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Protein Neddylation as an Immune Checkpoint: A Dual Role in Innate Immunity and Resistance to Cancer Immunotherapy

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Abstract

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The remarkable clinical benefits in cancer patients receiving immunotherapy have ignited the research enthusiasm on cancer immunotherapy. However, primary resistance to immune checkpoint blockade (ICB) therapy remains one of the major challenges to effective immunotherapy in many patients. A deeper understanding of tumor-intrinsic mechanisms is therefore essential to identify new therapeutic targets and improve ICB outcomes. This project aimed to uncover novel immune regulatory pathways in cancer cells that could enhance PD-1/PD-L1-targeted therapies.

In Paper I, we performed genome-wide CRISPR/Cas9 screens in a tumor-immune co-culture system, and we identified *NEDD8* as a key resistance gene to PD-1 blockade in triple-negative breast cancer (TNBC). *NEDD8* deletion in cancer cells increased tumor immunogenicity, enhanced antigen presentation (e.g., HLA-DR), and improved responses to anti-PD-1 and anti-PD-L1 therapy. In immunocompetent mice, *Nedd8* loss in tumors enhanced PD-1/PD-L1 efficacy, leading to CD8+ T cell-dependent tumor eradication, prolonged survival, and activation of multiple immune pathways.

In Paper II, integrated scRNA-seq and high-resolution spatial transcriptomics revealed that cancer-cell neddylation regulates immune cell infiltration and function. *Nedd8* deletion in cancer cells increased inflammatory myeloid cell infiltration and amplified adaptive responses to PD-1 blockade. High-content drug screening showed that protein neddylation sensitized human breast cancer cells to lipid metabolism inhibitors e.g., statin drugs and ferroptosis inducers. These compounds synergize with PD-1 blockade and protected the mice from tumor rechallenge in vivo.

In Paper III, we identified *NEDD8* as a myeloid-intrinsic immune checkpoint that restrains innate immune activation. *NEDD8* loss in human monocytic THP-1 cancer cells enhanced pro-inflammatory signaling and revealed mTORC1-dependent vulnerabilities. Statin treatment, which drives pathway changes similar to *NEDD8*-deficient human cells, amplified strong inflammatory responses in human primary CD14+ monocytes through mTOR signaling, and improved early anti-tumor responses in immunocompetent mice.

Altogether, this work uncovers previously unrecognized mechanisms by which *NEDD8* regulates tumor and myeloid immunity, providing new avenues to potentiate innate and adaptive anti-tumor responses and improve cancer immunotherapy outcomes.

Keywords: Tumor immunity, TNBC, NEDD8, PD-1/PD-L1 blockade, CRISPR/Cas9, Monocytes

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Papakyriacou I**, Kutkaite G, Rúbies Bedós M, Nagarajan D, Alford LP, Menden MP, Mao Y. Loss of NEDD8 in cancer cells causes vulnerability to immune checkpoint blockade in triple-negative breast cancer. *Nat Commun*. 2024 Apr 27;15(1):3581.

II. **Papakyriacou I***, Lu Y*, Jarvius M, Lapins M, Puigvert CJ, Mao Y. Cancer intrinsic protein neddylation modulates the intra-tumoral immune landscape to constrain immune checkpoint blockade therapy. *Cancer Immunology Research, under review*

III. **Papakyriacou I**, Lu Y, Alford PL, Mao Y. Targeting intracellular mechanisms to enhance sensitivity to TLR activation in myeloid cells, *manuscript ready for submission*

* Equally contributing first authors

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Other Published Work by the Author

- Shang Z, Li L, Jing C, Liu J, Zhang B, Rúbies Bedós M, **Papakyriacou I,** Mao Y, Chen Y. FGFR inhibition restores antitumor immunity and enhances PD-1 blockade in colorectal cancer models, *manuscript ready for submission*
- Tunalı G, Rúbies Bedós M, Nagarajan D, Fridh P, **Papakyriacou I**, Mao Y. IL-1 receptor-associated kinase-3 acts as an immune checkpoint in myeloid cells to limit cancer immunotherapy. *J Clin Invest*. 2023 Apr 3;133(7):e161084.

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Abbreviations

ACT	adoptive cell therapy
ADCC	antibody-dependent cell-mediated cytotoxicity
ADP	antibody-dependent phagocytosis
AI	artificial intelligence
ALN	axillary lymph nodes
APCs	antigen-presenting cells
BCR	B cell receptor
BL1/2	basal-like 1/2
BRCA1/2	breast cancer gene 1/2
CAR	chimeric antigen receptor
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
CRISPR	clustered regularly interspaced short palindromic repeats
CRLs	Cullin-RING E3 ubiquitin ligases
CT	computed tomography
ctDNA	circulating tumor DNA
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
CTLs	cytotoxic T lymphocytes
DAMPs	damage-associated molecular patterns
DC	dendritic cells
DCIS	ductal carcinoma in situ
dMMR	deficient mismatch repair
DSBs	double-stranded breaks
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FDA	food and drug Administration
GEP	gene expression profile
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDR	homology-directed repair
HER2	human epidermal growth factor receptor 2
ICB	immune checkpoint blockade
ICD	immunogenic cell death
IDC	invasive ductal carcinoma

IFN	interferon
IL	interleukin
IM	immunomodulatory
irAEs	immune-related adverse events
KO	knockout
LAG-3	lymphocyte activation gene-3
LAR	luminal androgen receptor
M	mesenchymal
mAbs	monoclonal antibodies
MDSC	myeloid-derived suppressor cell
MHC I/II	major histocompatibility complex I/II
MOI	multiplicity of infection
MRI	magnetic resonance imaging,
MSI-H	microsatellite instability-high
MSL	mesenchymal stem-like
NAE	NEDD8-activating enzyme E1
NEDD8	neuronal precursor cell-expressed developmentally down-regulated protein 8
NHEJ	nonhomologous end joining
NK	natural killer
NSCLC	non-small-cell lung cancer
OS	overall survival
OV	oncolytic viruses
PALB-2	partner and localizer of BRCA2
PAP	prostatic acid phosphatase
PD-1	programmed cell death protein 1
PD-L1	programmed death ligand 1
PFS	progression-free survival
PR	progesterone receptor
scFv	single-chain variable fragment
sgRNA	single-guide RNA
TAAs	tumor-associated antigens
TAMs	tumor-associated macrophages
TAP	transporter associated with antigen processing
TCRs	T cell receptors
TGF-β	transforming growth factor beta
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TILs	tumor-infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin-domain containing protein 3

TMB	tumor mutational burden
TMB-H	tumor mutational burden-high
TME	tumor microenvironment
TNBC	triple-negative breast cancer
TNF	tumor necrosis factor
tracrRNA	trans-activating crRNA
Tregs	regulatory T cells
TSAs	tumor-specific antigens
T-VEC	Talimogene laherparepvec
UPS	ubiquitin-proteasome system

1 Breast Cancer

Breast cancer is the most commonly diagnosed cancer globally and remains the second leading cause of cancer-related mortality among women, with an estimated 2.3 million new cases, and over 670,000 deaths reported in 2022. Although it predominantly affects women, around 0.5-1 per cent of breast cancer cases occur in men[1]. Its complex biological mechanisms and varied clinical presentations significantly hinder effective treatment and prevention, continuing to represent a significant public health challenge worldwide[2-4]. With the global incidence of breast cancer steadily increasing[5] and projections indicating a 38% rise in cases worldwide by 2050[1], understanding the complex nature of the disease is crucial for developing more effective treatment strategies. Efforts aimed at achieving a 2.5% annual reduction in breast cancer mortality could potentially prevent around 2.5 million deaths by 2040[1].Although significant advances have been made in early diagnosis and therapeutic approaches, breast cancer's etiology is shaped by a complex interplay of molecular, genetic, environmental, and lifestyle factors (**Figure 1**). Inherited genetic mutations, particularly in the breast cancer gene 1 (*BRCA1), BRCA2* and partner and localizer of BRCA2 (*PALB-2*) along with a family history, are estimated to account for approximately 5% to 10% of all cases[6,7]. Additionally, 20% to 30% of cases are associated with potentially modifiable risk factors, including alcohol consumption, smoking, obesity, no breastfeeding, late menopause, and hormone exposure[8,9] (**Figure 1**). Understanding these interconnected influences is essential in early detection and to improving patient outcomes.

Breast cancer diagnosis typically begins with a clinical risk assessment and physical examination for signs such as palpable lumps or skin changes. Suspicious findings are usually followed by imaging, most commonly mammography, which can detect tumors too small to be seen or felt. Additional imaging techniques, such as ultrasound, magnetic resonance imaging (MRI), or computed tomography (CT), may be used to further evaluate abnormal tissue[10]. In recent years, advancements in sequencing technologies and highly efficient experimental methodologies have significantly enhanced the detection and diagnostic accuracy of various tumors, including breast cancer. Notably, the integration of liquid biopsy with high-throughput sequencing has opened new

avenues for real-time cancer diagnostics[11,12] (**Figure 1**). Artificial intelligence (AI) is playing an increasingly pivotal role in clinical oncology, particularly in the diagnosis and management of breast cancer. It offers significant potential for improving early tumor detection, optimizing risk assessment, and enabling more precise, individualized treatment strategies[13-16].

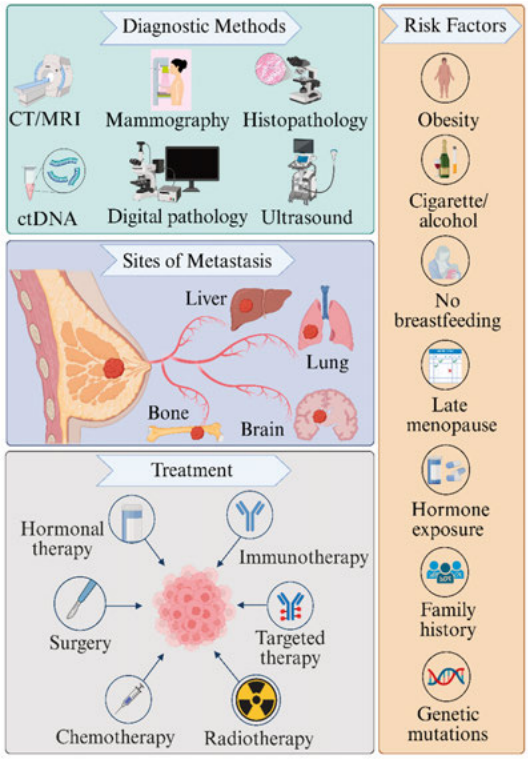


Figure 1. Overview of key factors in breast cancer development, diagnosis, and treatment. This schematic illustrates the multifactorial nature of breast cancer development and progression, including genetic mutations, hormonal factors, and lifestyle influences. Advances in diagnostic methods, such as liquid biopsy and digital pathology, are enhancing the detection and characterization of breast cancer. The figure also highlights the shift toward precision and individualized therapies in modern treatment, as well as common sites of metastasis. CT: computed tomography, MRI: magnetic resonance imaging, ctDNA: circulating tumor DNA. The figure was created in BioRender.com.

Conventional treatment modalities such as surgery, chemotherapy, radiotherapy, hormone therapy, and targeted therapy remain foundational in breast cancer management[17] (**Figure 1**). Medical treatments may be administered either before (neoadjuvant) or after (adjuvant) surgery, and are typically guided by the tumor's biological subtype. In recent years, immunotherapy has revolutionized breast cancer treatment, offering new therapeutic possibilities[18].

Despite the effectiveness of current treatments, approximately 30% of breast cancer patients eventually develop metastatic or recurrent disease[19-21]. Metastases most frequently observed in the bone, lung, liver, and brain (**Figure 1**)[22,23]. The risk of developing distant metastases is strongly influenced by the molecular subtypes of breast cancer[24].

1.1 Subtypes of Breast Cancer

Breast cancer is a heterogeneous disease that is classified into distinct molecular subtypes based on the expression of hormone receptors, namely estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). These subtypes include hormone receptor positive (luminal A and B), HER2-enriched, and basal-like or triple-negative breast cancer (TNBC)[23,25-28] (**Figure 2**). They are often classified histologically as ductal carcinomas, as they commonly originate in the lumen of the milk-transporting ducts of the breast.

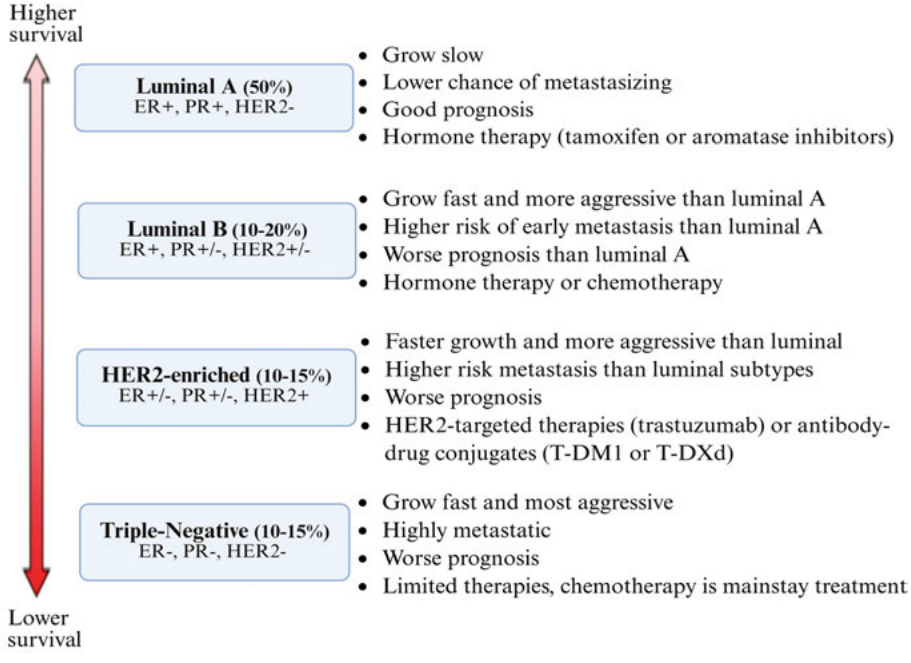


Figure 2. Summary of breast cancer subtypes characteristics. This figure presents the major breast cancer subtypes, along with their approximate prevalence (%) among all diagnosed cases. Classification is based on receptor status, with each subtype displaying distinct clinical characteristics, prognoses, and treatment responses. Subtypes are arranged from top to bottom according to increasing severity, with those at the bottom associated with poorer clinical outcomes. ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor 2, T-DM1: trastuzumab emtansine, T-DXd: trastuzumab deruxtecan. The figure was created in BioRender.com.

Ductal carcinoma in situ (DCIS) is a non-invasive, non-life-threatening disease. It is considered a stage 0 neoplastic lesion confined to the milk ducts, with no invasion into surrounding breast tissue or distant sites (**Figure 3**). It accounts

for approximately 15% to 20% of all diagnosed breast cancer cases[29-31]. DCIS represents a critical early form of breast cancer, as it carries an elevated risk of progression to invasive ductal carcinoma (IDC) if left untreated.

IDC is the most prevalent form of breast cancer, accounting for approximately 80% to 85% of all breast cancer diagnoses. IDC originates when cancer cells proliferating within the lumen of the milk ducts penetrate the ductal basement membrane and invade the surrounding breast tissue. This form of cancer can be life-threatening and potentially fatal. It commonly spreads through the axillary lymph nodes (ALNs) or bloodstream, facilitating distant metastasis to organs leading to the development of metastatic breast cancer[32,33] (**Figure 3**), with a higher incidence observed in TNBC cases[34].

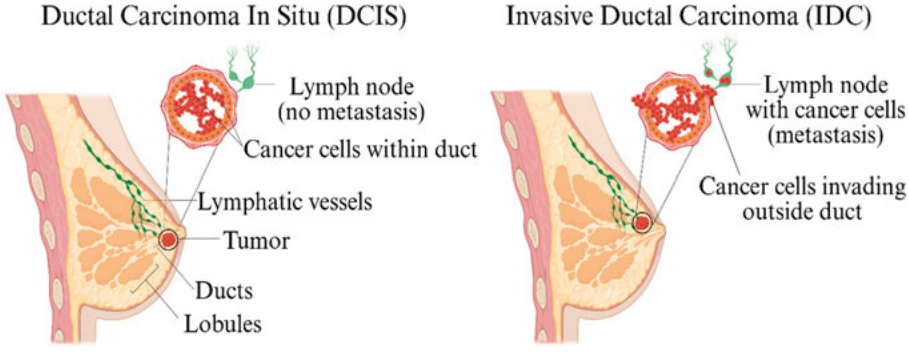


Figure 3. Comparison between ductal carcinoma in situ and invasive ductal carcinoma. The illustration shows that in ductal carcinoma in situ (DCIS), abnormal cells remain within the duct lining, while in invasive ductal carcinoma (IDC), cancer cells spread beyond the ducts into surrounding breast tissue. The figure was created in BioRender.com.

2 Triple-Negative Breast Cancer

TNBC represents approximately 10%–15% of all breast cancer cases, and is considered the most aggressive and invasive subtype. It is defined by the absence of ER and PR, and lacks overexpression or amplification of HER2[35]. TNBC typically exhibits rapid growth and carries an increased risk of recurrence following treatment compared to other forms of breast cancer. Consequently, it is associated with lower survival rates, with approximately 77% overall 5-year survival rate, which is 8-16% lower than that for non-TNBC[36]. TNBC is most prevalent in Hispanic and African American women under the age of 40, and its risk is influenced by a combination of factors, including genetic predisposition[37,38].

TNBC frequently arises in individuals carrying germline *BRCA1* mutations[39]. Comprehensive landscapes of somatic mutation in TNBC patient samples have identified *TP53* as the most commonly mutated gene, followed by *PIK3CA*, with recurrent mutations also observed in *PTEN*, *KMT2C*, and *RB1*[40]. These genetic alterations underpin the substantial heterogeneity observed in TNBC, which comprises distinct molecular subtypes defined by unique gene expression profiles. Notably, six molecular subgroups have been identified e.g., basal-like (BL1 and BL2), immunomodulatory (IM), luminal androgen receptor (LAR), mesenchymal (M) and mesenchymal stem-like (MSL), each associated with specific phenotypic characteristics, clinical outcomes, and pharmacological sensitivities[41,42].

Owing to the absence of hormone receptors and HER2 amplification, TNBC lacks targeted therapeutic options, making treatment particularly challenging. Chemotherapy remains the standard-of-care therapy; however, patients with advanced disease frequently develop resistance, leading to limited treatment efficacy and poor clinical outcomes[43]. Notably, TNBC is recognized as the most immunogenic breast cancer subtype, exhibiting elevated levels of tumor-infiltrating lymphocytes (TILs)[44-46]. Literature studies demonstrated TILs as a favorable prognostic indicator, correlating with reduced recurrence risk, and improved clinical outcomes in both adjuvant and neoadjuvant chemotherapy-treated cohorts[46-49]. The immunogenic nature of TNBC, along with the expression of programmed death ligand 1 (PD-L1), has opened new avenues for immunotherapies targeting the programmed cell death protein 1 (PD-

1)/PD-L1 axis, a mechanism frequently exploited by cancer cells to evade anti-tumor immune responses. In particular, the KEYNOTE-355 trial showed that adding pembrolizumab (anti-PD-1) to chemotherapy (nab-paclitaxel, paclitaxel, or gemcitabine plus carboplatin) significantly improved both progression-free survival (PFS) and overall survival (OS), leading to approval for the treatment of PD-L1-positive metastatic TNBC patients[50,51]. The Food and Drug Administration (FDA) has also approved pembrolizumab in combination with chemotherapy as neoadjuvant therapy, followed by adjuvant monotherapy for patients with high-risk early-stage TNBC[51]. Furthermore, the IMpassion130 trial demonstrated improved PFS with atezolizumab (anti-PD-L1) in combination with nab-paclitaxel for advanced or metastatic TNBC[52,53]. Thus, the FDA has approved atezolizumab with chemotherapy for the treatment of PD-L1 positive advanced or metastatic TNBC[54]. Despite these therapeutic advancements, TNBC remains a significant clinical challenge due to its aggressive biology, high metastatic potential, genetic background, and the lack of robust biomarkers for patient stratification[55].

3 The Immune System: The Natural Weapon Against Diseases

The immune system is a complex and dynamic network of cells, soluble factors, and organs that work together to defend the body against pathogens and maintain physiological homeostasis. It consists of two main branches: the innate and adaptive immune systems.

Innate immunity recognizes and responds to pathogens in a nonspecific and rapid manner. This system serves as the body's first line of defense and comprises: (1) physical and chemical barriers such as the skin and mucous membranes; (2) innate immune cells including phagocytes (e.g., macrophages, neutrophils), dendritic cells (DC), and natural killer (NK) cells; (3) circulating proteins like those of the complement system and other inflammatory mediators; and (4) cytokines, which regulate and coordinate immune cell activity. DCs possess the ability to capture, process, and present antigens to lymphocytes of the adaptive immune system. These cells, known as antigen-presenting cells (APCs), serve as a critical bridge between innate and adaptive immunity (**Figure 4**).

The adaptive immune system provides a highly specific response mediated by both T and B lymphocytes. It requires time to develop, about 5-7 days after initial antigen exposure for full activation. In particular, B cells contribute to the humoral immune response through their B-cell receptors (BCRs), which recognize specific antigens. Upon binding to their cognate antigen, B cells become activated and differentiate into plasma cells that secrete antigen-specific antibodies (**Figure 4**). In contrast, T cells drive the cell-mediated immune response through direct interactions with infected or abnormal cells, and coordination of other immune cells. Importantly, a hallmark of adaptive immunity is immunological memory, which provides long-lasting protection by facilitating a faster and more robust response upon subsequent exposures to the same antigen[56,57].

APCs are essential for the initiation and development of adaptive immunity. They perform three critical functions in T cell activation. First, APCs process protein antigens into peptides and present them on major histocompatibility complex (MHC) molecules for recognition by T cells via their T cell receptors (TCRs). Second, APCs provide additional signals, termed

costimulatory signals, which are necessary for full T cell activation. Key costimulatory molecules expressed by APCs include cluster of differentiation 80 (CD80) and CD86, which engage CD28 on naïve T cells to promote their activation and differentiation. Third, T cell activation and phenotype are critically regulated by the cytokine milieu, including diverse interleukins (ILs) (**Figure 4**). In the absence of requisite costimulatory signals or appropriate cytokine environments during antigen presentation, T cells may undergo functional inactivation or enter a state of anergy, characterized by unresponsiveness to subsequent antigenic stimulation. The two principal T cell subsets are $CD4^+$ helper T cells, which coordinate immune activation and regulation, and $CD8^+$ cytotoxic T cells, which mediate targeted destruction of infected or malignant cells; where these lymphocytes subsets recognize antigens through MHC class II and MHC class I molecules, respectively[58,59](**Figure 4).**

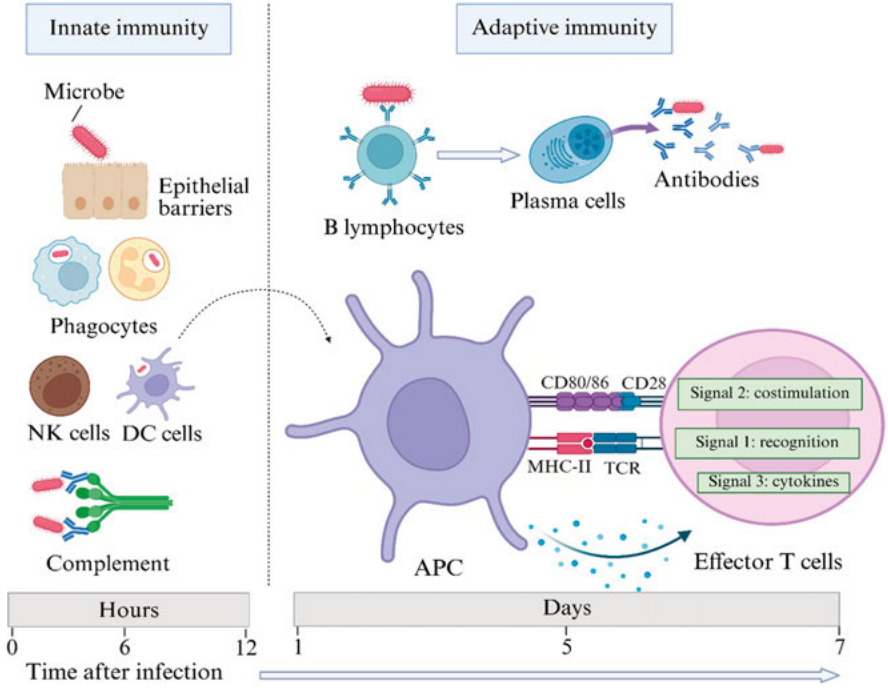


Figure 4. Innate and adaptive immunity. Innate immunity acts as the first line of defense against infection, while adaptive immunity develops later through lymphocyte activation. Antigen-presenting cells (APCs) e.g., dendritic cells (DC) initiate T cell responses by presenting antigen-derived peptides (first signal), providing costimulatory molecules (second signal), and secreting cytokines (third signal), all of which are essential for full T cell activation. NK: natural killer, TCR: T cell receptor, MHC: major histocompatibility complex, CD: cluster of differentiation. The figure was created in BioRender.com.

The innate and adaptive immune responses operate as components of a highly integrated host defense system, in which diverse cells and molecular pathways act cooperatively. Many pathogenic microbes have evolved strategies to evade or resist innate immune defenses, thereby requiring the more specialized and potent functions of the adaptive immune system for effective clearance. Moreover, innate immune responses not only initiate but also shape and influence the magnitude and nature of subsequent adaptive immune responses.

In addition to its role in defending against pathogens, the immune system plays a pivotal role in immune surveillance, where both innate and adaptive immune components collaborate to recognize and eliminate malignant or abnormal cells[60].

3.1 Tumor Immunology: Immune Surveillance and Evasion

The possibility that cancers can be targeted and eliminated by the immune system has driven extensive research in tumor immunology. The concept of immune surveillance, first proposed by Macfarlane Burnet in the mid-20th century, states that a fundamental function of the immune system is to detect and eliminate clones of transformed cells before they develop into clinically detectable tumors, as well as to control the growth of established tumors[61]. Although the concept of immune surveillance was once subject to debate, it is now widely accepted that the immune system can recognize and respond to many types of tumors. Leveraging these anti-tumor immune responses continues to be a key focus in the field of tumor immunology. Furthermore, a defining characteristic of tumor progression is the ability of cancer cells to evade or inhibit immune detection and destruction, enabling their sustained growth and metastasis. This dynamic interaction between the immune system and tumor cells is encapsulated in the concept of cancer immunoediting, which comprises three distinct phases: elimination, equilibrium, and escape[62].

The elimination phase represents the critical initial stage of immunosurveillance, during which both innate and adaptive immune responses are activated to efficiently target and eliminate tumor cells[63]. This phase is illustrated by the dynamic cancer-immunity cycle, which highlights the continuous interaction between the immune system and tumor cells[64] (**Figure 5**). The initiation of the anti-tumor immune response is driven by key oncogenic processes, such as tissue remodeling, including invasion and angiogenesis. These processes result in tissue damage and the release of damage-associated molecular patterns (DAMPs), which activate innate immune cells and lay the foundation for the initiation of adaptive immune responses aimed at

eliminating the tumor. As shown in **Figure 5**, tumor antigens, such as tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs), are released from cancer cells and are essential for specific immune recognition and the targeted elimination of malignant cells (Step 1)[63-65]. APCs, for example DCs, capture and process the tumor-derived antigens and migrate to the lymph nodes (Step 2), where they present these antigens to naïve T cells. This interaction leads to the priming and activation of tumor-specific helper $CD4^+$ T cells and cytotoxic $CD8^+$ T lymphocytes (CTLs) via cross-presentation (Step 3). These activated T cells proliferate and differentiate into effector cells capable of targeting and eliminating tumor cells. The activated T cells then exit the lymph nodes and travel through the bloodstream to the tumor site (Step 4), guided by chemokines and adhesion molecules that facilitate their migration into the tumor microenvironment (TME) (Step 5). Upon reaching the TME, T cells recognize and engage with cancer cells (Step 6). CTLs then kill the tumor cells (Step 7), leading to the release of additional tumor antigens that further stimulate the immune system, creating a positive feedback loop. This amplifies the anti-tumor immune response and contributes to the generation of additional tumor-specific immune responses, perpetuating the cycle of immune activation[64].

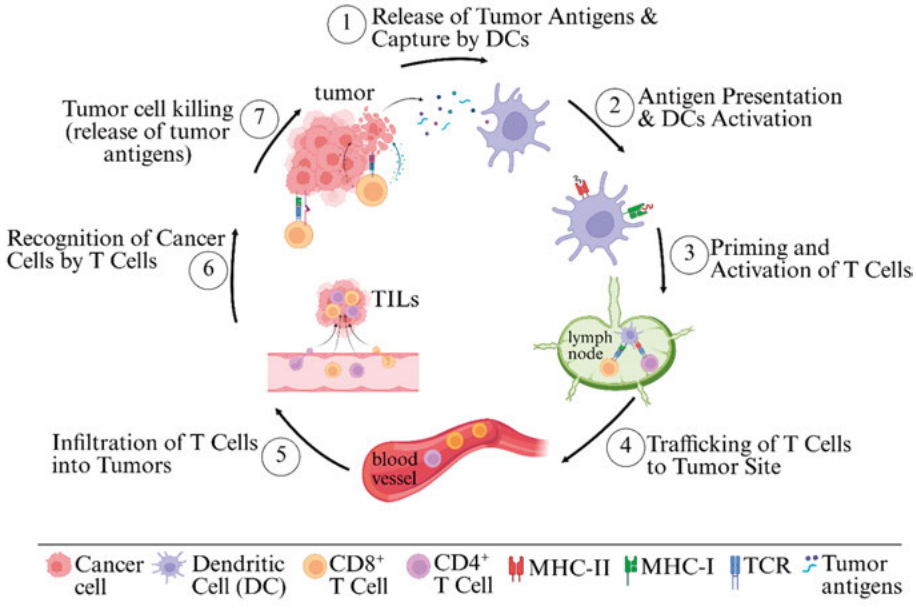


Figure 5. The cancer-immunity cycle. First described by Chen and Mellman (2013)[64], the cancer-immunity cycle comprises a series of coordinated immune events that enable the recognition and elimination of tumor cells. It begins with the release of tumor antigens, which are captured by antigen-presenting cells (APCs), primarily dendritic cells. These APCs process and present the antigens via MHC molecules to naïve T cells in secondary lymphoid organs, leading to T cell priming and activation. Activated effector T cells then migrate to the tumor site, infiltrate the tumor microenvironment, and mediate cytotoxic activity against tumor cells. This results in further antigen release, sustaining and amplifying the cycle of anti-tumor immunity. TILs: tumor infiltrated lymphocytes, TCR: T cell receptor, MHC: major histocompatibility complex, CD: cluster of differentiation. The figure was created in BioRender.com.

The equilibrium phase represents a prolonged period of dynamic balance between immune surveillance and tumor persistence, during which the immune system contains tumor growth without fully eradicating it. In this state of functional dormancy, tumor cells are kept in check through ongoing immune pressure, primarily mediated by T cells and cytokines. Although tumor mass does not expand, it is not eliminated either. This phase, likely the longest in the cancer immunoediting process, may last for years. Over time, however, continued immune selection can drive the emergence of tumor cell variants with genetic and phenotypic alterations that enable them to evade immune detection, setting the stage for the escape phase[66].

The escape phase represents the final stage of the cancer immunoediting process, during which tumor cells that have acquired immune-evasive characteristics proliferate uncontrollably and establish clinically detectable

malignancies. These tumor variants evade immune recognition through various mechanisms that impair immune detection and cytotoxic activity[67].

3.2 Resistance Mechanisms: Tumor Strategies for Immune Evasion

Many tumors have evolved diverse mechanisms to evade or resist host immune responses (**Figure 6**). A central objective of tumor immunology is to elucidate these immune escape strategies with the aim of developing interventions that enhance tumor immunogenicity and strengthen anti-tumor immune responses. Immune evasion can occur through multiple mechanisms, such as:

Central tolerance and tumor mutational burden

Tumor antigen tolerance represents a significant barrier to effective anti-tumor immunity. TAAs are often overexpressed in malignancies or expressed during specific stages of cellular differentiation, with limited expression in normal adult tissues. However, because TAAs are non-mutated self-antigens, they are subject to central tolerance during T cell development in the thymus. This process eliminates high-affinity T cells specific for self-antigens, thereby narrowing the peripheral T cell repertoire and contributing to the weak or absent T cell responses frequently observed in clinical trials[68-71]. In parallel, tumors exhibit varying levels of tumor mutational burden (TMB), which is defined as the number of genetic and epigenetic alterations within the tumor genome. This variability influences the generation of tumor-specific neoantigens, with low-TMB tumors typically producing fewer immunogenic targets, further limiting effective T cell-mediated responses[72] (**Figure 6**).

Impairment of antigen presentation and cytokine signaling

A common immune evasion strategy employed by tumors involves the downregulation or loss of key components involved in antigen presentation. This includes reduced expression of MHC class I molecules, β2-microglobulin, and elements of the antigen processing machinery such as the transporter associated with antigen processing (TAP) and immunoproteasome subunits. These defects collectively impair the presentation of tumor antigens to CTLs, thereby enabling tumor cells to evade immune detection. Such alterations are believed to arise under selective pressure from the host immune system, promoting the survival of tumor variants that are less recognizable by T cell-mediated immunity[73]. Notably, mutations in Janus kinases *JAK1* and *JAK2*,

critical mediators of interferon-gamma (IFNγ) signaling, can abolish tumor cell responsiveness to IFNγ secreted by activated T cells. This insensitivity compromises the induction of antigen processing and MHC class I expression, further facilitating immune escape[74] (**Figure 6**).

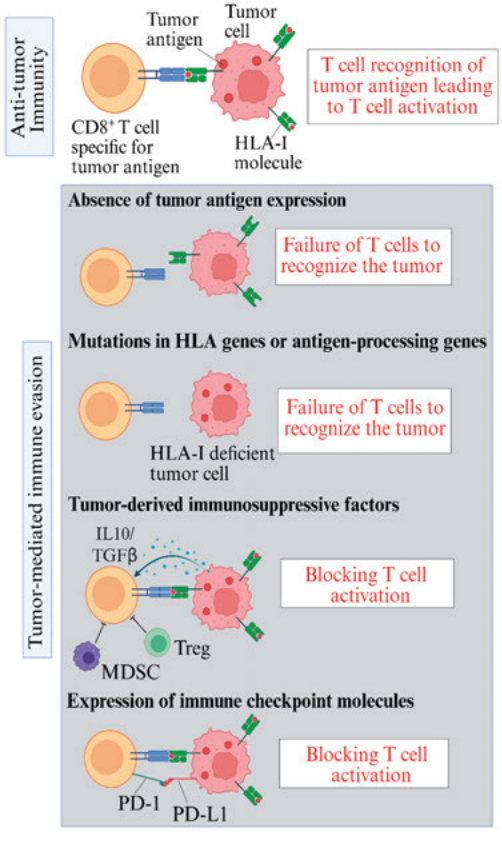


Figure 6. Mechanisms of tumor immune evasion. Anti-tumor immune response is initiated when T cells recognize tumor-associated antigens and become activated. However, tumors can evade immune detection and elimination through several mechanisms, including the loss or downregulation of tumor antigens and major histocompatibility complex (MHC) molecules, secretion of immunosuppressive factors that directly inhibit immune responses or recruit immunosuppressive cells, and upregulation of immune checkpoint molecules that inhibit T cell activity. PD-1: programmed cell death protein 1, PD-L1: programmed death-ligand 1, MDSC: myeloid-derived suppressor cell, Tregs: regulatory T cells, TGF-β: transforming growth factor beta, IL: interleukin. The figure was created in BioRender.com.

Tumor-derived immunosuppression in the tumor microenvironment

Tumor-derived factors play a crucial role in dampening anti-tumor immune responses. In particular, immunosuppressive cytokines e.g., transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) are frequently overexpressed in the tumor microenvironment by tumor cells. These cytokines inhibit the activation, proliferation, and effector functions of immune cells[75,76]. In parallel, tumor-derived factors facilitate the induction and recruitment of immunosuppressive cell populations into the TME, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-

associated macrophages (TAMs). These cells play pivotal roles in dampening anti-tumor immune responses. For example, Tregs suppress effector T cell functions through the secretion of immunoregulatory cytokines such as IL-10 and TGF-β[77]. MDSCs exert potent immunosuppressive effects by depleting nutrients essential for T cell proliferation and activity e.g., L-arginine, and generating reactive oxygen and nitrogen species[78]. TAMs, particularly those with an M2-like phenotype, further contribute to immune evasion by promoting tissue remodeling, angiogenesis, and the release of anti-inflammatory mediators[79]. Moreover, tumor cells can resist immune-mediated cytotoxicity by upregulating anti-apoptotic proteins or acquiring mutations that disrupt apoptotic signaling pathways, including functional impairments in death receptors such as Fas and TRAIL receptors, further supporting their survival and progression[80].

Upregulation of immune checkpoint molecules

The upregulation of immune checkpoint molecules, including PD-1 and its ligand PD-L1, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), as well as additional inhibitory receptors such as lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucin-domain containing protein 3 (TIM-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT), constitutes a major immune evasion strategy employed by tumors. Under physiological conditions, these immune checkpoints are critical for maintaining self-tolerance and modulating immune activation to prevent autoimmunity. However, in the context of malignancy, tumor cells exploit these regulatory pathways to inhibit effector T cell function. Engagement of these inhibitory receptors induces T cell exhaustion, impairs cytokine production and cytotoxic activity, and contributes to immune suppression within the tumor microenvironment, thereby facilitating tumor immune escape and progression[81,82]. Immune checkpoint molecules will be discussed in more depth in the context of immune checkpoint blockade (ICB) therapy.

4 Cancer Immunotherapy

The immunological treatment of cancer has revolutionized oncology by offering tumor-specific targeting with reduced systemic toxicity compared to conventional therapies. Unlike chemotherapy and radiotherapy, which indiscriminately affect proliferating cells, immunotherapy leverages the immune system's ability to selectively recognize and eliminate malignant cells via tumor-specific or tumor-associated antigens. Immunotherapeutic approaches are generally classified as either active, aiming to boost endogenous anti-tumor responses, or passive, involving the administration of monoclonal antibodies (mAbs) or adoptively transferred lymphocytes. A major advantage of immunotherapy is its capacity to induce durable immunological memory, enhancing long-term tumor control. Nonetheless, challenges such as interpatient variability and the immunosuppressive tumor microenvironment limit efficacy and highlight the need for ongoing refinement. Over the past decade, several immunotherapeutic strategies have entered clinical practice, including adoptive cell therapy (ACT), oncolytic viruses (OV), cancer vaccines, targeted mAbs, and ICB therapy, each with distinct mechanisms and applications in oncology.

ACT involves the isolation of autologous immune cells with tumor-specific reactivity, followed by their ex vivo expansion and reinfusion into the patient. To enhance therapeutic efficacy, these cells can be genetically modified to improve antigen specificity, persistence, and cytotoxic function. The three principal modalities of ACT include chimeric antigen receptor (CAR) T cells, T cell receptor (TCR)-engineered T cells, and TILs[83]. Among these, CAR-T cell therapy has shown the most significant clinical success, particularly in hematologic malignancies. CARs consist of a single-chain variable fragment (scFv) derived from a monoclonal antibody, fused to intracellular signaling domains such as CD3ζ and costimulatory molecules (e.g., CD28 or 4-1BB), enabling T cell activation upon antigen recognition in an MHC-independent manner. This strategy has received FDA approval and is now integrated into the standard-of-care therapy for several hematologic cancers, including CD19-targeted CAR-T cell therapy for relapsed or refractory non-Hodgkin lymphoma[84-86]. While the clinical application of CAR-T cells in solid tumors remains in the experimental stages, ongoing clinical trials and advances in CAR design, such as the use of bispecific CARs, and combination therapies,

are actively being explored to overcome barriers such as tumor heterogeneity and the immunosuppressive tumor microenvironment, with the aim of extending the success of this modality to a broader range of cancers[87].

OVs represent a rapidly advancing class of cancer immunotherapies that exploit the ability of certain viruses to selectively infect and lyse tumor cells while sparing normal tissues. This selectivity arises from their innate or engineered capacity to induce immunogenic cell death (ICD), thereby stimulating antitumor immune responses. Both naturally occurring and genetically engineered OVs are developed to enhance tumor targeting, replication efficiency, safety, and immunogenicity[88]. A prominent example is Talimogene laherparepvec (T-VEC), a genetically modified herpes simplex virus type 1, engineered to selectively replicate in tumor cells. Mechanistically, T-VEC expresses granulocyte-macrophage colony-stimulating factor (GM-CSF) to enhance systemic antitumor immunity by recruiting and promoting the maturation of DCs. In 2015, T-VEC became the first oncolytic virus to receive FDA approval in the Western world for the treatment of recurrent melanoma[88,89]. T-VEC has also been investigated in other cancer types, and in combination with immune checkpoint blockade therapy[90,91]. Additional clinical trials involving other oncolytic viruses have also demonstrated encouraging therapeutic potential[92].

Cancer vaccines aim to harness and direct the immune system to recognize and eliminate tumor cells by TAAs or neoantigens. Several types of cancer vaccines have been explored, including peptide-based, dendritic cell-based, viral vector-based, and nucleic acid-based vaccines, each offering unique advantages in antigen delivery and immune activation. Among the numerous tumor-associated antigen-targeted cancer vaccines that have advanced to clinical evaluation, only sipuleucel-T (Provenge) has demonstrated a definitive overall survival benefit. Sipuleucel-T is an autologous DC-based vaccine that targets prostatic acid phosphatase (PAP), an antigen expressed in prostate cancer. It provides a modest survival advantage of approximately four months, and received FDA approval in 2011 for the treatment of metastatic castration-resistant prostate cancer. However, its adoption in clinical practice has been limited, likely due to the high cost, logistical complexity of autologous vaccine production, and relatively modest efficacy[93,94]. Despite such challenges, particularly those related to antigen specificity and platform development, cancer vaccines continue to evolve as a promising component of cancer immunotherapy. Several clinical studies are currently ongoing to evaluate their safety and efficacy[93,95].

A major class of immunotherapy involves mAbs that specifically target antigens expressed on the surface of cancer cells. Upon binding to these antigens,

the antibodies trigger tumor cell death through several immune mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antibody-dependent phagocytosis (ADP)[96]. The first mAb approved for cancer treatment was rituximab, an anti-CD20 antibody that specifically binds to CD20, a receptor expressed on B lymphocytes and certain hematologic malignancies. Since its approval in 1997, rituximab has been recognized as the first targeted cancer immunotherapy and is now widely used in clinical oncology[97]. Several other mAbs have since been developed and integrated into cancer care, including bevacizumab (anti-VEGF-A), trastuzumab and pertuzumab (anti-HER2), among others. In addition, mAbs can bind to ligands or receptors to inhibit their natural functions, as exemplified by immune checkpoint blockade therapies.

4.1 Checkpoint Blockade: Turning the Key to Immune Activation

Immune checkpoints are inhibitory receptors that maintain immune homeostasis by attenuating T cell activity upon engagement with their ligands on healthy cells. These receptors act as critical "brakes" on the immune system, preserving self-tolerance and preventing autoimmunity[98,99]. However, tumor cells can exploit this regulatory mechanism by upregulating checkpoint ligands, thereby suppressing anti-tumor T cell responses and facilitating immune evasion. ICB therapy, primarily involving mAbs, blocks these inhibitory interactions and effectively "releases the brakes" on the immune system. This restores T cell effector function and promotes the activation and expansion of tumor-specific cytotoxic T cells capable of recognizing and eliminating malignant cells (**Figure 7**)[100]. Nowadays, ICB therapy has generated remarkable clinical outcomes in patients with multiple cancer types, and is now one of the most extensively researched areas in oncology[101].

The PD-1/PD-L1 and CTLA-4 pathways are the most thoroughly characterized and clinically validated immune checkpoint axes, forming the foundation of many current immunotherapeutic strategies. PD-1 is expressed on activated T cells during the effector phase in peripheral tissues and binds to its ligands, PD-L1 or PD-L2. These ligands are present on myeloid cells, endothelial cells as well as on various tumor cells. PD-1 engagement transmits inhibitory signals that suppress T cell activity by blocking TCR signaling, thereby downregulating the immune response[102] (**Figure 7A**). Moreover, CTLA-4, a homolog of the co-stimulatory receptor CD28, is expressed on both effector and Treg cells. It competes with CD28 for binding to B7 molecules (CD80 and CD86) on APCs, but with significantly higher affinity.

CTLA-4 expression is upregulated shortly after T cell priming in the lymph nodes. Upon binding to B7, CTLA-4 transmits an inhibitory signal that attenuates T cell activation and proliferation[103] (**Figure 7B**). Further, LAG-3 is another checkpoint molecule that negatively regulate T cell function. LAG-3 is expressed on various immune cells, particularly on activated $CD4^+$ and $CD8^+$ T cells. It interacts with MHC class II molecules on antigen-presenting cells but with significantly higher affinity than CD4, and thus transmitting inhibitory signals that suppress T cell activation and effector function[104]. In addition, galectin-3 and LSECtin in the tumor microenvironment act as additional LAG-3 ligands, suppressing $CD8^+$ T cell-mediated anti-tumor immunity[105].

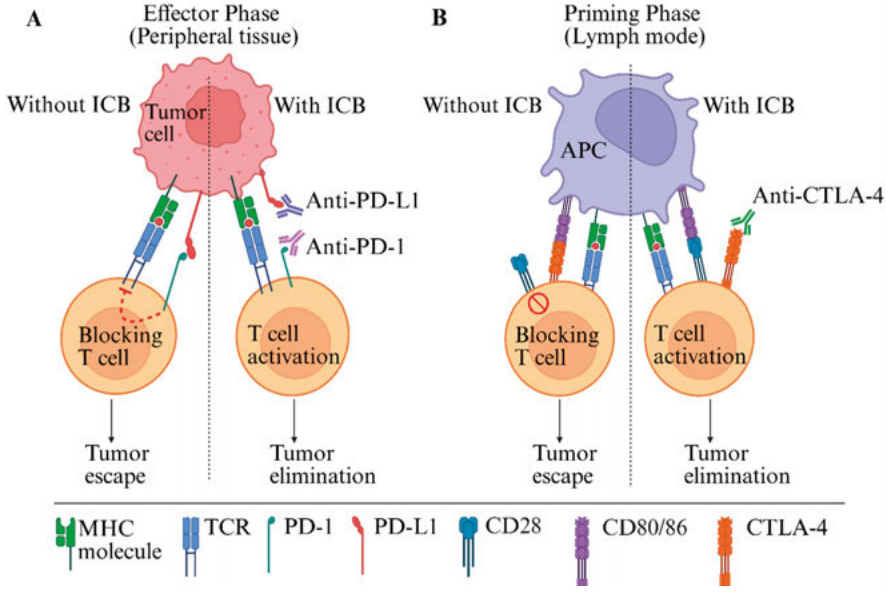


Figure 7. Immunological mechanisms of PD-1/PD-L1 and CTLA-4 immune checkpoint inhibition. Immune checkpoint blockade restores anti-tumor T cell function by targeting key inhibitory pathways. **(A)** PD-1 is an inhibitory receptor expressed on activated T cells, including tumor-infiltrating lymphocytes, and regulates effector function predominantly in peripheral tissues. Engagement of PD-1 by its ligands, PD-L1 or PD-L2, inhibits proximal T cell receptor (TCR) signaling pathways, resulting in reduced cytokine production, impaired proliferation, and T cell exhaustion. Therapeutic blockade of PD-1 or PD-L1 reactivates suppressed effector T cells and facilitates localized anti-tumor immunity. **(B)** CTLA-4, a CD28 homolog, competes with CD28 for binding to the costimulatory ligands CD80/CD86 on antigen-presenting cells (APCs), thereby attenuating T cell activation during the priming phase in secondary lymphoid organs. Anti-CTLA-4 monoclonal antibodies disrupt this interaction, favoring CD28-mediated costimulation, and consequently enhance T cell proliferation and expansion. PD-1: programmed cell death protein 1, PD-L1: programmed death-ligand 1, CTLA-4: cytotoxic T lymphocyte-associated antigen 4, ICB: immune checkpoint blockade, MHC: major histocompatibility complex. The figure was created in BioRender.com.

The era of ICB therapy began with the FDA approval of ipilimumab, an anti-CTLA-4 mAb, for the treatment of melanoma in 2011[106]. This was followed by the approval of anti-PD-1 therapies, with pembrolizumab (Keytruda) receiving approval in 2014 for metastatic melanoma, and nivolumab (Opdivo) shortly thereafter in 2014[107]. Moreover, LAG-3 became the latest immune checkpoint to be targeted by an FDA-approved therapy. Opdualag, a combination therapy nivolumab (anti-PD-1) and relatlimab (anti-LAG-3), is approved for unresectable or metastatic melanoma treatment in 2022[108]. Nowadays, numerous ICB therapies have been approved, either as monotherapies or in combination with other ICB or conventional treatments such as chemotherapy in multiple tumor types, which are summarized in **Table 1**.

ICB therapy can lead to immune-related adverse events (irAEs) as a result of systemic immune hyperactivation, potentially impacting multiple organs and tissues. Frequently observed irAEs include dermatologic toxicities such as pruritus and rash, gastrointestinal inflammation e.g., colitis, and endocrine dysfunctions (hypophysitis and thyroiditis). Although less common, serious irAEs may involve the nervous system (e.g., encephalitis and peripheral neuropathy), musculoskeletal system (myositis and arthritis,) as well as inflammatory conditions affecting the kidneys (nephritis), heart (myocarditis), liver (hepatitis), and lungs (pneumonitis)[109,110]. However, the development of irAEs has been linked to stronger immune activation and improved treatment outcomes in multiple tumor types[111]. In non-small-cell lung cancer (NSCLC) patients who develop irAEs tend to exhibit higher response rates, as well as prolonged progression-free and overall survival[112].

Given the heterogeneity in response to immune checkpoint blockade therapy across patients with different tumor types, the integration of biomarkers is essential for predicting therapeutic outcomes, detect resistance, and guiding personalized treatment decisions to optimize clinical benefit and minimize toxicity. Currently, several biomarkers have been associated with the efficacy of immune checkpoint blockade therapy. For example, high PD-L1 expression is correlated with improved responses in certain cancers, such as NSCLC and TNBC[50,113]. Furthermore, clinical studies have demonstrated that tumors with tumor mutational burden-high (TMB-H) serve as a biomarker associated with increased efficacy of ICB therapy, likely due to enhanced immunogenicity from a higher number of neoantigens[114]. Microsatellite instability-high (MSI-H) is a key genomic feature often linked to TMB-H. Tumors exhibiting deficient mismatch repair (dMMR) accumulate uncorrected DNA errors, resulting in the MSI-H phenotype, e.g., in gastric, colorectal and endometrial adenocarcinomas[115,116]. Accordingly, MSI-H tumors have shown exceptional responses to ICB therapy[114]. This prompted the FDA to grant accelerated approval in

2017 for the use of the anti-PD-1 antibody (pembrolizumab) in both adult and pediatric patients with dMMR/MSI-H solid tumors, marking the first time the agency approved a treatment based on a shared molecular biomarker rather than the anatomical origin of the tumor[117]. Additionally, TILs, particularly $CD8^+$ cytotoxic T cells, along with gene expression profiles (GEPs) associated with IFNγ signature has been linked to improved clinical outcomes. Notably, an 18-gene T cell-inflamed gene expression profile was identified as a robust predictor of response to pembrolizumab across various tumor types, demonstrating greater predictive power than PD-L1 expression alone[118]. Overall, these results highlight the importance of strategic biomarker selection to improve the evaluation of ICB efficacy.

Table 1: Immune checkpoint blockade therapies approved by the FDA				
Drug	Target	Indication		
		Advanced disease	Adjuvant	Neoadjuvant
Nivolumab	PD-1	Melanoma RCC Urothelial ESCC NSCLC Head/neck cancer Hodgkin's lymphoma CRC (MSI or dMMR)	Melanoma Urothelial Esophageal	NSCLC[b]
Pembrolizumab	PD-1	Melanoma Hodgkin's lymphoma Skin cancer, SCC NSCLC Head/neck SCC Gastric Urothelial RCC CRC (MSI or dMMR) Cervical Endometrial (MSI or dMMR) Merkel cell Any tumor with TMB, MSI, and dMMR	Melanoma, stage 2b-3 TNBC, stage 2-3 NSCLC, stage 1B-3A RCC	TNBC, stages 2-3[b]
Cemiplimab	PD-1	Skin cancer, SCC, NSCLC Skin cancer, basal cell	-	-
Atezolizumab	PD-L1	NSCLC Melanoma Liver cancer SCLC TNBC[b] Urothelial[b]	NSCLC	-
Durvalumab	PD-L1	NSCLC SCLC Cholangiocarcinoma Liver cancer	NSCLC	-
Avelumab	PD-L1	Merkel cell Urothelial	-	-
Ipilimumab[a]	CTLA-4	NSCLC Mesothelioma ESCC RCC HCC Melanoma CRC (MSI or dMMR)	-	-
Relatlimab[a]	Lag-3	Melanoma	-	-

RCC: renal cell carcinoma, SCLC: small-cell lung cancer, NSCLC: non-small-cell lung cancer, ESCC: esophageal squamous cell cancer, CRC: colorectal cancer, SCC: squamous cell cancer, HCC: hepatocellular cancer, dMMR: deficient mismatch repair, TMB: tumor mutational burden, MSI: microsatellite instability. [a] In combination with nivolumab, [b] In combination with chemotherapy.

ICB therapies are expected to play an increasingly pivotal role in the future of cancer treatment. While the majority of current regulatory approvals are limited to advanced or metastatic settings, the therapeutic potential of ICB in earlier stages of disease is being actively investigated. The rationale for this shift lies in the opportunity to prevent disease recurrence and progression to metastasis through early immunologic intervention. In the adjuvant setting, ICB aims to eliminate residual microscopic disease following surgical resection, thereby reducing the likelihood of relapse. More recently, neoadjuvant administration of ICB has gained substantial interest, supported by emerging clinical evidence. Notably, a study involving patients with locally advanced dMMR colon cancer demonstrated that neoadjuvant nivolumab with ipilimumab resulted in major pathological responses in 95% of cases, with 68% achieving complete pathological remission and no recurrence observed over a median follow-up period of 26 months. These findings suggest that ICB may offer a chemotherapy-sparing approach with durable clinical benefit, representing a potential paradigm shift in the management of early-stage malignancies.

Furthermore, ongoing investigation into additional checkpoint targets, including TIM-3 and TIGIT, may further expand the therapeutic scope and efficacy of ICB strategies in the coming years[119,120].

Despite the clinical success of ICB therapy, a significant proportion of patients exhibit either primary or develop acquired resistance against this therapy[81]. Recently, CRISPR/Cas9-based loss-of-function screening has emerged as a powerful, unbiased approach for identifying novel regulators of immune evasion and resistance mechanisms.

5 The CRISPR Technique: Precision Engineering of the Genome

Gene editing is a precise molecular technique that enables targeted modifications to the genome, including insertions, deletions, and base substitutions[121,122]. Nowadays, gene-editing technology is largely dominated by the third-generation clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, which offers unprecedented precision, efficiency, and ease of use compared to earlier methods[123].

The CRISPR/Cas9 system, which naturally evolved in bacteria and archaea as an RNA-guided adaptive immune mechanism, allows these organisms to incorporate fragments of invading pathogen genomes into their CRISPR loci, creating a genetic memory for future defense. Upon subsequent infections, endogenous CRISPR-Cas9 complexes, utilize this stored information to specifically recognize and bind complementary foreign DNA sequences, inducing site-specific double-stranded breaks (DSBs) that result in the degradation of the invading genetic material.

Recognizing the precision and programmability of this microbial defense system, researchers repurposed CRISPR/Cas9 for genome editing in eukaryotic cells. Its transformative potential became evident in 2013, marking a pivotal moment in genome engineering. Cong et al.[124] demonstrated the successful application of CRISPR-mediated gene editing in mammalian cells, laying the groundwork for its use in medicine, agriculture, and beyond. Concurrently, other landmark studies[125-127] advanced the system by designing a chimeric single-guide RNA (sgRNA), and by refining the guide sequence to 20 base pairs.

By introducing a sgRNA containing a sequence complementary to the target gene, researchers can direct the Cas9 endonuclease to specific genomic loci with high precision. The sgRNA consists of two key components: the CRISPR RNA (crRNA), which determines target specificity, and the trans-activating crRNA (tracrRNA), which provides the structural scaffold for Cas9 binding. Upon locating the target site, the Cas9 protein induces a double-stranded break in the DNA[128]. The resulting break is then repaired by one of two main cellular pathways: the error-prone nonhomologous end joining (NHEJ), or the more precise homology-directed repair (HDR) which requires a donor DNA template[129]. NHEJ is commonly leveraged for gene knockout

(KO) applications, as its inherent inaccuracy often results in insertions or deletions that disrupt the reading frame, leading to premature stop codons or activation of nonsense-mediated decay (**Figure 8**). In contrast, HDR enables precise genome editing by incorporating specific sequence modifications using an exogenously supplied donor template[130] (**Figure 8**). These foundational achievements not only confirmed the functionality of CRISPR components in eukaryotic systems but also streamlined the technology, dramatically enhancing its versatility and accelerating its widespread adoption across multiple fields of research and biotechnology.

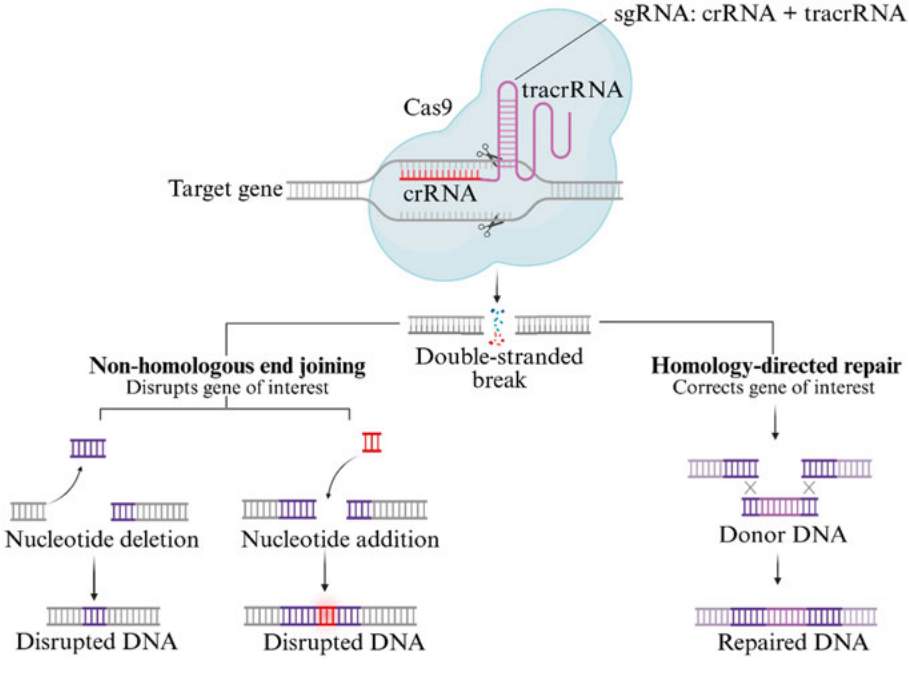


Figure 8. CRISPR-Cas9 gene editing mechanism. The CRISPR-Cas9 system employs a Cas9 nuclease guided by a single-guide RNA (sgRNA) sequence that matches a specific genomic target. Cas9 introduces a double-strand break (DSB) at the target site, which can be repaired by one of two pathways: non-homologous end joining (NHEJ), an error-prone process that may result in insertions or deletions, or homology-directed repair (HDR), which uses a supplied DNA template to enable accurate modification of the genome. crRNA: CRISPR RNA, TracrRNA: trans-activating crRNA. The figure was created in BioRender.com.

5.1 When CRISPR Met Immunology: The Ultimate Power Couple of Cancer Treatment

Building on these foundational discoveries, CRISPR-based technologies have revolutionized biomedical research by providing a powerful and versatile platform for unbiased genetic screening and functional genomics across a wide range of disciplines, including immunology. Specifically, CRISPR-mediated gene editing enables the precise targeting of key regulatory genes to modulate immune responses in disease contexts. Additionally, it supports high-throughput genetic screens that uncover novel immune modulators, elucidate causal relationships between genes and phenotypic outcomes, and generate comprehensive maps of gene-gene interactions.

The development of guide RNA libraries[131-133] has enabled high-throughput screening of numerous genes simultaneously using a pooled format. In such screens, targeted gene knockout, repression, or activation is followed by selection for a phenotype of interest. The resulting cell populations are then compared to unselected control pools to identify 'hit' genes that influence the phenotype. Pooled CRISPR screens are widely employed in immunological research due to their high efficiency, scalability, and suitability for genome-wide discovery efforts. In a typical pooled screen (**Figure 9A**), cancer cells are first transduced with a vector to enable constitutive expression of the Cas9 protein. A genome-wide library of sgRNAs is then introduced into this bulk population, typically via lentiviral or retroviral transduction. Each cell ideally receives only one sgRNA, inducing a unique genetic perturbation. The multiplicity of infection (MOI), defined as the ratio of viral particles to target cells, is carefully controlled, usually maintained below 0.5, to ensure that most cells incorporate a sgRNA, and to minimize confounding effects from multiple integrations. After allowing sufficient time for gene knockout, the cancer cell population is exposed to a defined selection pressure relevant to the biological question under investigation. High-throughput sequencing is then used to quantify the enrichment or depletion of specific sgRNAs, enabling the identification of genes that positively or negatively influence phenotypes such as cell survival, proliferation, or immune function[134,135]. By employing this powerful approach in cancer immunology, researchers can identify cancer-intrinsic genetic regulators that modulate immune cell function within the tumor microenvironment, providing critical insights into mechanisms of immune evasion and novel therapeutic targets.

5.2 Genome-Wide CRISPR Screen: Identifying Novel Tumor-Intrinsic Regulators of Antitumor Immunity

Identifying novel immune-evasive mechanisms used by cancer cells is critical for deepening our understanding of tumor-immune interactions and improving antitumor immune responses. Recent CRISPR-based functional genomic screens have offered unprecedented insights into tumor-intrinsic pathways that inhibit immune cell activity, uncovering promising targets to overcome immune resistance and enhance immunotherapy efficacy. In particular, pooled genome-wide CRISPR/Cas9 knock-out screens have been performed in cancer cells under immune pressure using co-culture system (**Figure 9A**). These studies have identified key tumor-intrinsic regulators of susceptibility to human lymphocytes[136,137], cytotoxic T cells[138-150] or NK cells[141,151-154](summarize in **Table 2A**). To promote precise and strong interactions with T cells, researchers have used tumor models that express engineered antigens such as OVA, NY-ESO-1, and GFP. These antigens are specifically recognized by CTLs that express transgenic T cell receptors[138-141,147,150]. In contrast, some CRISPR screen studies have utilized natural tumor antigens to direct T cell responses, for instance, Pmel-1 T cells, which recognize a native melanoma antigen (gp100) with relatively low TCR affinity, offering greater physiological relevance[144-146,148,149]. Additionally, engineered T cells, including CAR T cells, have been used to apply selective immune pressure and identify tumor-intrinsic resistance mechanisms specific to adoptive cell therapies[142,143]. Across these CRISPR-based studies, several key pathways essential for tumor cell cytotoxicity of CTL[138,147,148,150], CAR T cells[142,143], and NK cells[151-153] have been uncovered. These include components of the IFNγ signaling axis, tumor antigen presentation machinery, death receptor pathways (e.g., Fas signaling), as well as costimulatory and adhesion molecules. Furthermore, disruption of previously unknown resistance pathways such as NF-κB signaling[144,146], autophagy[146], chromatin remodeling[144], and TNF signaling[139] has been shown to enhance antitumor immune responses. Beyond cell-based cytotoxicity, CRISPR/Cas9 screens have also been applied to identify tumor-intrinsic determinants of resistance to ICB therapy in vitro. These efforts have revealed modulators of ICB efficacy, including regulators of epigenetic modifiers[136] and protein neddylation[137] which represent potential targets for overcoming immunotherapy resistance.

Given the complexity of the TME and its role in modulating immune evasion and resistance to immunotherapy, various CRISPR screening strategies have been employed to uncover tumor-intrinsic factors that influence anti-tumor immune responses in vivo (**Figure 9B**) (summarize in **Table 2B**). These approaches include the use of both immunocompromised and

immunocompetent mouse models[146,155-164], adoptive T cell transfer[148,165], and treatment with ICB therapies[155-157,161,162,166-170]. Early in vivo CRISPR screens demonstrated that loss of tumor-intrinsic gene, such as phosphatase *Ptpn2* sensitized melanoma to anti-PD-1 therapy by enhancing JAK-STAT signaling[155]. Similarly, deficiency of the epigenetic regulator *Asf1a* in lung cancer increased sensitivity to PD-1 blockade by promoting M1 macrophage polarization and T cell activation via GM-CSF upregulation[157]. Conversely, *Cop1* supported immune evasion by inducing chemokines that recruited immunosuppressive macrophages[160].

Together, these data highlight the power of CRISPR screens to uncover actionable targets for cancer immunotherapy, as exemplified by the development of a small-molecule inhibitor of *PTPN2,* which is now progressing toward clinical evaluation[171].

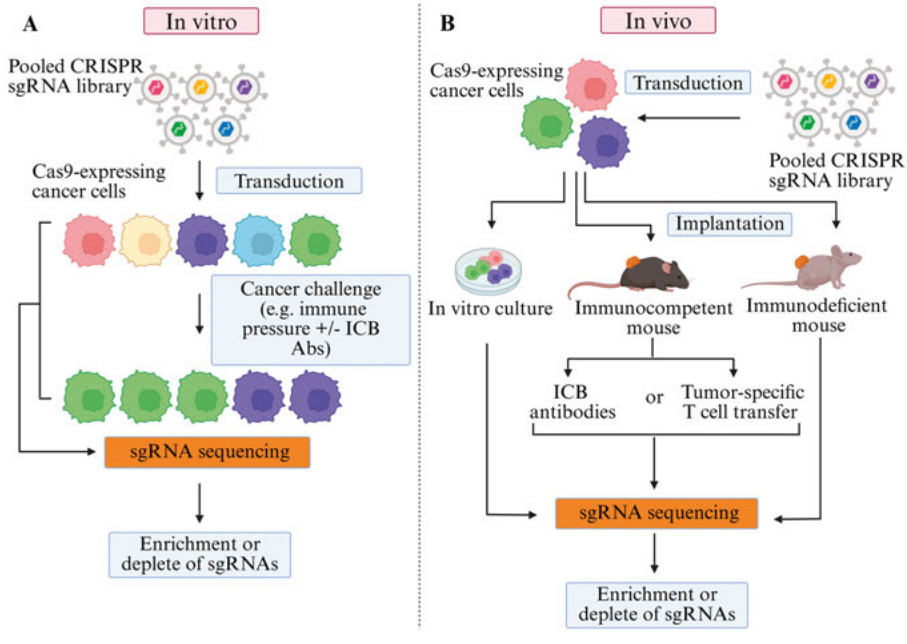


Figure 9. CRISPR screen to identify tumor-intrinsic regulators of anti-tumor immunity. A) In vitro CRISPR screening is performed by transducing Cas9-expressing cancer cells with a pooled sgRNA library and exposing them to immune checkpoint blockade (ICB) therapy to identify genes that modulate anti-tumor immune responses. **B)** For in vivo studies, Cas9-expressing tumor cells are transduced with the sgRNA library, and then are cultured in vitro or implanted into either immunodeficient or immunocompetent mice, modeling the absence or presence of immune pressure, respectively. Immunocompetent mice are treated with ICB antibodies or tumor-specific T cells to evaluate genes regulating immunotherapy response. Tumors are collected and subjected to sgRNA sequencing. The enriched gRNAs represent genes that are required for the treatment while the depleted gRNAs represent genes that confer resistance to the treatment. The figure was created in BioRender.com.

Table 2A. Overview of <u>in vitro</u> CRISPR screening studies				
Cell types	**Cancer challenge**	**Task**	**Top hits**	**Ref**
Human Mel624 NY-ESO-1^{+} melanoma cells	NY-ESO-1 TCR-transduced CD8^{+} T cells Vs cancer alone	Genes in tumor cells essential for EFT	*APLNR*	138
IFNGR1-deficient D10 human melanoma cells	MART-1 transduced CD8^{+} T cells Vs control CD8^{+} T cells	Genes in tumor cells confer resistance to T cell killing independent of *IFNG* pathway	Components in TNF pathways	139
Human A375 NY-ESO-1^{+} melanoma cells	NY-ESO-1 TCR-transduced CD8^{+} T cells Vs control CD8^{+} T cells	Genes in tumor cells confer resistance to T cell cytotoxicity	*CD274, MCL1, JUNB & B3GNT2*	140
Human MART-1^{+} D10 melanoma cells	MART-1^{+} transduced CD8^{+} T cells or NK cells (KHYG-1) Vs untreated	Genes in tumor cells confer resistance to T cell or NK cytotoxicity	LUBAC components	141
Human IMR-32 *MYCN*^{Amp} NB cells	Pool lymphocytes treated with anti-PD-1 Abs (nivolumab) Vs without Abs	Genes in tumor cells confer resistance to anti-PD-1 therapy	*H2AFY*	136
Human ST1 and KK1 ATLL cancer cells	Human YT1 (NK cell line) treated with CCR4 mAbs (mogamulizumab) Vs without Abs	Genes in tumor cells enhance direct or ADCC NK cytotoxicity	*CD48*	151
Human HCT-116 colon carcinoma cells	Human NK cells (KHYG-1) treated with Vs without anti-NKp44 mAbs Vs NKp44-deficient KHYG-1 cells	Genes in tumor cells essential for NKp44-mediated tumor killing by NK	*ICAM-1*	152
Human CD19^{+} Nalm6 ALL cells	CART-19 cells	Genes in tumor cells essential to CART19 cells	Death receptor signaling molecules	142
Human K562 erythroleukemia cells	Primary NK cells	Genes in tumor cells essential and resistance to NK cytotoxicity	*NCR3LG1* and antigen presentation or IFNγ signaling	153
Human U87 glioblastoma cells	EGFR-CART cells	Genes in tumor cells essential to CART cells	*IFNγR1*, *JAK1* or*JAK2*	143
Human MDA-MB-231 TNBC cells	Pool lymphocytes treated with anti-PD-1 Abs (nivolumab) Vs without Abs	Genes in tumor cells confer resistance to anti-PD-1 therapy	*NEDD8*	137
Mouse B16F10 melanoma cells	Pmel-1/OT-1 T cells Vs control T cells (irrelevant specificity)	Genes in tumor cells confer resistance to T cell killing	SWI/SNF[1]	144
Mouse Hepa1-6-OVA	OT-1 T cells Vs WT CD8^{+} T cells	Genes in tumor cells confer resistance to T cell cytotoxicity	*Birc2*	145
Multiple mouse tumor cell lines positive with HA or OVA	OT-1 CD8^{+} T or CL4 CD8^{+} T cells Vs control CD8^{+} T cells	Genes in tumor cells confer resistance to T cell killing	*Atg3, Atg7, Atg5 & Fitm2*	146
Mouse B16F10 melanoma cells	Mouse NK cells (CD27^{+}CD11b^{+})	Genes in tumor cells confer resistance to NK cytotoxicity	IFNγ signaling and/or antigen presentation components	154
Mouse SIYRYYGL-expressed B16F10 melanoma cells (B16.SIY)	Mouse SIY-specific CD8^{+} TCR transgenic T cells isolated from *Rag2–/–* mice	Genes in tumor cells essential for CD8^{+} cytotoxicity	*Ifngr2* and *Jak1*	147

Mouse B16/OVA cells	OT-1 T cells	Genes in tumor cells essential to T cell cytotoxicity	*Jak1*	[148]
Mouse MC38 colon cells expressing melanoma antigen gp100 (MC38/gp100)	Pmel T cells Vs cancer cell alone	Genes in tumor cells confer resistance to anti-tumor immunity	*Prmt1 & Ripk1*	[149]
Mouse A20 lymphoma cells (Ag^+ A20 GFP or Ag^- A20 mCherry cells)	GFP-specific (JEDI) CD8 T cells	Genes in tumor cells essential for T cell cytotoxicity	*Fas*	[150]

EFT: effector function of $CD8^+$ T cells, LUAD: lung adenocarcinoma, LUBAC: linear ubiquitination chain assembly complex (*RNF31*, *RBCK1* and *SHARPIN*), NB: neuroblastoma, ATLL: adult T-cell leukemia/lymphoma, HA: haemagglutinin, OVA: ovalbumin, ALL: acute lymphoblastic leukemia, CML: chronic myeloid leukemia. [1]Chromatin remodeling complex components.

Table 2B. Overview of in vivo CRISPR screening studies

Cell types	Cancer challenge	Task	Top hits	Ref
Mouse CMT167 lung cancer cells	C57BL/6 mice treated with anti-PD-1 Abs Vs control IgG	Genes in tumor cells confer resistance to anti-PD-1 therapy	*Tubb3*	[166]
Multiple mouse tumor cell lines	WT mice treated with/without anti-PD-1/ anti-CTLA-4 Abs Vs non-treated (NSG) mice	Genes in tumor cells confer resistance to anti-tumor immunity and/or ICB Abs	IFNγ signaling pathway	[161]
Mouse B16 melanoma cells	C57BL/6 mice treated with GVAX or in combination with anti-PD-1 Vs non-treated (*Tcra*$^{-/-}$)	Genes in tumor cells confer resistance to anti-PD-1 Abs	*Ptpn2*	[155]
Mouse B16 melanoma cells	C57BL/6 mice treated with GVAX or in combination with anti-PD-1 Vs non-treated (*Tcra*$^{-/-}$)	Genes in tumor cells confer resistance to anti-PD-1 Abs	*Adar1*	[155] [167]
Mouse B16 melanoma and Lewis lung carcinoma	C57BL/6J mice treated with anti-PD-1 (B16) or anti-PD-1+anti-CTLA-4 (LLC) Vs non-treated (NSG) mice	Genes targeting epigenetic regulators confer resistance to ICB Abs	*Setdb1*	[156]
Mouse KrasG12D/Trp53−/− (KP) mutant lung cancer	B6-*Rag1*−/− and B6 WT mice treated with anti-PD-1 Abs Vs IgG control	Genes targeting epigenetic regulators confer resistance to anti-PD-1 Abs	*Asf1a*	[157]
Mouse 4T1 TNBC cells	BALB/c mice Vs 4T1 cells transduced with gRNAs cultured in vitro	Genes in tumor cells confer resistance to anti-tumor immunity	*Cd28*	[163]
Mouse MC38 colon cancer cells	C57BL/6 J mice Vs B6-*Rag1*$^{-/-}$ mice	Genes in tumor cells confer resistance to anti-tumor immunity	*Mbtps1*	[164]
Mouse 4T1-mOva TNBC cells	BALB/c treated with/without ovalbumin Vs BALC/c *Foxn1-/-* (nude)	Genes in tumor cells confer resistance to anti-tumor immunity	*Cop1*	[160]
Mouse B-ALL cancer cells	Irradiated C57BL/6 mice treated with mouse CART19 cells or control mouse CART targeting hEGFRvIII Vs cancer cells	Genes in tumor cells confer resistance to CAR-T cells	IFNγR/JAK/STAT & antigen presentation pathways	[165]

	transduced with gRNAs cultured in vitro			
Mouse autochthonous liver cells[1]	Mice treated with anti-PD-1 or anti-CTLA-4 Vs non-treated (PBS)	Genes in tumor cells confer resistance to ICB therapy	*Kmt2d*	[168]
Multiple mouse tumor cell lines	WT mice Vs immunodeficient (SCID or *Rag1*$^{-/-}$)	Genes in tumor cells block tumorigenesis in the presence of adaptive immunity	*Gna13*	[159]
Mouse *Kras*^{G12D} */Trp53*^{R172H} mutant KP pancreatic cells	C57BL/6J WT or immunodeficient mice Vs gRNAs cultured in vitro	Metabolic genes in tumor cells confer resistance to anti-tumor immunity	*Atg7*	[158]
KPCY pancreatic cells (6419c5 tumor cell clone)	C57BL/6 WT treated with combination therapy GAFCP Vs non-treated	Genes in tumor cells confer resistance to combination therapy GAFCP	*Kdm3a*	[169]
Mouse renal cells (Renca)	BALB/c mice treated with/without anti-PD-1 and anti-CTLA-4 Vs NSG mice	Genes in tumor cells confer resistance to ICB therapy	MHC class I antigen presentation molecules, and autophagy components	[162]
Human MDA-MB-231 TNBC cells	Humanized mice treated with anti-PD-1 Abs (pembrolizumab) Vs control IgG	Genes in tumor cells confer resistance to anti-PD-1 therapy	*TPST2*	[170]

TPST2: tyrosylprotein sulfotransferase-2, NSG: nod scid *Il2rg*−/−, GVAX: granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting irradiated tumor cell vaccine, mOva: membrane-bound ovalbumin, MHC: major histocompatibility complex, GAFCP: gemcitabine (G), abraxane (A), CD40 agonistic antibody (F), CTLA4 blocking antibody (C) and PD1 blocking antibody (P). [1]Adeno-associated virus-CRISPR-mediated pooled mutagenesis.

6 Neddylation: A Molecular Gatekeeper of Protein Homeostasis

Neddylation is an essential post-translational modification that regulates protein function and stability in eukaryotic cells. It involves the reversible covalent attachment of the ubiquitin-like protein NEDD8 (neuronal precursor cell-expressed developmentally down-regulated protein 8) to specific lysine residues on substrate proteins[172].

NEDD8 is a highly conserved protein primarily localized in the nucleus, and is widely expressed across various adult tissues. Among ubiquitin-like proteins, NEDD8 shares the highest sequence similarity with ubiquitin, with their human orthologues exhibiting approximately 59% amino acid identity[173,174]. Despite this close resemblance, NEDD8 and ubiquitin perform distinct and non-redundant cellular functions due to subtle structural differences that govern their specific interactions[175]. Central to NEDD8's unique cellular roles is its conjugation to substrate proteins via a tightly regulated enzymatic cascade. This process involves the NEDD8-activating enzyme E1 (NAE), a heterodimer of NAE1 (APPBP1) and UBA3, followed by transfer to NEDD8-conjugating enzymes E2, and finally substrate-specific NEDD8 E3 ligases[176-180]. Briefly, mature NEDD8 is adenylated and activated in an ATP-dependent reaction catalyzed by NAE[181]. The activated NEDD8 is then transferred to E2 enzymes UBE2M (UBC12) or UBE2F via a trans-thiolation mechanism[182,183], before being covalently attached to a lysine residue on the substrate protein in an E3 ligase-mediated step (**Figure 10**)[180].

Cullin proteins (Cul-1, 2, 3, 4A, 4B, and 5) are the most extensively characterized substrates of the neddylation pathway and serve as scaffold components within Cullin-RING E3 ubiquitin ligases (CRLs), a principal class of E3 ubiquitin ligases[184]. CRLs mediate the ubiquitination and subsequent proteasomal degradation of approximately 20% of cellular proteins via the ubiquitin-proteasome system (UPS)[184-186]. The assembly of CRL complexes is driven by cullins functioning as molecular scaffolds that facilitate the coordinated recruitment of adaptor proteins, substrate receptors, and RING domain-containing E3 ligase subunits[184,187-189]. Neddylation activates CRLs through the covalent attachment of NEDD8 to a conserved lysine residue on the cullin scaffold[190,191], inducing a conformational change to an open, active state. This

structural rearrangement enhances substrate accessibility to the CRL complex, promoting efficient ubiquitin transfer and subsequent recognition and degradation of ubiquitinated substrates by the UPS[192-194]. Conversely, CRL activity is stringently regulated by deneddylation, a reversible process mediated by specific deneddylases such as CSN5, NEDP1, and DEN1. These enzymes catalyze the removal of NEDD8 from protein substrates, resulting in inactivation of the protein neddylation process and contributing to the dynamic maintenance of cellular homeostasis[195-197] (**Figure 10**).

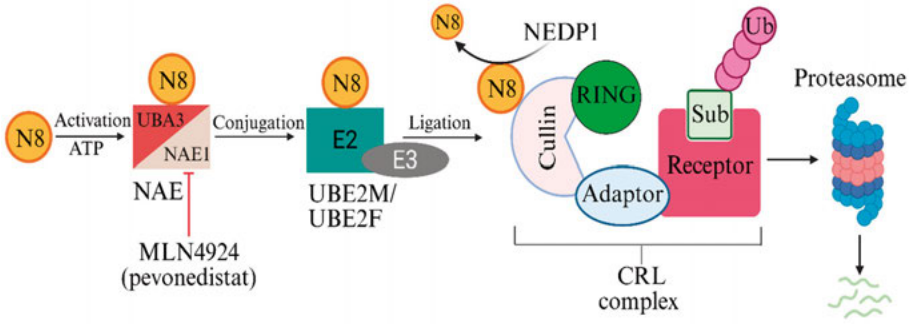


Figure 10. The neddylation pathway. Neddylation is initiated by the E1 activating enzyme complex NAE, composed of the catalytic subunit UBA3 and the regulatory subunit NAE1. Activated NEDD8 is subsequently transferred to one of two E2 conjugating enzymes, UBE2M or UBE2F. E3 ligases, such as RBX1 and RBX2, then facilitate the covalent conjugation of NEDD8 to specific target proteins, particularly Cullins. Neddylated cullins assemble into Cullin-RING ligase (CRL) complexes, which drive the ubiquitination of substrate proteins, marking them for proteasomal degradation. This modification is reversed by deneddylases like NEDP1, which remove NEDD8 to modulate CRL function and maintain substrate homeostasis. The figure was created in BioRender.com.

Many CRL substrates are functionally essential regulatory proteins, including signal transducers, cell cycle regulators, transcription factors, tumor suppressors, and oncoproteins. Notable examples include the cyclin-dependent kinase inhibitors p21 and p27, the cell cycle regulator cyclin E, the DNA replication licensing factor CDT1, transcription factors such as HIF-1α and NRF2, and the proto-oncogene c-Myc[172,198]. Beyond CRL substrates, a variety of non-cullin proteins have also been recognized as substrates of neddylation. In most cases, neddylation appears to inhibit their functional activity through diverse mechanisms, including modulation of protein stability, interference with protein-protein or protein-DNA interactions, and changes in subcellular localization[176]. For example, the tumor suppressor p53 is neddylated by MDM2, a modification that represses p53 transcriptional activity without promoting its degradation[199]. Several other non-cullin proteins have been identified as NEDD8 substrates, including the tumor suppressor pVHL, the oncoprotein Hu

antigen R (HuR), receptor proteins such as the epidermal growth factor receptor (EGFR) and the type II TGF-β receptor, as well as the transcription factor E2F-1, among others[176,198].

6.1 Hijacking Neddylation: Post-Translational Reprogramming in Tumorigenesis

Emerging evidence has revealed a strong association between dysregulated neddylation and tumorigenesis. Literature data indicates that NEDD8 and key enzymes of the neddylation pathway are frequently overexpressed in a variety of human cancers, a pattern that correlates with disease progression and poor clinical outcomes[185,200-211]. Therefore, hyperactivation of the neddylation pathway may act as an oncogenic driver in tumorigenesis by facilitating the degradation of cancer-related regulatory proteins, such as the tumor suppressors p21 and p27[212]. Given its central role in promoting malignant transformation, protein neddylation has emerged as a promising target for anti-cancer therapy, with efforts focused on inhibiting key enzymatic components of the pathway.

Pharmacological inhibition of the neddylation pathway represents an active area of research, with MLN4924 (also known as pevonedistat) identified as a potent and highly selective small-molecule inhibitor of NAE (**Figure 10**). Pevonedistat disrupts protein neddylation by blocking the initial activation step of the enzymatic cascade[186]. In particular, pevonedistat is an ATP analog that selectively binds to the adenylation site of the NAE, mimicking the NEDD8-adenylate intermediate. It forms a stable covalent adduct with NEDD8, thereby locking the enzyme in an inactive state and preventing further progression of the neddylation cascade[176]. A primary cellular consequence of pevonedistat treatment is DNA re-replication, attributed to the stabilization of the DNA replication licensing factor CDT1, a known CRL substrate. In addition to CDT1 accumulation, pevonedistat leads to the buildup of various CRL substrates, triggering a range of cellular responses, including apoptosis, autophagy, cell cycle arrest and senescence, in a cell type-dependent manner[186,213-215]. While other NAE inhibitors have also been developed e.g., TAS4464, pevonedistat remains the most extensively studied compound in this class. Consistent with its mechanism of action, pevonedistat has demonstrated potent anti-tumor activity across various preclinical cancer models[216-220]. Due to its promising efficacy, pevonedistat has been evaluated in combination with standard chemotherapies across various malignancies[221-224]. However, its combination with azacitidine failed to show significant clinical benefit in myeloid malignancy patients, as demonstrated by the negative results of the Phase III PANTHER trial (NCT03268954)[225]. Separately, the

development of TAS4464 was halted in a Phase 1 trial for multiple myeloma due to severe liver toxicity (NCT02978235), with similar dose-limiting toxicities observed in another trial involving patients with solid tumors[226].

6.2 Neddylation and Immune Cell Dynamics in Tumors

Several lines of evidence suggest that neddylation plays a regulatory role in modulating immune cell functions. Specifically, inhibition of protein neddylation suppresses macrophage proliferation, inflammatory responses, and pro-inflammatory cytokine production[227-229]. In lung cancer cells, pevonedistat-mediated neddylation inhibition downregulates immune-related pathways such as TNF and NF-κB signaling, resulting in decrease expression of chemokines critical for recruiting myeloid-derived suppressor cells[212]. Furthermore, neddylation regulates DC function by modulating cytokine production, co-stimulatory molecule expression, and cell survival. In particular, inhibition of neddylation via pevonedistat or knockdown of the E3 ligase component *RBX2* suppresses inflammatory cytokines and induces DC apoptosis[230-233]. Moreover, pevonedistat-mediated inhibition of the NEDD8-activating enzyme in multiple myeloma enhance NK cell-mediated anti-tumor activity by inducing upregulation of NKG2D ligands on tumor cells[234,235]. Xu et al. demonstrate that neddylation promotes the degradation of nuclear cGAS, thereby suppressing type I IFN production. Inhibition of this pathway enhances nuclear cGAS activity, leading to cGAS-dependent type I IFN responses and improved priming of innate immunity[236].

Inhibition of protein neddylation, either through NAE inhibitors or siRNA targeting the *UBE2M* E2 enzyme, also impairs adaptive immunity such as $CD4^+$ T cell proliferation[237] and TCR signaling[238]. Consistently, genetic deletion of *RBX2* markedly reduces T cell activation and cytokine production. These findings are supported by both in vitro and in vivo studies using the neddylation inhibitor pevonedistat[239]. Moreover, neddylation is crucial for the differentiation of follicular T helper cells[240], and for effective antibacterial T cell immune responses[241], highlighting its broad impact on immune cell function and signaling. Notably, disrupting neddylation in Treg cells impairs their suppressive function, leading to an inflammatory phenotype, further underscoring the essential role of this pathway in maintaining immune homeostasis.

The impact of protein neddylation on cancer immunogenicity has also been investigated using pevonedistat. Treatment with this inhibitor induces proteome instability and significantly enhances the efficacy of anti-PD-1 antibodies in dMMR colon cancer cells[242]. Similarly, pevonedistat upregulates PD-L1

expression in glioblastoma cells and synergizes with ICB therapy to improve outcomes in mouse models[220]. Notably, the combination of pevonedistat and ICB therapy is currently being evaluated in a phase I clinical trial involving dMMR colon cancer patients (NCT04800627).

Collectively, these findings highlight the essential role of neddylation in regulating diverse immune cell functions, including activation, proliferation, cytokine production, and effector responses. Interestingly, neddylation appears to have a dual role in immune regulation, with its effects varying depending on the cellular context. Although pevonedistat effectively targets this pathway to modulate immune activity, some of its effects may result from off-target mechanisms[137], potentially contributing to both its immunomodulatory and anti-tumor actions. Therefore, careful monitoring of immune responses is crucial when employing pevonedistat or other neddylation inhibitors. Optimizing treatment through metronomic or intermittent dosing, as well as targeted delivery strategies[243] may improve therapeutic outcomes while limiting adverse effects.

7 Aim of the Thesis

Immune checkpoint blockade (ICB) therapy is designed to restore and amplify anti-tumor immune responses, enabling the immune system to recognize and eliminate cancer cells. However, clinical benefits are only recorded in a subset of cancer patients. In addition, suppressive myeloid cells can limit anti-tumor immune responses and are recognized as one of the major barriers for efficacious cancer immunotherapy. Therefore, deep mechanistic understanding of the immunosuppressive network in cancer or myeloid cells could lead to new treatment options for patients. In this work, we aimed to investigate cancer-intrinsic resistance mechanisms in triple-negative breast cancer that impair responses to ICB therapy to enhance anti-tumor immunity and improve therapeutic outcomes. Further, we aimed to deeply explore the functional role of this resistance mechanism within myeloid cells, with the goal of clarifying its contribution to immune suppression and therapeutic resistance in the tumor microenvironment.

8 Summary of Investigation

Paper I

Title: Loss of NEDD8 in cancer cells causes vulnerability to immune checkpoint blockade in triple-negative breast cancer

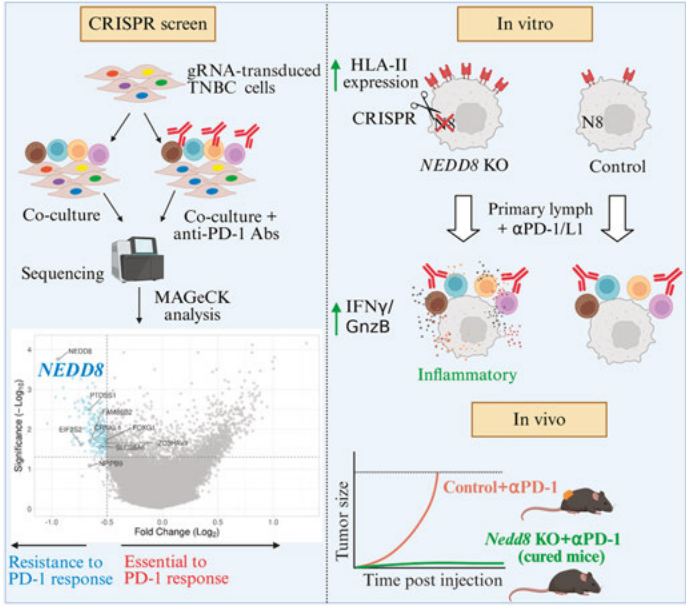
Aim: To identify and validate novel suppressive mechanisms in triple-negative breast cancer cells upon the anti-PD-1 therapy.

Background: Triple-negative breast cancer (TNBC) is a clinically aggressive subtype of breast cancer with limited effective therapeutic options[43]. Immune checkpoint blockade (ICB) targeting the PD-1/PD-L1 axis has shown therapeutic potential, offering a promising immunotherapeutic strategy[50]. However, clinical responses remain suboptimal, potentially due to tumor-intrinsic mechanisms that suppress anti-tumor immune responses[244]. Therefore, a deeper understanding of these tumor-intrinsic resistance mechanisms is critical for enhancing the efficacy of immunotherapy and guiding the development of novel combination strategies involving checkpoint inhibitors in TNBC.

Major findings: To achieve our study's goal, we performed genome-wide CRISPR screens integrated with a Tumor Immune Co-Culture System (TICS) in the presence of an FDA-approved anti-PD-1 immune checkpoint inhibitor, nivolumab. This approach identified *NEDD8* as one of the most significantly depleted genes in TNBC cells treated with anti-PD-1, suggesting a key role in resistance to PD-1 blockade. Targeted deletion of *NEDD8* using CRISPR/Cas9 in cancer cells enhanced anti-tumor immunity and increased sensitivity to both PD-1 and PD-L1 (durvalumab) blockade in human TNBC co-culture assays with primary lymphocytes. Proteomic analysis confirmed increased immunogenicity in *NEDD8* KO cells, including upregulation of HLA-DR molecules. In a murine TNBC model, *Nedd8* loss led to enhanced responses to PD-1 therapy, durable tumor regression, and $CD8^+$ T cell-dependent survival benefits. Surprisingly, despite *NEDD8* being an essential gene[245], TNBC KO cells remained proliferative. Follow-up CRISPR screens and proteomic profiling indicated activation of compensatory pathways such as

mTOR signaling, DNA repair, and ubiquitination, contributing to cellular adaptation. Collectively, our findings demonstrate that *NEDD8* functions as a context-dependent essential gene in TNBC and reveal its role in mediating intrinsic resistance to immune checkpoint blockade therapy.

Visual summary for paper I:



Paper II

Title: Cancer intrinsic protein neddylation modulates the intra-tumoral immune landscape to constrain immune checkpoint blockade therapy.

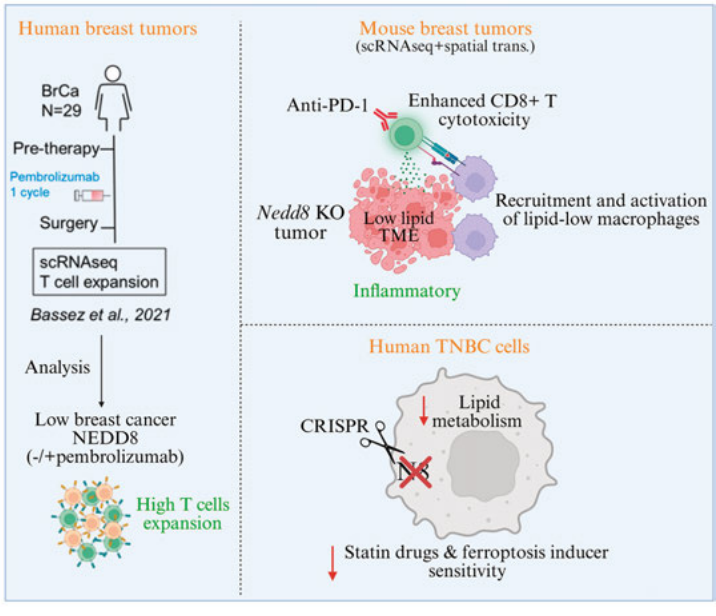
Aim: To dissect the immune regulatory network orchestrated by neddylation in TNBC cells and explore how NEDD8 shapes tumor immunogenicity following anti-PD-1 therapy.

Background: Emerging evidence from Paper I has identified protein neddylation as a key cancer-intrinsic suppressor of anti-PD-1 therapy, highlighting its potential role in immune evasion. Neddylation, a multi-enzymatic process involving conjugation of NEDD8 to target substrates, regulates multiple biological mechanisms, including those driving tumorigenesis[176]. Despite growing recognition of its role in oncogenesis, how neddylation shapes tumor-immune dynamics in response to immune checkpoint blockade remains poorly understood. Therefore, understanding the functional link between cancer-intrinsic protein

neddylation and immune cross-talk upon PD-1 blockade therapy is critical for identifying novel strategies to enhance immunotherapy responses in cancer.

Major findings: Analysis of public single-cell RNA-seq data from breast cancer patients[246] revealed that low protein neddylation in pre-treatment tumors undergoing anti-PD-1 therapy was associated with enhanced T cell expansion. In mice bearing neddylation-deficient EO771 tumors, PD-1 blockade induced strong anti-tumor responses and immune memory. Furthermore, single-cell data from both patients and tumor-bearing mice showed that elevated neddylation impaired antigen presentation and rewired lipid metabolism in tumor cells and macrophages. In contrast, *Nedd8* KO in tumor cells significantly enhanced $CD8^+$ T cell cytotoxicity and macrophage activation following PD-1 blockade. Single-cell spatial transcriptomics revealed distinct spatial-temporal changes and the formation of stimulatory macrophage niches in neddylation-deficient murine tumors. Moreover, genetic deletion of neddylation in human breast cancer cells caused loss of sensitivity to statins and ferroptosis inducers. Notably, combining simvastatin or RSL3 with PD-1 blockade strongly synergized to enhance anti-tumor immunity and immune memory in tumor-bearing mice. Therefore, our study uncovers a novel function of protein neddylation in modulating lipid metabolism and tumor immunogenicity, offering actionable approaches to reprogram the tumor microenvironment for improved immunotherapy outcomes.

Visual summary for paper II



Paper III

Title: Targeting intracellular mechanisms to enhance sensitivity to TLR activation in myeloid cells

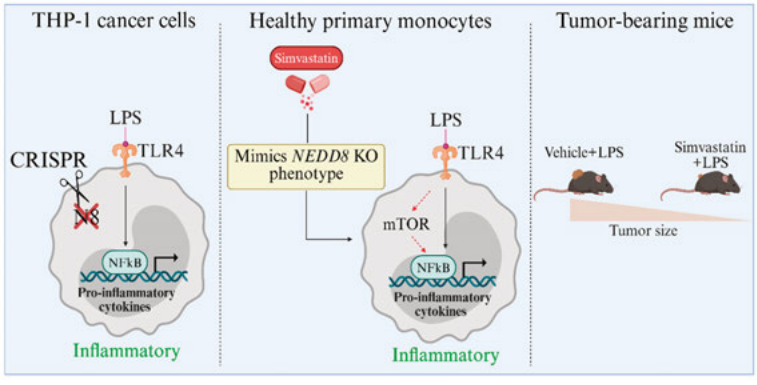
Aim: To investigate the role of *NEDD8* in regulating innate immune responses within myeloid cells and to determine how neddylation influences myeloid-driven immunosuppression in the tumor microenvironment.

Background: Myeloid cells are pivotal modulators of the tumor immune microenvironment and play a dual role in shaping responses to cancer immunotherapy. However, tumors exploit this plasticity by secreting factors that recruit and reprogram myeloid cells toward immunosuppressive phenotypes, thereby attenuating anti-tumor immune responses. The accumulation of such suppressive myeloid populations correlates with poor clinical outcomes and contributes to resistance against immune checkpoint blockade therapy through mechanisms including the inhibition of effector immune cell infiltration and suppression of cytotoxic immune activity[247,248]. Thus, delineating the molecular pathways that govern myeloid cell function in tumors is critical for identifying novel therapeutic targets. Since neddylation has been implicated as a cancer-intrinsic mechanism of resistance to ICB therapy, its specific role in regulating myeloid cell function remains undefined. We therefore hypothesized that NEDD8 modulates the immunoregulatory behavior of myeloid cells within the tumor microenvironment.

Major findings: Genetic deletion of *NEDD8* in the human monocytic THP-1 cell line significantly enhanced TLR-driven innate immune activation, resulting in elevated expression of pro-inflammatory cytokines and immune-stimulatory surface molecules. Importantly, the statin drug, simvastatin, recapitulated the effects of *NEDD8* knockout in human primary CD14+ monocytes and induced robust secretion of inflammatory cytokines, including IL-1β, IFN-γ, and TNF-α in vitro. Integrated CRISPR screen and proteomic analyses revealed that *NEDD8* loss upregulated innate immune stimulatory pathways while rendering components of the mTORC1 signaling pathway essential in THP-1 cells. Consistently, pharmacological inhibition of mTOR-signaling pathway significantly attenuated simvastatin-induced cytokine secretion in primary monocytes, indicating a critical role for mTOR signaling downstream of *NEDD8* depletion. In tumor-bearing mice, simvastatin treatment significantly delayed the progression of C1498 monocytic leukemia following TLR stimulation, demonstrating enhanced innate anti-tumor immunity in vivo. Collectively, these findings establish *NEDD8* as a myeloid-intrinsic immune

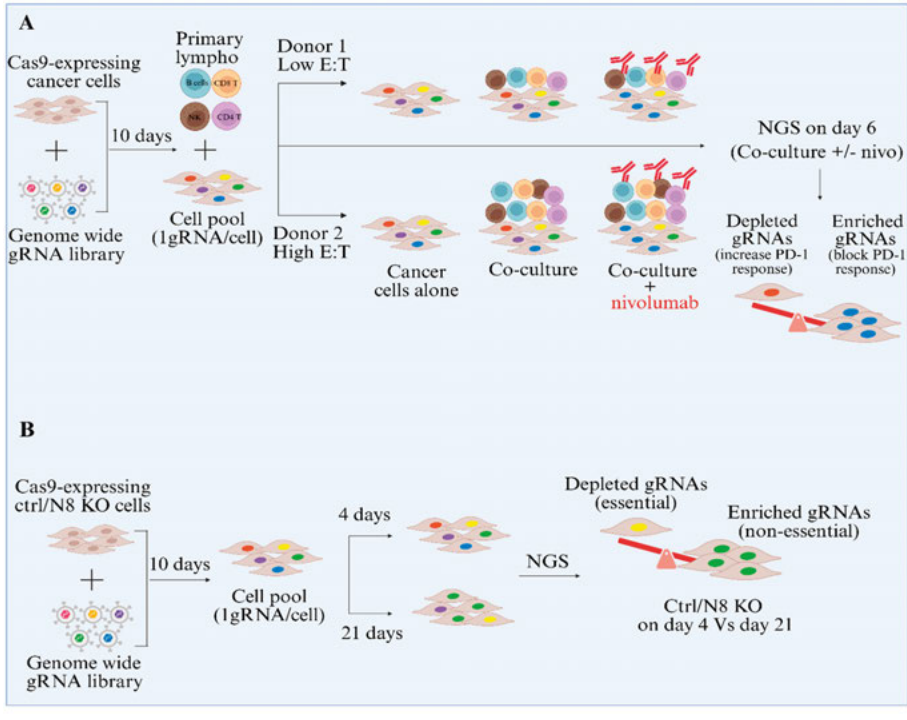
checkpoint that restrains innate immune activation and define an actionable pathway for its modulation.

Visual summary for paper III:



9 Key Methodologies Used in the Project

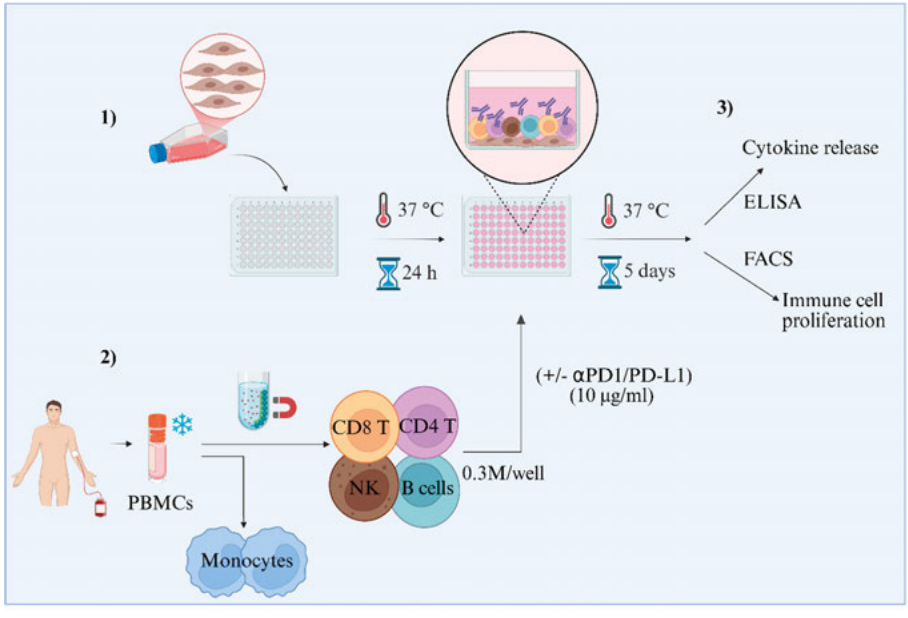
Genome-wide CRISPR screens



A) To identify cancer-intrinsic mechanisms of resistance to anti-PD-1 therapy, an optimized genome-wide CRISPR screen[249] is performed in MDA-MB-231 TNBC cells. Cas9-expressing cells are transduced with a genome-wide gRNA library (one gene knockout per cell) and cultured for 10 days. Primary human lymphocytes are then co-cultured at low (2.5:1) or high (7:1) lymphocyte-to-cancer (L2C) ratios, with or without 10 µg/ml nivolumab. After 6 days, lymphocytes are removed, and cancer cells are harvested for gRNA profiling by next-generation sequencing (NGS). gRNA enrichment or depletion is determined by fold change and p value, compared to the no-antibody co-culture control.

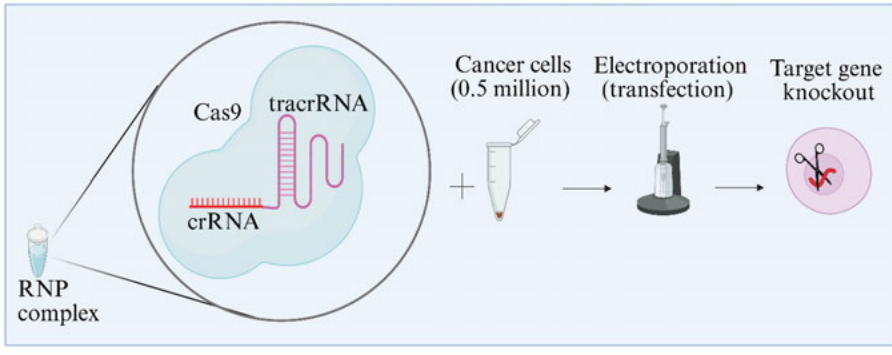
B) To confirm the loss of essentiality in *NEDD8* KO cells and identify functional genes that support cell proliferation, gRNA library-transduced control or *NEDD8* KO cancer cells are harvested on days 4 and 21 post-transduction. gRNA enrichment or depletion in both cell lines is analyzed by comparing gRNA frequencies between the two time points.

Tumor Immune co-Culture System (TICS)



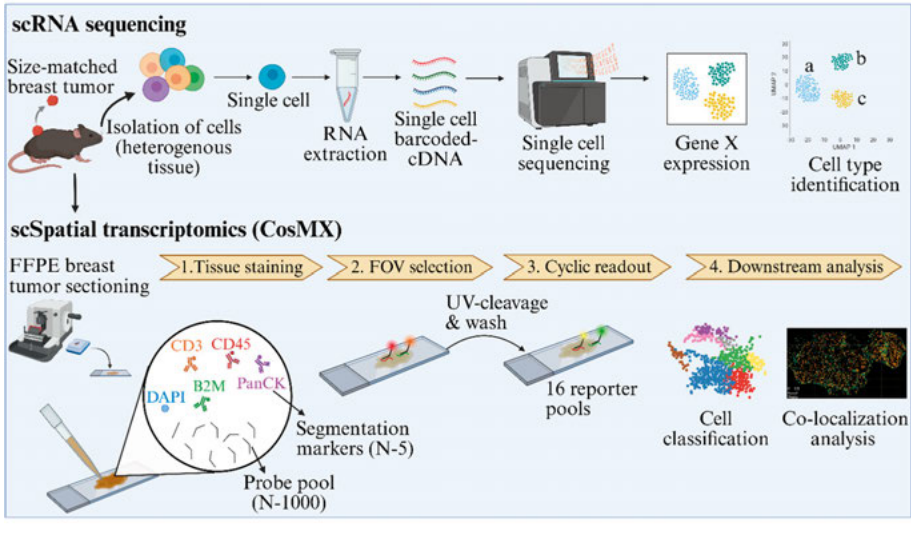
To investigate the role of *NEDD8* in response to anti-PD-1/PD-L1 therapy, an optimized TICS assay[250] is established using control/*NEDD8* KO cell line pair or the neddylation inhibitor pevonedistat. **1)** Cancer cells are seeded in 96-well plates and incubated overnight. **2)** Primary human lymphocytes isolated from healthy peripheral blood mononuclear cells (PBMCs) are added to the tumor-loaded plate, along with PD-1/PD-L1 antibodies (10 µg/ml). **3)** After 5 days of co-culture, supernatants are collected to measure IFNγ and granzyme B secretion by ELISA, and lymphocyte proliferation is assessed by flow cytometry.

Target gene deletion using CRISPR/Cas9



To inactivate target genes, 20 bp gene-specific crRNA is paired with tracrRNA to form a guide RNA, which is then complexed with recombinant Cas9 protein. The Cas9-gRNA complex, known as RNP complex, is introduced into cancer cells via Neon electroporation using a protocol optimized for each cell line. Control cells are transfected without crRNA molecules. Target protein levels are evaluated at least 10 days after transfection by Western Blotting.

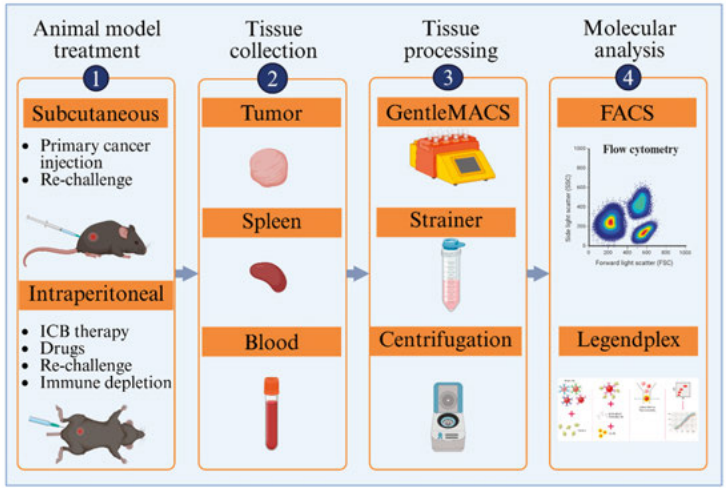
ScRNA sequencing and scSpatial transcriptomics (CosMX)



ScRNA seq is established to investigate how tumor-intrinsic protein neddylation influences antitumor immune response during PD-1 checkpoint inhibition. Briefly, tissues are first dissociated into a single-cell suspension. Individual cells are then captured, barcoded, and sequenced to generate

transcriptomic profiles for high-resolution analysis of cellular diversity. Moreover, to map the spatial organization and cellular composition of the tumor microenvironment, single-cell spatial transcriptomics is performed using the CosMX platform. FFPE sections of murine breast tumors are stained with fluorescent antibodies and dyes to define cell types and boundaries, alongside *in situ hybridization* (ISH) probes targeting 1,000 mRNAs. Selected fields of view (FOVs) are imaged through sequential fluorescence cycles using 16 fluorophore-tagged reporter pools, with fluorophores removed after each cycle via UV cleavage. Spatial transcript data are registered to precise tissue coordinates, enabling cell classification and colocalization analysis based on gene expression and clustering algorithms.

In vivo mouse models



Immunocompetent C57BL/6NTac mice are injected subcutaneously (s.c.) with isogenic murine cancer cell lines. Once tumors become detectable, mice are treated intraperitoneally (i.p.) with immune checkpoint blockade (ICB), small-molecule inhibitors, or depleting antibodies to assess anti-tumor responses. Tumor volumes are recorded continually until defined endpoints. At these time points, tumors and relevant organs are harvested and processed into single-cell suspensions. For cell surface or intracellular proteins, viable cells are stained with fluorochrome-conjugated antibodies following live/dead discrimination. Marker expression is quantified by flow cytometry (e.g., CytoFlex LX). Cytokines secretion is analyzed by multiplex Legendplex assay using serum isolated from blood. For re-challenge studies, cancer cells were implanted s.c. or i.p. into cured mice.

10 Limitations and Future Perspective of the Study

While our study provides novel insights into the role of *NEDD8* in tumor immunity and its potential to enhance responses to ICB therapy, some limitations should be considered when interpreting the findings. Our investigation was confirmed to a breast cancer model, and thus the generalizability of our observations to other solid tumor types remains unknown. Given the substantial heterogeneity of tumor immune microenvironments, it is critical to evaluate whether *NEDD8* inhibition exhibits similar immunomodulatory and therapeutic effects in additional preclinical cancer models, such as melanoma, lung carcinoma, or colorectal cancer. Furthermore, our current approach relied on genetically inhibition of *NEDD8* using CRISPR/Cas9 technique, due to the absence of compounds that specifically or directly inhibit NEDD8. The absence of selective NEDD8-targeting compounds, together with the known immunosuppressive effects of pevonedistat, limits the immediate translational potential of our findings. The development of more precise molecular tools or inhibitors that directly and selectively modulate NEDD8 activity would enable a clearer delineation of its specific functional contributions and supports future clinical studies. Moreover, our study primarily focused on the role of NEDD8 in modulating innate immune responses, particularly in inflammatory cytokine secretion and remodeling of the tumor microenvironment. However, the role of *NEDD8* in the adaptive immune system, especially in lymphocyte activation, proliferation, and effector function, remains largely unexplored. Given the central importance of adaptive immunity in mediating tumor cell cytotoxicity, future studies should address how NEDD8 influences T cell biology, both in homeostasis and in the context of cancer.

To address these limitations and enhance the translational relevance of our findings, future work will focus on developing more targeted therapeutic strategies that improve the specificity and safety profile of NEDD8 pathway inhibition. We are conducting high-throughput screening of structurally diverse and previously uncharacterized compounds to identify novel small-molecule inhibitors that directly or indirectly target the neddylation pathway. Since the clinical-stage neddylation inhibitor pevonedistat exhibits off-target effects and systemic toxicity due to its broad inhibition outside the neddylation pathway, there is a critical need to improve drug delivery and reduce exposure to non-

tumor tissues. To this end, we propose engineering a lipid nanoparticles (NLPs)-based delivery system in which NLP-loaded pevonedistat is conjugated to antibodies recognizing tumor-associated antigens. This targeted approach would facilitate the preferential accumulation of the inhibitor within the tumor microenvironment, thereby minimizing off-target liabilities and enhancing therapeutic efficacy. Such a strategy holds promise not only for improving the safety profile of protein neddylation inhibition but also for enabling more effective combination regimens with immunotherapies such as ICB. In parallel, we are conducting ongoing chemoproteomics and CRISPR-based functional genomic screens aimed at systematically identifying off-target protein interactors and essential genes modulating sensitivity to pevonedistat. These complementary approaches will provide an unbiased, proteome- and genome-wide view of pevonedistat's activity, offering crucial insights into the molecular basis of its toxicity and informing the rational design of more selective NEDD8-targeting agents with improved safety and efficacy profiles.

Furthermore, ongoing efforts are focused on elucidating the underlying mechanisms responsible for the enhanced immunogenicity observed in *NEDD8* KO cells. While we have demonstrated that *NEDD8* KO increases surface antigen presentation, the mechanistic basis for this effect remains to be fully defined. Given the role of the neddylation pathway in post-translational protein regulation, we hypothesize that *NEDD8* loss may alter the processing or stability of intracellular proteins, thereby influencing the pool of peptides available for MHC class I or class II presentation. To investigate this, we are conducting mass spectrometry-based immunopeptidomic analyses to characterize the repertoire of peptides presented on the surface of *NEDD8* KO cells. Preliminary data from these ongoing studies suggest that loss of *NEDD8* may indeed reshape the immunopeptidome, supporting the notion that neddylation plays a critical role in shaping tumor antigenicity. These insights may uncover novel pathways that contribute to immune recognition and could inform the development of new strategies to enhance tumor immunogenicity in the context of immunotherapy.

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I dedicate this thesis to every cancer patient and to all those whose lives have been affected by this disease. To contribute, even in a small way, to the ongoing fight against cancer is both a responsibility and a profound privilege. It is my hope that this work brings us one step closer to deeper understanding, more effective treatments, and, ultimately, a cure.

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