms different immune cell populations within the TME remains controversial and worth further exploration.

Another aspect is the antitumor immune response orchestrated by T cells. Replicating DC-T cell coculture under varied lactate concentrations and culture periods can help us understand the metabolic modulation effects and immune evasion mechanisms. We will also study DC antigen presentation machinery by studying T cell activation signals, including antigen recognition, co-stimulation, and cytokine signaling. Additionally, leveraging metabolic profiling techniques enables the assessment of metabolic fluxes and nutrient uptake in both cancer cells and immune

cells within the cell culture media. This approach offers potential insights into the metabolic dependencies and vulnerabilities of TNBC tumors.

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