Dissecting the predictive and therapeutic value of HLA-DR-expressing cytotoxic T lymphocytes in Breast Cancer

RUTE MARIA SILVA SALVADOR

A thesis submitted in fulfilment of the requirements for the Doctoral Degree in Health Sciences in the speciality of Biomedicine

September, 2024

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The research presented in this thesis was conducted at NOVA Medical School - Faculdade de Ciências Médicas, Universidade Nova de Lisboa, between 2019 and 2024. This work was supported by a PhD fellowship from the Portuguese Foundation for Science and Technology (SFRH/BD/148422/2019 and COVID/BD/153648/2024). Additional funding was provided by the Terry Fox Research Grant 2019 from Liga Portuguesa Contra o Cancro, the Clinical Investigation Prize 2019 from Pfizer, and iNOVA4Health (UIDB/04462/2020). This work was accomplished under the supervision of Maria de Guadalupe Gonçalves Cabral (PhD) and António Alfredo Coelho Jacinto (PhD).

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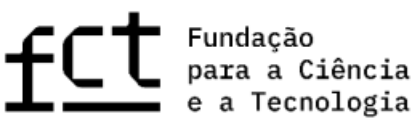

SCIENTIFIC OUTPUTS

Part of the content of this thesis has already been published in a peer-reviewed journal:

- Saraiva, DP; Azeredo-Lopes, S; Antunes, A; **Salvador, R**; Borralho, P; Assis, B; Pereira, IL; *et al*. Expression of HLA-DR in Cytotoxic T Lymphocytes: A Validated Predictive Biomarker and a Potential Therapeutic Strategy in Breast Cancer. Cancers (Basel). **2021** Jul 30;13(15):3841. http://dx.doi.org/10.3390/cancers13153841.

Additionally, the majority of the work presented in this thesis is available as a preprint and has been submitted to a peer-reviewed journal:

- **Salvador, R;** Correia, BF; Grosa, D; Martins, T; Soares Baal, SC; Saraiva, DP; Cristovão-Ferreira, S; Pereira, IL; Rebelo de Almeida, C; Fior, R; Jacinto, A; Mathias, C; Braga, S; Cabral, MG. Enhancing HLA-DR in Cytotoxic T Lymphocytes is crucial for the development of efficient adoptive T cell Therapies for Breast Cancer. *bioRxiv*. https://doi.org/10.1101/2024.09.03.610810

Furthermore, the content of this thesis has been partially presented at the following scientific conferences:

- Selected poster presentation at the XLVII SPI Annual Meeting, Lisbon, **2022**
- Selected oral communication at the 4th Symposium on Immunomodulation in Cancer & Regeneration, Porto, **2022**
- Selected oral communication at the 1st NMS Research Week, Lisbon, **2023**

During the course of this thesis, I also contributed to other studies, resulting in several peer-reviewed publications:

- Ferreira, AC; Mendes, M; Silva, C; Cotovio, P; Aires, I; Navarro, D; Caeiro, F; **Salvador, R**; *et al*. Bone densitometry versus bone histomorphometry in renal transplanted patients: a cross-sectional study. Transplant International 34 6 (**2021**): 1065-1073. http://dx.doi.org/10.1111/tri.13888. 10.1111/tri.13888

- Ferreira, AC; Cotovio, P; Aires, I; Mendes, M; Navarro, D; Silva, C; Caeiro, F; **Salvador, R**; *et al*. The Role of Bone Volume, FGF23 and Sclerostin in Calcifications and Mortality; a Cohort Study in CKD Stage 5 Patients. Calcified Tissue International 110 2 (**2021**): 215-224. http://dx.doi.org/10.1007/s00223-021-00910-8. 10.1007/s00223-021-00910-8

- Saraiva, DP; Correia, BF; **Salvador, R**; de Sousa, N; Jacinto, A; Braga, S; Cabral, MG. Circulating low density neutrophils of breast cancer patients are associated with their worse prognosis due to the impairment of T cell responses. Oncotarget 12 24 (**2021**): 2388-2403. http://dx.doi.org/10.18632/oncotarget.28135. 10.18632/oncotarget.28135

- Coelho, S; Cabral, MG; **Salvador, R**; Andrade, C; Martins, A; Correia, BF; Freitas, P; Cruzado, JM; Jacinto, A. Urinary immune cell phenotype of severe AKI in critically ill patients. International Urology and Nephrology (**2022**): http://dx.doi.org/10.1007/s11255-021-03088-y. 10.1007/s11255-021-03088-y

- Ferreira, AC; Mendes, M; Silva, C; Cotovio, P; Aires, I; Navarro, D; Caeiro, F; Ramos, R; **Salvador,** R; Correia, BF; Cabral, MG; Nolasco, F; Ferreira, A. Improvement of Mineral and Bone Disorders After Renal Transplantation. Transplantation (**2022**). 106, 5, p. e251-e261. https://doi.org/10.1097/TP.0000000000004099

- Ferreira, AC; Mendes, M; Silva, C; Cotovio, P; Aires, I; Navarro, D; Caeiro, F; Ramos, R; **Salvador, R**; *et al*. Biochemical Clusters as Substitutes of Bone Biopsies in Kidney Transplant Patients. Calcified Tissue International (**2024**): http://dx.doi.org/10.1007/s00223-023-01173-1

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ABBREVIATIONS AND ACRONYMS

2D - Two-Dimensional

3D - Three-Dimensional

AC - Adjuvant Chemotherapy

ADC - Antibody-Drug Conjugates

ALI - Air-Liquid Interface

APCs - Antigen-Presenting Cells

ATC - Adoptive T Cell Therapy

BC - Breast Cancer

BL1 - Basal-like 1

BL2 - Basal-like 2

BRCA1 - Breast Cancer Gene 1

BRCA2 - Breast Cancer Gene 2

CAR - Chimeric Antigen Receptor

CAR-T - Chimeric Antigen Receptor T-cell Therapy

CCL - Chemokine (C-C Motif) Ligand

CCL4 - C-C motif chemokine ligand 4

CCR4 - C-C Chemokine Receptor 4

CCR5 - C-C chemokine receptor type 5

CDK - Cyclin-Dependent Kinase

CTLA4 - Cytotoxic T-Lymphocyte–Associated Antigen 4

CTLs - Cytotoxic T Lymphocytes

CXCL - Chemokine (C-X-C Motif) Ligand

CXCR6 - C-X-C motif chemokine receptor 6

DAPI - 4',6-Diamidino-2-Phenylindole

DCs - Dendritic Cells

DFS - Disease-Free Survival

DMSO - Dimethyl Sulfoxide

DMEM - Dulbecco's Modified Eagle Medium

DPBS - Dulbecco's Phosphate-Buffered Saline

E3 - Embryonic Medium

ECM - Extracellular Matrix

EDTA - Ethylenediaminetetraacetic Acid

EGFR - Epidermal Growth Factor Receptor

ELISA - Enzyme-Linked Immunosorbent Assay

EMT - Epithelial-to-mesenchymal transition

ER - Estrogen Receptor

ERBB2 - Erb-B2 Receptor Tyrosine Kinase 2

FA - Formaldehyde

FACS - Fluorescence Activated Cell Sorting

FasL - Fas Ligand

FBS - Fetal Bovine Serum

FGFR - Fibroblast Growth Factor Receptor

FoxP3 - Forkhead Box Protein 3

GF - Growth Factors

GRB7 - Growth Factor Receptor-Bound Protein 7

HDAC - Histone Deacetylase

HDACi - Histone Deacetylase Inhibitors

HER2 - Human Epidermal Growth Factor Receptor 2

HIV - Human Immunodeficiency Virus

HRT - Hormone Replacement Therapy

ICI - Immune Checkpoint Inhibitors

IFN - Interferons

IFN-γ - Interferon Gamma

IL - Interleukin

LDH - Lactate dehydrogenase

lncRNAs - Long non-coding RNAs

MCTSs - Multicellular Tumor Spheroids

MDC - Macrophage-Derived Chemokine

MDSCs - Myeloid-Derived Suppressor Cells

MHC - Major Histocompatibility Complex

M-MDSCs - Monocytic Myeloid-Derived Suppressor Cells

NACT - Neoadjuvant Chemotherapy

NK - Natural Killer

ORR - Overall Response Rate

OS - Overall Survival

OXPHOS - Oxidative phosphorylation

P/S - Penicillin-Streptomycin

PARP - Poly-(ADP-ribose) Polymerase

PBMCs - Peripheral Blood Mononuclear Cells

PD-1 - Programmed Death-1

PD-L1 - Programmed Death-Ligand 1

PDTOs - Patient-Derived Tumor Organoids

PMN-MDSCs - Polymorphonuclear Myeloid-Derived Suppressor Cells

pCR - Pathological Complete Response

PR - Progesterone Receptor

RBC - Red Blood Cells

rIL-2 - Recombinant Interleukin-2

ROS - Reactive Oxygen Species

RPMI - Roswell Park Memorial Institute Medium

scRNA-seq - Single-Cell RNA Sequencing

SEER - Surveillance, Epidemiology, and End Results

siRNA - Small Interfering RNA

sTCR - Synthetic T-Cell Receptor

TARC - Thymus and Activation-Regulated Chemokine

TAMs - Tumor-Associated Macrophages

TANs - Tumor-Associated Neutrophils

TILs - Tumor-Infiltrating Lymphocytes

TIME - Tumor Immune Microenvironment

TME - Tumor Microenvironment

TKIs - Tyrosine Kinase Inhibitors

Th - Helper T Cells

TNF - Tumor Necrosis Factor

TNF-α - Tumor Necrosis Factor Alpha

TNBC - Triple-Negative Breast Cancer

TNM - Tumor, Nodes, Metastasis

Treg - Regulatory T Cells

VEGF - Vascular Endothelial Growth Factor

WHO - World Health Organization

ABSTRACT

Breast cancer (BC) remains the most prevalent cancer in women worldwide, posing a significant public health challenge. Projections indicate an increase in BC incidence and mortality rates in the coming years, underscoring the urgent need for innovative and effective therapeutic strategies.

The treatment landscape for BC is rapidly evolving, with neoadjuvant chemotherapy (NACT) continuing as a standard approach for managing high-risk or inoperable tumors. However, with a success rate below 50%, the effectiveness of NACT is limited, particularly in cases of chemotherapy-resistant tumors. While emerging therapies like immune checkpoint inhibitors show promise, especially in treating triple-negative breast cancer, their applicability across all BC subtypes remains limited. Consequently, novel approaches such as adoptive cellular therapy, which involves the *ex vivo* expansion of patient-derived T lymphocytes, are being investigated.

Building on our previous research, which identified cytotoxic T lymphocytes (CTLs) with high HLA-DR expression within the BC tumor microenvironment as predictive of favorable responses to NACT, this study aims to further elucidate the role of HLA-DR expression in CTLs by investigating how HLA-DR expression enhances CTLs' anti-tumor efficacy, offering potential advancements in T cell-based treatments. We hypothesize that increasing the frequency and functionality of HLA-DR-expressing CTLs can improve BC treatment outcomes, particularly in cases where conventional therapies are ineffective. This research addresses a critical unmet need by introducing a novel approach to enhance the effectiveness of T cell-based therapies. By optimizing HLA-DR expression in CTLs, we aim to deliver more personalized and effective immunotherapies for BC, especially for patients with resistant or difficult-to-treat tumors.

To achieve this, we refined a protocol for the *ex vivo* expansion of HLA-DR-expressing CTLs and employed a 3D co-culture platform to assess the effects of various immune modulators on CTLs cytotoxicity against BC cells. Moreover, we investigated the role of metabolic modulation in enhancing CTLs function. Additionally, to deepen our understanding of HLA-DR regulation, we performed a bioinformatic analysis of single-cell RNA sequencing (scRNA-seq) data from BC patients.

Our findings revealed that HLA-DR expression is crucial for CTLs to effectively target and eliminate tumor cells. Additionally, we found that blocking HLA-DR or depleting CD4+ T cells significantly impair CTLs activation and cytotoxicity, indicating that antigen presentation through HLA-DR and the involvement of CD4+ T cells are key mechanisms driving enhanced anti-tumor responses. Furthermore, our optimized *ex vivo* expansion protocol demonstrated that short-term stimulation, rather than long-term, significantly increases HLA-DR expression and consequently enhances CTLs functionality, emphasizing the importance of prioritizing cell quality over quantity for improved therapeutic efficacy. Additionally, we discovered that anti-PD-1 treatment of peripheral blood mononuclear cells further elevated HLA-DR levels in CTLs, thereby augmenting their anti-tumor potential.

Notably, our *in silico* analysis also identified 34 co-expressed genes shared between PD-1 and HLA-DR in CTLs, including several non-coding RNAs, suggesting a potential PD-1-mediated regulatory pathway influencing HLA-DR expression.

In conclusion, the present study underscores the importance of enhancing HLA-DR expression in CTLs as a promising strategy for improving T cell-based therapies for BC. We highlight the synergistic benefits of combining anti-PD-1 treatment with short-term stimulation, offering new avenues for more effective and personalized treatments for BC patients. These findings pave the way for future clinical applications aimed at improving outcomes in BC therapy.

Keywords: breast cancer; cytotoxic T lymphocytes; HLA-DR; 3D co-cultures; immunomodulation; immunotherapy; adoptive T cell therapy

RESUMO

O cancro da mama (CM) continua a ser o cancro mais prevalente entre as mulheres em todo o mundo, representando um desafio significativo para a saúde pública. As projeções indicam um aumento na incidência e nas taxas de mortalidade por CM nos próximos anos, sublinhando a necessidade urgente de estratégias terapêuticas inovadoras e eficazes.

O panorama do tratamento do CM está a evoluir rapidamente, com a quimioterapia neoadjuvante (QNA) a manter-se como uma abordagem padrão para o tratamento de tumores de alto risco ou inoperáveis. No entanto, com uma taxa de sucesso inferior a 50%, a eficácia da QNA é limitada, especialmente em casos de tumores resistentes à quimioterapia. Embora terapias emergentes, como os inibidores de pontos de controlo imunológicos, tenham mostrado potencial, especialmente no tratamento do cancro da mama triplo-negativo, a sua aplicabilidade a todos os subtipos de CM permanece limitada. Consequentemente, abordagens inovadoras, como a terapia celular adotiva, que envolve a expansão *ex vivo* de linfócitos T derivados do próprio paciente, estão a ser investigadas.

Com base na nossa investigação anterior, que identificou a importância dos linfócitos T citotóxicos (LTCs) com alta expressão de HLA-DR no microambiente tumoral do CM como fator preditivo de respostas favoráveis à QNA, este estudo visa aprofundar o papel da expressão de HLA-DR nos LTCs, investigando como é que esta expressão de HLA-DR potencia a eficácia antitumoral do LTCs, oferecendo potenciais avanços para os tratamentos baseados em células T. Hipotetizamos que o aumento da frequência e da funcionalidade dos LTCs que expressam HLA-DR pode melhorar os resultados no tratamento do CM, particularmente em casos onde as terapias convencionais são ineficazes. Esta investigação responde a uma necessidade crítica, ao introduzir uma abordagem inovadora para melhorar a eficácia das terapias baseadas em células T. Ao otimizar a expressão de HLA-DR nos LTCs, pretendemos proporcionar imunoterapias mais personalizadas e eficazes para o CM, especialmente para pacientes com tumores resistentes ou difíceis de tratar.

Para alcançar este objetivo, refinámos um protocolo para expansão *ex vivo* de LTCs que expressam HLA-DR e utilizámos uma plataforma avançada de co-cultura 3D para avaliar os efeitos de vários moduladores imunológicos na citotoxicidade dos LTCs contra células

de CM. Além disso, investigámos o papel da modulação metabólica na melhoria da função dos LTCs. Adicionalmente, para aprofundar a nossa compreensão da regulação do HLA-DR, realizámos uma análise bioinformática de dados de sequenciação de RNA de célula única (scRNA-seq) de pacientes com CM.

Os nossos resultados revelaram que a expressão de HLA-DR é crucial para que os LTCs possam efetivamente direcionar e eliminar as células tumorais. Adicionalmente, verificámos que o bloqueio do HLA-DR ou a depleção de células T CD4+ prejudica significativamente a ativação e citotoxicidade dos LTCs, indicando que a apresentação de antigénios através do HLA-DR e a presença de células T CD4+ são mecanismos-chave que impulsionam respostas antitumorais melhoradas. Além disso, o nosso protocolo otimizado de expansão *ex vivo* demonstrou que uma estimulação de curto prazo, em vez de longo prazo, aumenta significativamente a expressão de HLA-DR e melhora a funcionalidade dos LTCs, enfatizando a importância de priorizar a qualidade celular ao invés da quantidade para melhorar a eficácia terapêutica. Adicionalmente, descobrimos que o tratamento com anti-PD-1 de células mononucleares do sangue periférico aumentou os níveis de HLA-DR nos LTCs, amplificando assim o seu potencial antitumoral.

Notavelmente, a nossa análise *in silico* identificou também 34 genes co- expressos entre PD-1 e HLA-DR nos LTCs, incluindo vários RNAs não codificantes, sugerindo uma potencial via regulatória mediada por PD-1 que influencia a expressão de HLA-DR.

Em conclusão, o presente estudo sublinha a importância de melhorar a expressão de HLA-DR nos LTCs como uma estratégia promissora para melhorar as terapias baseadas em células T para o CM. Destacamos os benefícios sinérgicos de combinar o tratamento com anti-PD-1 com a estimulação de curto prazo, oferecendo novas vias para tratamentos mais eficazes e personalizados para pacientes com CM. Estes resultados abrem caminho para futuras aplicações clínicas destinadas a melhorar os resultados na terapia do CM.

Palavras-Chave: cancro da mama; linfócitos T citotóxicos; HLA-DR; co-culturas 3D; imunomodulação; imunoterapia; terapia celular adotiva com células T

TABLE OF CONTENTS

CHAPTER II

CHAPTER III

LIST OF FIGURES

Chapter I

Chapter II

Chapter III

Chapter IV

LIST OF TABLES

Chapter I

Chapter II

Chapter III

CHAPTER I

Introduction and Aims

1. Cancer

Cancer is a complex and prevalent disease, affecting nearly 20 million new individuals annually and causing almost 10 million deaths worldwide[1]. The National Cancer Institute defines cancer as "a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body"[2]. This broad definition underscores the diverse nature of cancer, which varies widely in its molecular pathology and biological aggressiveness, complicating management and making it a life-threatening condition[3].

Research indicates that cancer has a variety of causes, including genetic mutations (whether somatic, inherited, or resulting from DNA replication errors), and epigenetic alterations like oncogene hypomethylation, tumor suppressor gene silencing through hypermethylation, and chromatin remodeling. Additionally, viral infections and toxin exposure contribute to cancer development, with external risk factors and lifestyle choices further increasing the likelihood of early cancer onset[4].

Cancer development, or carcinogenesis (Figure I-1), is a complex process shaped by a series of intricate molecular events influenced by gene-environment interactions. This process, also known as tumorigenesis or oncogenesis, encompasses sequential genetic mutations and/or epigenetic modifications that lead to uncontrolled cell proliferation and disturbance of normal cellular balance. These changes affect cellular metabolism and behavior, disrupting the regulation of cell division, prolonging cell lifespan, altering communication between neighboring cells, and facilitating evasion of the immune system. Consequently, cancer cells can divide and proliferate[3,5].

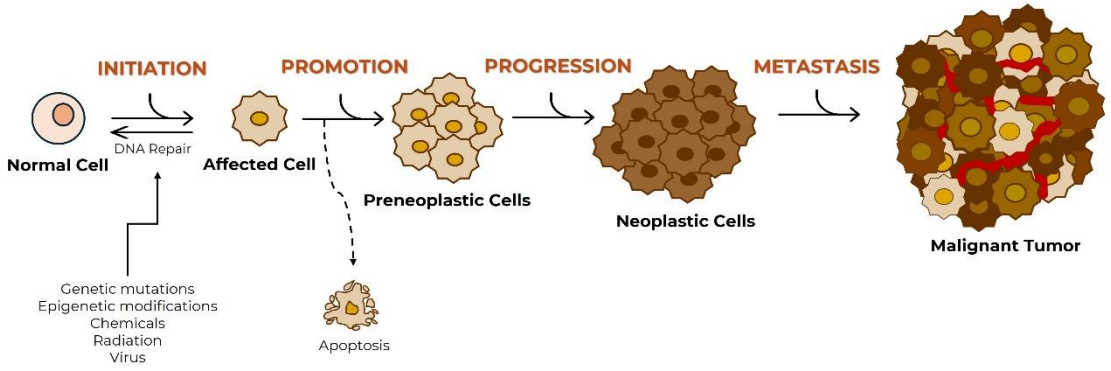


Figure I-2: Carcinogenesis. Cancer development includes different phases namely initiation, promotion, progression, and metastasis. Adapted from Siddiqui, Imtiaz & Sanna, *et al*[6].

In multicellular organisms, fundamental processes regulate cell proliferation and death. However, cancer cells resist these regulatory contexts, leading to adaptation, proliferation, invasion of nearby tissues, and resistance to the body's defense mechanisms[7].

Historically, cancer was viewed as a group of abnormally proliferating cells that had lost the ability to undergo apoptosis. However, it is now understood that cancer is more complex and comprises various cell types, including tumor cells, fibroblasts, normal stromal cells, endothelial cells, and immune cells. Together, these different cell types form the tumor microenvironment. Cancer progression depends not only on the attributes of tumor cells but also on the complex interactions within this microenvironment. To elucidate the biology of cancer, Hanahan and colleagues proposed six hallmarks that define tumor origin, growth, and dissemination[8]. Later they added more hallmarks, providing a comprehensive understanding of the multifaceted nature of cancer[9]. These hallmarks will be explored in detail in the next subsection.

1.1. Hallmarks of Cancer

In 2000, Hanahan and Weinberg introduced the concept of cancer hallmarks (Figure I-2), delineating six fundamental biological characteristics acquired by cancer cells that are crucial for the stepwise progression of human tumors. These hallmarks encompass sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Essentially, they form the foundation of tumor behavior—uncontrolled proliferation spurred by the release of growth factors, resistance to growth-inhibiting signals, evasion of apoptosis, promotion of angiogenesis for enhanced nutrient supply, and invasion of surrounding tissues with the potential for distant spread[8]. However, recognizing the significance of the tumor microenvironment in shaping tumor biology, Hanahan and Weinberg later proposed two enabling characteristics and two additional emerging hallmark capabilities[9].

In 2011, they emphasized that genomic instability, leading to random mutations, and the inflammatory state of malignant lesions driven by immune cells, are indispensable for malignant cells to acquire the hallmark traits, enabling their survival, proliferation, and dissemination. Furthermore, they identified the reprogramming of cellular energy metabolism to support continuous proliferation and the active evasion of immune surveillance as emerging hallmarks[9] (Figure I-2).

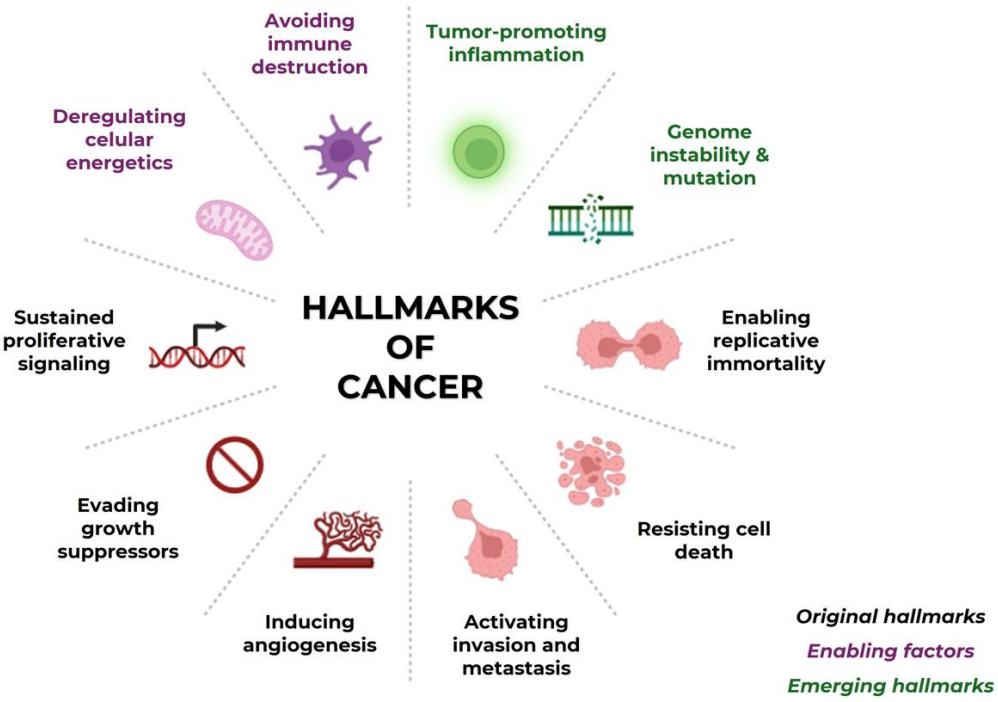


Figure I-2: Hallmarks of Cancer. Adapted from Hanahan *et al.*[8,9]. Figure created in BioRender.

More recently, in 2022, Hanahan proposed two prospective new hallmarks and enabling characteristics to deepen our understanding of cancer complexity (Figure I-3). These prospective hallmarks include unlocking phenotypic plasticity and the role of senescent cells. Phenotypic plasticity allows cancer cells to escape terminal differentiation constraints, while senescent cells contribute to cancer development by transmitting specific molecular signals that facilitate the acquisition of other hallmarks. Furthermore, the proposed enabling characteristics include non-mutational epigenetic reprogramming, where genome alterations solely through epigenetic mechanisms play a role in tumor progression, and polymorphic microbiomes, as the variability of

microbiomes among individuals diversely influences cancer phenotypes, either promoting or inhibiting progression[10].

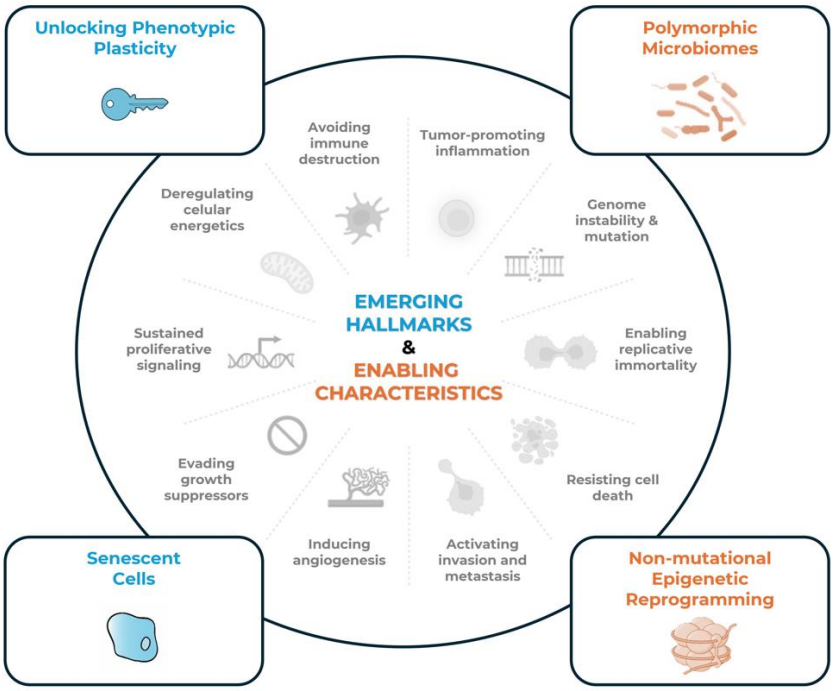


Figure I-3: Hallmarks of Cancer: New Dimensions. Adapted from Hanahan *et al.*[10]. Figure created with the help of BioRender.

Recognizing the key role of the immune system in cancer development, recent research underscores the necessity of examining not only cancer cells' characteristics but also the diverse cell types within the tumor microenvironment, particularly immune cell populations, and their interactions with malignant cells. Indeed, cancer progression is intricately influenced by these interactions, highlighting the complexity of tumor biology.

1.2. Immune Surveillance Against Cancer

The concept of immune surveillance against tumors suggests that the immune system constantly monitors and eliminates emerging tumor cells. However, tumor cells can develop various strategies to evade this immune

surveillance. Evidence supporting immune surveillance comes from both animal models and clinical observations[11].

Indeed, the idea that immune cells can control and eliminate tumor cells dates back to the early 20th century by Paul Ehrlich. Later, in the 1950s, the concept of immune surveillance through recognition of tumor-specific antigens was created. This hypothesis gained experimental support from studies with immunosuppressed mice, which showed an increase in spontaneous or chemically induced tumors. Additionally, immunodeficient mice demonstrated higher susceptibility to viral-related tumors. However, early technical limitations led some groups to question these findings, and the concept of tumor immune surveillance was temporarily left behind[11,12].

It wasn't until the mid-1970s through the 1980s and 1990s that tumor immune surveillance was revisited. Experiments with nude mice, which have inhibited immune systems, confirmed that these mice developed more chemically induced tumors. During these decades, researchers also discovered that antigen-presenting cells could process and present tumor antigens to other immune cells, and the capacity to develop effector and memory immune cells was revealed[11,12].

Clinical evidence also supports the occurrence of tumor immune surveillance. For instance, patients with immunodeficiencies, such as those with HIV or those who have undergone organ transplants and are on immunosuppressive drugs, have a higher incidence of cancer[11,12]. Additionally, older individuals have an increased incidence of cancer, which can be attributed to the age-related decline in immune system function, leading to reduced tumor immune surveillance. Furthermore, patients with cancers such as ovarian, colorectal, lung, and breast cancer have shown longer survival rates when there is a higher percentage of lymphocytes infiltrating the tumor[12].

Despite the immune system's surveillance efforts, some cancer cells manage to bypass these defenses. The field of Immuno-Oncology has grown significantly over the years to better understand this complex relationship between cancer and the immune system. Today, immune cells and their released cytokines are considered crucial elements in either tumor

suppression or progression. In the next section, this intricate tumor-immune crosstalk will be discussed in greater detail.

2. Cellular Dynamics of Tumor-Immune Interactions

The tumor microenvironment (TME) is a heterogeneous and complex ecosystem that plays a crucial role in tumor development, progression, and response to therapies. Tumor cells are central to cancer, driving its occurrence and development, but they do not act alone. They recruit a variety of other cells, forming the dynamic and constantly evolving TME. This environment includes both cellular and non-cellular elements, encompassing malignant cells, immune cells, stromal cells, fibroblasts, endothelial cells, blood vessels, extracellular matrix (ECM) components, proteolytic enzymes (notably matrix metalloproteinases or MMPs), chemokines, and cytokines[13,14].

An integral component of the TME is the tumor immune microenvironment (TIME), consisting of both innate and adaptive immune cells. Tumor-infiltrating immune cells are crucial within the TIME, encompassing innate immune cells such as macrophages, natural killer cells, and dendritic cells, along with adaptive immune cells like T cells and B cells. These immune cells secrete various signals that play vital roles in tumor development and progression[13].

Understanding the interactions within the TME, particularly the role of immune cells, is essential for advancing cancer therapies. The type, location, and density of these immune cells significantly impact the overall survival of cancer patients and their response to treatment[15]. Therefore, the complexity of the TME necessitates comprehensive exploration to develop strategies that effectively enhance the immune system's ability to combat cancer. In the following sections, we will delve into the role of the most notable immune cell populations in tumor progression and/or regression.

2.1. The Origin and Differentiation of Immune Cells

The origin of immune cells lies in the hematopoietic stem cells (HSCs), primarily located in the bone marrow. These cells have the ability to differentiate into all the various cell types that make up the immune system. This differentiation process is crucial for the development and function of both the innate and adaptive immune responses (Figure I-4)[16,17].

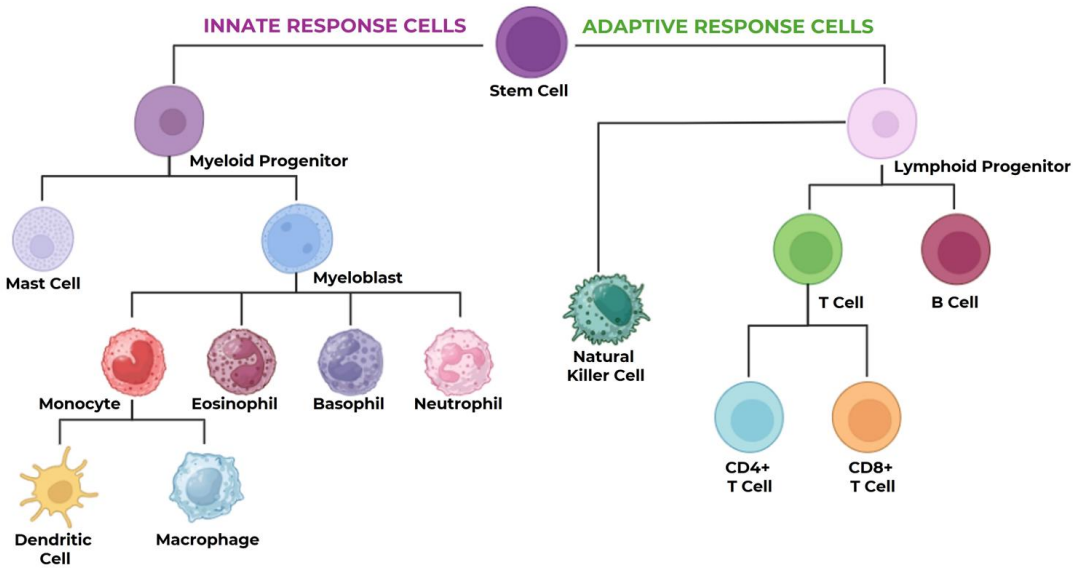


Figure I-4: Lineage tree representation of cells of the immune system. Immune cells originate from hematopoietic stem cells (HSCs). HSCs differentiate into lymphoid and myeloid progenitors, which subsequently branch into the more specialized cell types involved in adaptive and innate immunity. Adapted from Torang, A, *et al.*[18]. Figure created with the help of BioRender.

Indeed, the immune system is a complex and coordinated network of cells that can be broadly classified into two categories: innate and adaptive immune cells. Innate immune cells, which provide the first line of defense, include myeloid cells such as eosinophils, neutrophils, basophils, monocytes, and mast cells (Figure I-4). These cells are responsible for immediate, non-specific responses to a wide range of pathogens. They act quickly to recognize and eliminate invaders through various mechanisms, including phagocytosis, release of antimicrobial substances, and initiation of inflammatory responses. On the other hand, adaptive immune cells are specialized cells that provide a more tailored and long-lasting immune response. These include B lymphocytes and T lymphocytes, which are capable of recognizing specific

antigens presented by pathogens (Figure I-4). B lymphocytes are primarily responsible for producing antibodies that neutralize pathogens, while T lymphocytes can directly kill infected cells or help coordinate the overall immune response[16–19].

The differentiation of HSCs into these specialized immune cells involves a stepwise process where HSCs first become either lymphoid or myeloid progenitors. Lymphoid progenitors give rise to the adaptive immune cells, including B and T lymphocytes, as well as natural killer (NK) cells, which have roles in both innate and adaptive immunity[19]. Myeloid progenitors differentiate into the various myeloid cells of the innate immune system (Figure I-4)[16–19].

This hierarchical differentiation ensures that the body maintains a balance of immune cells to respond promptly and efficiently to a variety of threats[20].

2.2. The role of T Lymphocytes in the Tumor Microenvironment

T cells play a crucial role in clearing tumor cells, but within this population, there are various types, each with distinct functions in the tumor microenvironment (TME). These T cells can be categorized into CD4+ T cells, which include helper T cells (Th) and regulatory T cells (Tregs), and CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs)[21].

Th cells consist of different subtypes such as Th1, Th2, Th9, Th17, and Th22 determined mainly by the cytokines they release (Figure I-5). Th cells infiltrate the TME and support the activation of CTLs[22]. Their role depends on the presentation of tumor antigens by antigen-presenting cells (APCs), like dendritic cells or macrophages. After recognizing and processing antigens, APCs present them with class II major histocompatibility molecules (MHC II) on their surface, stimulating Th cells through a series of signals. These signals can induce interferon gamma (IFN-γ) production and subsequent CTLs stimulation[23]. Indeed, Th cells exhibit variable roles in tumor activity depending on their subsets (Figure I-5). For instance, the Th1 subtype, which produces IFN-γ and tumor necrosis factor alpha (TNF-α), is known for its anti-tumor properties by enhancing CTLs function and inhibiting angiogenesis.

Their presence in tumors is correlated with better prognosis in breast, colorectal, ovarian, gastric, and hepatocellular cancers[24]. Given this correlation, current research aims to use Th1 cells to enhance the effectiveness of therapies, such as adoptive T-cell therapy. On the other hand, Th2 cells secrete interleukin 4 (IL-4), interleukin 6 (IL-6), and interleukin 10 (IL-10), potentially playing dual roles in tumor progression. However, their production of the anti-inflammatory cytokine IL-10, which stimulates pro-tumor immune cells, identifies Th2 cells as poor prognostic predictors in pancreatic, gastric, ovarian, cervical cancer, and melanoma[21–24].

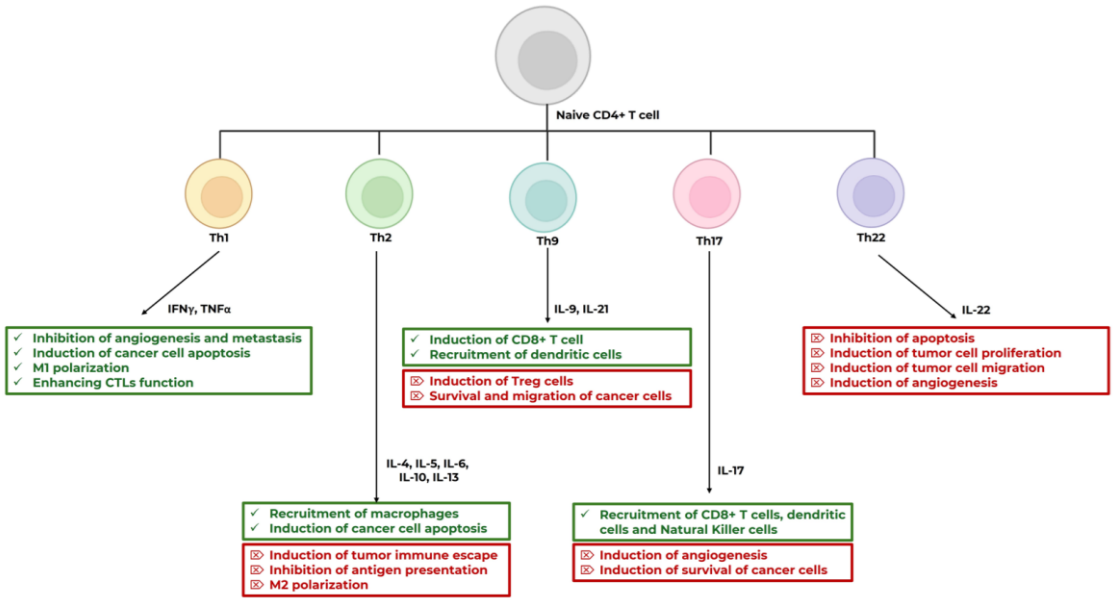


Figure I-5: CD4+ T helper subsets and their roles. Based on the cytokine environment, naive CD4+ T cells differentiate into various T helper subsets, each with distinct cytokine expression profiles and functions in tumor immunology. Adapted from Andreu-Sanz, D *et al.*[22].

Tregs represent a specific subset of CD4+ T cells, distinguished by the expression of the transcription factor Forkhead box protein 3 (FoxP3)[23,25]. This marker plays a crucial role in the specification of Tregs lineage and the regulation of their suppressive signals[25]. In physiological conditions, Tregs are indispensable for maintaining immune homeostasis and preventing autoimmune diseases by preserving self-tolerance[26]. However, their immunosuppressive nature can also promote cancer progression, as they possess the ability to suppress the function of other immune cells, namely CTLs, macrophages, and Th1 cells (Figure I-6). The Treg immunosuppression mechanism is mediated through various pathways, including the expression

of cytotoxic T-lymphocyte–associated antigen 4 (CTLA4) on their surface and the secretion of immunosuppressive cytokines like IL-10, IL-35 and TGF-β[23,27] (Figure I-6). Furthermore, it is important to note that Tregs possess a variety of chemokine receptors, with C-C chemokine receptor 4 (CCR4) being particularly prominent, enabling them to respond to chemokines released by tumor cells and migrate to the TME. Indeed, the chemokines macrophage-derived chemokine (MDC/CCL22) and thymus and activation-regulated chemokine (TARC/CCL17), produced by tumor cells, play a role in attracting Tregs to the TME through chemotaxis (Figure I-6). Thus, the accumulation of Tregs in the TME is associated with a poor prognosis in cancer and reduced efficacy of anti-cancer therapies[28]. Consequently, Tregs are often linked with unfavorable outcomes in certain cancers, such as pancreatic, lung, renal, ovarian, melanoma and breast. However, conflicting evidence suggests that Tregs may play a protective role in some contexts, potentially leading to a favorable prognosis in cancers like head and neck, bladder, and colon[24].

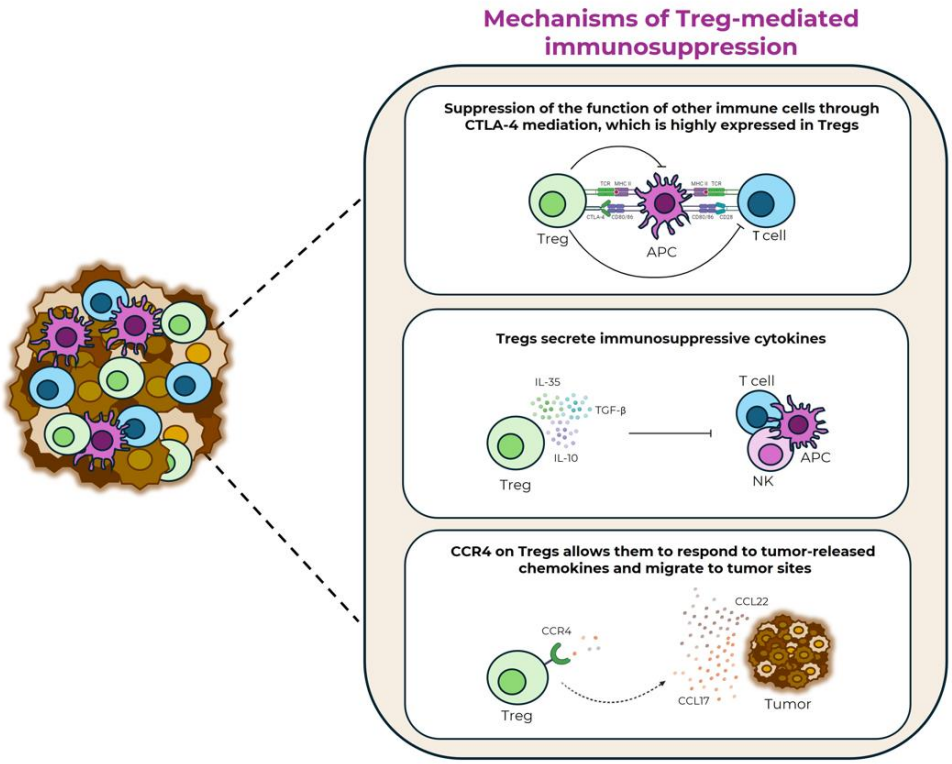


Figure I-6: Mechanisms of Treg-mediated immunosuppression. Tregs suppress the activity of antigen-presenting cells (APCs) and T cells via immune checkpoints. Additionally, Tregs release immunomodulatory cytokines such as IL-10, IL-35, and TGF-β that inhibit the activation of T cells, natural killer (NK) cells, and APCs. Furthermore, Tregs express CCR4, enabling them to respond to chemokines released by tumors (CCL17 and CCL22) and migrate to tumor sites. Adapted from Li, Q *et al.*[29]. Figure created with the help of BioRender.

CTLs play a key role in mediating the immune responses for the elimination of tumor cells within the TME and also in pathogen clearance[30]. CTLs recognize antigens presented by class I major histocompatibility complex (MHC-I) molecules, expressed by all nucleated cells, making them essential for targeting and destroying cancer cells, often more effectively than Th1 cells. In fact, CTLs eliminate tumor cells through two primary mechanisms: the release of perforin and granzyme B, and the Fas/FasL interaction (Figure I-7). The granule exocytosis pathway involves the release of perforin, which creates pores in the membrane of target cells, allowing granzyme B to enter and induce apoptosis by cleaving substrates in the cytoplasm. The Fas/FasL pathway involves Fas ligand (FasL) on the surface of CTLs binding to Fas on tumor cells, triggering a caspase cascade that leads to apoptosis (Figure I-7). Additionally, CTLs secrete TNF-α and IFN-γ, which enhance cytotoxicity against tumor cells[30–32]. The presence of CTLs in the TME is associated with a favorable prognosis in cancers such as breast and colorectal cancer[33], and there is a correlation between intratumoral CTLs and reduced tumor size[31].

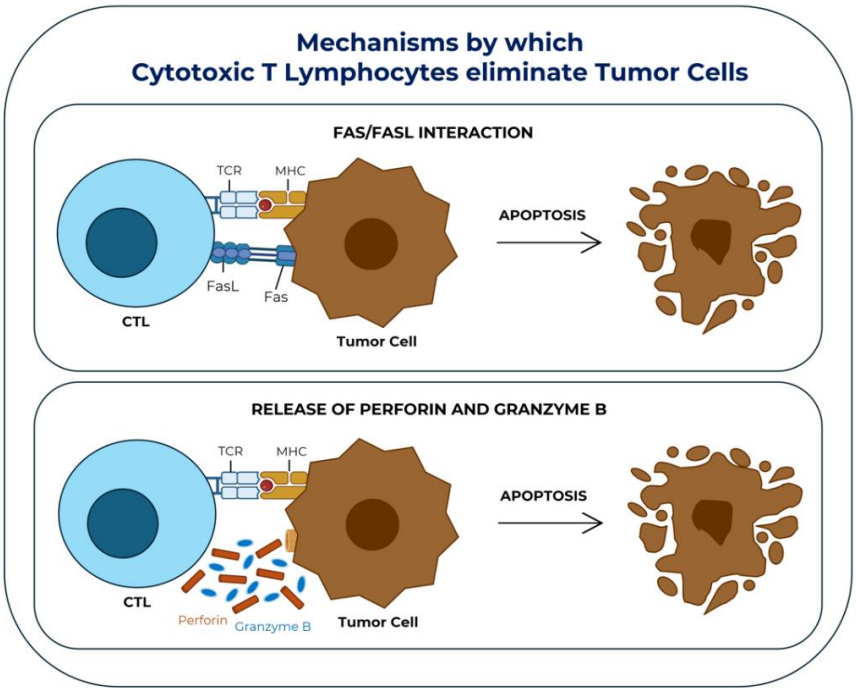


Figure I-7: Mechanisms by which Cytotoxic T Lymphocytes (CTLs) eliminate tumor cells. CTLs target and destroy tumor cells via two main mechanisms: the Fas/FasL interaction and the release of perforin and granzyme B. The interaction between Fas on tumor cells and FasL on CTLs trigger programmed cell death, while perforin and granzyme work together to induce apoptosis in the target cells. Adapted from Trapani, J *et al.*[34]. Figure created with the help of BioRender.

The process of cancer cell elimination by CTLs is the first step of immunoediting (detailed further in section 2.4), where CTLs target and destroy immunogenic tumor cells[31]. However, it is necessary to recognize that CTLs interact with other cells within the tumor microenvironment (Figure I-8). Therefore, as previously referred, CTLs' anti-tumor activity can be suppressed by tumor cells, Tregs, or other immune cells through various immune escape mechanisms. Indeed, tumor cells, directly or by reprograming the surrounding cells, can support immunosuppressive activities, facilitating unchecked growth and invasion. CTLs may have both positive interactions with immunostimulatory cells, including NK cells, M1 macrophages, CD4+ T cells, and dendritic cells (DCs), and negative interactions with immunoinhibitory cells, including cancer-associated fibroblasts (CAFs), cancer cells, Tregs, and M2 macrophages (Figure I-8). Consequently, the interplay between CTLs and other cells within the TME significantly affects tumor progression and the effectiveness of immune responses[31]. This dynamic will be further examined in subsection 2.3, with a particular focus on the role of various myeloid cells in the TME.

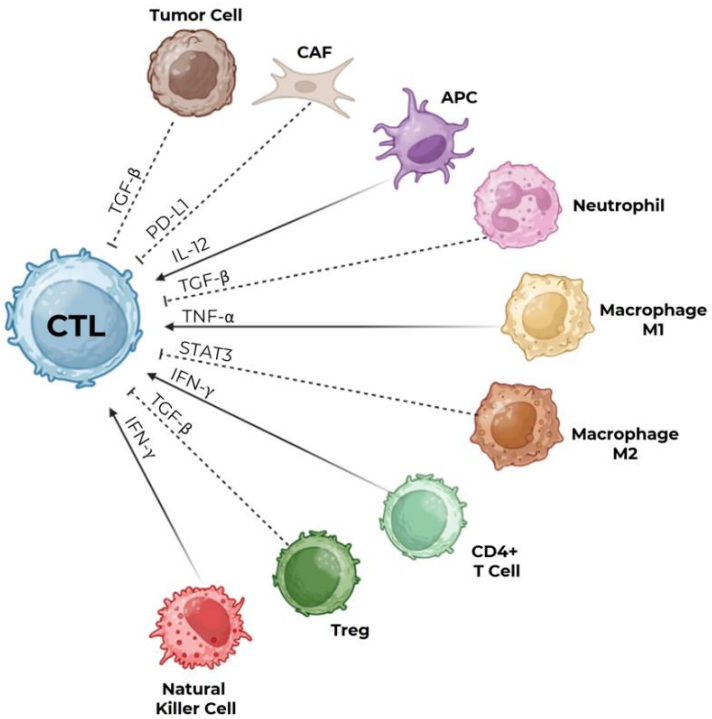


Figure I-8: Cross-talks between Cytotoxic T Lymphocytes (CTLs) and other cells in the tumor microenvironment. CTLs positively interact with immunostimulatory cells such as natural killer (NK) cells, anti-tumor macrophage type 1 (M1) cells, CD4+ T cells, and antigen-presenting cells (APCs). Conversely, they negatively interact with immunoinhibitory cells including cancer-associated fibroblasts (CAFs), cancer cells, regulatory T cells (Tregs), and pro-tumor M2 macrophages. Adapted from Farhood, B, *et al.*[34]. Figure created in BioRender.

2.2.1. The T Cell Activation

T cell activation is a crucial process in the immune response, enabling the body to recognize and combat pathogens and abnormal cells, such as cancer cells. This process begins when a T cell receptor (TCR) on the surface of a T cell binds to a specific antigen presented by an antigen-presenting cell (APC), such as a dendritic cell. The antigen is displayed on the APC's surface via major histocompatibility complex (MHC) molecules. The activation requires three key signals: the first signal involves the TCR binding to the antigen-MHC complex, the second signal comprises co-stimulatory interaction between molecules on the T cell and the APC, and the third signal encompasses cytokines released during the interaction. These signals trigger a cascade of intracellular events, leading to T cell proliferation, differentiation, and the acquisition of effector functions[35].

In the context of cancer, T cells, particularly cytotoxic T lymphocytes (CTLs), can infiltrate the tumor microenvironment (TME) but are often suppressed by various mechanisms, inhibiting their cytotoxic response. The ability of CTLs to become and remain activated is essential for their anti-tumor capacity.

CTLs activation can be observed by the upregulation of distinct activation markers such as CD69, CD25, and HLA-DR. CD69 is an early activation marker, upregulated within 2-4 hours of stimulation, leading to increased intracellular calcium levels and production of IL-2, IL-4, and TNF-α. CD25 (IL-2 receptor) is upregulated 12-24 hours after activation, resulting in higher levels of IFN-γ and TNF-α, which initiate cell proliferation and differentiation. HLA-DR, a class II MHC molecule, is upregulated in activated T cells 24-48 hours post-activation. HLA-DR expression in T cells is associated with enhanced IFN-γ production and is believed to play a role in forming T cell-T cell synapses, amplifying IFN-γ production and other CTLs-related functions through rapid positive feedback[36,37].

This intricate process of activation and subsequent interactions within the TME underscore the complexity and importance of CTLs functions in cancer immunity. By enhancing the efficacy of CTLs through activation, there is a significant potential for targeted immunotherapies to bolster anti-tumor responses.

2.3. The Influence of Myeloid Cells in the Tumor Microenvironment

Although T cells are considered the most prominent anti-tumor cells, myeloid cells also play a crucial role in either tumor clearance or progression. These myeloid cells include macrophages, dendritic cells, myeloid-derived suppressor cells, and neutrophils.

In the tumor microenvironment (TME), macrophages are referred to as tumor-associated macrophages (TAMs). These TAMs can be categorized into M1 and M2 subtypes, influenced by environmental factors. M1 polarization occurs in an IFN-γ-rich environment, making M1 macrophages pro-inflammatory. Activated by IFN-γ, lipopolysaccharide, and IL-1β, M1 macrophages can recognize and destroy malignant cells via phagocytosis and cytotoxicity[38]. Particularly, M1 macrophages express nitric oxide synthase (iNOS) and reactive oxygen species (ROS), as well as cytokine IL-12[39]. Upon activation, M1 macrophages release pro-inflammatory cytokines such as IL-12, IL-23, and TNF-α, which enhance antigen presentation and promote Th1 responses[40] (Figure I-9). In opposition, M2 polarization occurs with stimuli like IL-4, IL-10, and IL-13, fostering tumor growth and promoting TME remodeling. M2 macrophages produce growth factors, immunosuppressive factors, pro-angiogenic molecules, and proteases, resulting in an anti-inflammatory phenotype[38]. Indeed, they secrete cytokines such as TGF-β, which promote immunosuppressive responses in the TME, inhibit effector T cell functions, and induce angiogenesis and tumor invasion[39] (Figure I-9). Effectively, M1 and M2 macrophages have distinct chemokine profiles: M1 macrophages express Th1 cell-attracting chemokines such as CXCL9 and CXCL10, while M2 macrophages express chemokines like CCL17, CCL22, and CCL24[40] (Figure I-9). The role of TAMs as a prognostic factor varies by cancer type. TAMs are associated with poor survival in gastric, breast, bladder, ovarian, thyroid, and oral cancers, but correlate with favorable outcomes in colorectal cancer. This dichotomy in macrophage action is essential for maintaining tissue homeostasis however, tumors can exploit these mechanisms to induce more anti-inflammatory or pro-tumor responses by TAMs[41].

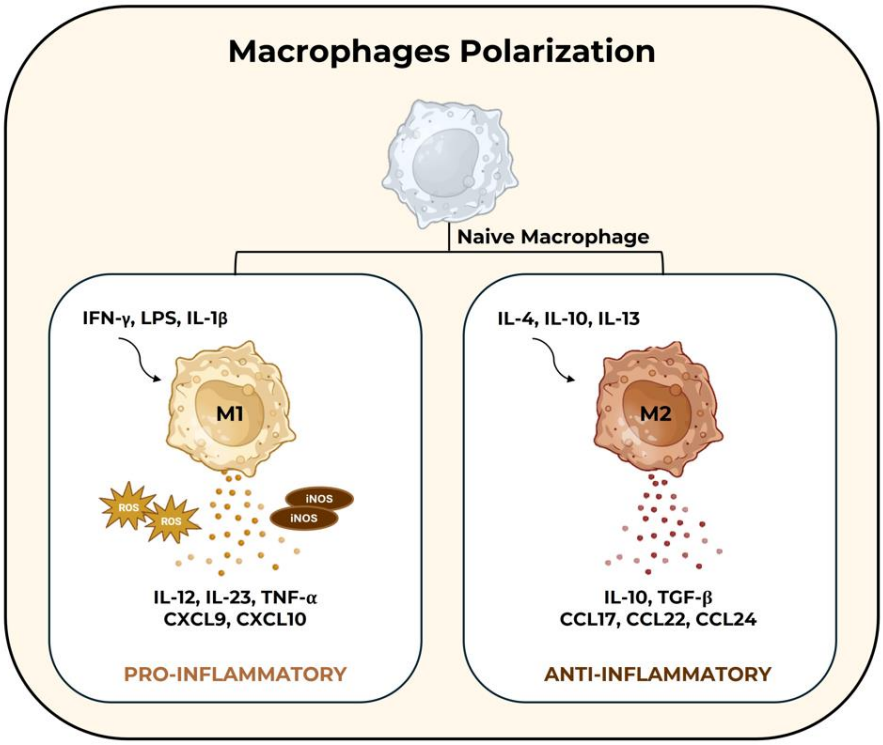


Figure I-9: Macrophages Polarization: M1 and M2 subtypes. Different stimulations trigger the generation of M1 and M2 macrophages. M1 and M2 macrophages differ phenotypically and in their production of pro-inflammatory and anti-inflammatory cytokines. Figure created with the help of BioRender.

Dendritic cells (DCs) are highly specialized antigen-presenting cells (APCs) that patrol the local environment through the extensive expression of membrane and cytosolic receptors. These receptors recognize various danger signals, including pathogens and altered cells such as tumor cells. Upon antigen uptake, activated DCs process and present both self and non-self-antigens to naive T lymphocytes, priming antigen-specific immune responses[42]. DCs are generally categorized into conventional DCs, which include DC1s and DC2s. DC1s specialize in processing and presenting intracellular antigens, shaping anti-tumor immune responses by cross-presenting tumor-associated antigens to CD8+ T lymphocytes via major histocompatibility complex class I (MHC I) signaling. DC2s efficiently present MHC II-associated antigens to CD4+ T cells, promoting Th1, Th2, and Th17 polarization[43]. In addition to antigen presentation, DCs produce cytokines such as IFN-γ, TNF-α, IL-6, and IL-12, which constitute the third signal required to activate T cell responses already explained above in section 2.2.1[42]. However, the anti-tumor function of DCs can be impaired in the TME. The presence of

IL-10 in the TME can inhibit cytokine production by DCs. Furthermore, hypoxic conditions and lower pH in the TME are known to impair normal DCs function. In some cases, DCs can become tolerogenic, contributing to immune non-responsiveness and tumor tolerance instead of promoting anti-tumor immunity[44].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that play a significant role in the negative regulation of immune responses in cancer and chronic inflammation. MDSCs are typically divided into two subtypes: polymorphonuclear MDSCs (PMN-MDSCs), which are closely related to neutrophils, and monocytic MDSCs (M-MDSCs), which are similar to monocytes[45]. These cells expand in environments rich in IL-6 and IL-10 and are associated with pathological conditions such as cancer. Indeed, tumor-derived factors abnormally produce and recruit MDSCs to tumor sites, where they help to establish a microenvironment that suppresses host immunity. MDSCs exert their suppressive role through the release of ROS, nitric oxide (NO), and arginase, leading to the inhibition of T cell responses. Additionally, MDSCs promote tumor angiogenesis and metastasis by producing vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), such as MMP-9, which can cleave the extracellular matrix and facilitate tumor cell invasion and migration. By releasing these factors, MDSCs not only support the immunosuppressive environment but also assist in tumor progression through enhanced angiogenesis and metastasis[46].

Neutrophils are the most abundant leukocytes in circulation, representing about 50-70% of all white blood cells, and play an essential role in host defense[47]. They act as the first line of defense in the innate immune system due to their rapid response to acute inflammation or infection[47,48]. Neutrophils can eliminate pathogens via both intra and extracellular processes, such as phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs)[49]. Classically, neutrophils were thought to be a homogenous population of cells focused solely on antimicrobial functions. However, recent research has revealed their significant role in modulating immune responses and maintaining immune homeostasis. Neutrophils are now understood to be a heterogeneous population with diverse functions in both healthy and

pathological conditions, including cancer, infections, and autoimmune disorders[50,51]. They can produce pro-inflammatory factors, such as IL-1β, TNF-α, and IL-12, as well as anti-inflammatory molecules like TGF-β, depending on the stimulus[52]. This allows neutrophils to interact and modulate other immune cells, including macrophages, DCs, and adaptive immune cells like B and T lymphocytes[48]. Tumor-associated neutrophils (TANs) represent a subset of neutrophils capable of infiltrating the tumor microenvironment in certain types of cancer. They can be classified into N1 (anti-tumor) and N2 (pro-tumor) subtypes (Figure I-10), similar to M1/M2 macrophage polarization. N2 neutrophils, which can be induced by TGF-β, are particularly associated with cancer. They release ROS, which can inhibit effector T cell function and promote cancer-related inflammation and progression. N2 neutrophils also produce VEGF, inducing angiogenesis, and initiate TGF-β-mediated metastasis[53,54] (Figure I-10). Neutrophils are recognized as a poor prognostic factor in several cancers, especially when considering the neutrophil/lymphocyte ratio (NLR)[55]. Neutrophils perform a dual role in cancer, being capable of promoting or suppressing disease development, depending on specific signals from the TME[56]. This dual role underscores the complexity of their function and highlights their significant impact on cancer progression and patient outcome.

Additionally, it is also important to highlight the emerging overlap between the pro-tumorigenic functions of neutrophils and PMN-MDSCs. Initially, these cell types were thought to have distinct roles in cancer, with MDSCs being primarily associated with T cell suppression, and neutrophils, particularly N2 subsets, driving tumor growth through angiogenesis, tumor proliferation, invasion, and metastasis. However, recent evidence has shown that N2 neutrophils also possess T cell suppressive functions, while PMN-MDSCs can promote tumor progression through mechanisms independent of T cells. This functional overlap has uncleared the distinction between PMN-MDSCs and pro-tumorigenic neutrophils, as both contribute to tumor growth and immune suppression within the tumor microenvironment. Although PMN-MDSCs and neutrophils are considered distinct populations, the technical challenges of isolating and characterizing these cells, particularly in cancer patients, complicate their distinction. Thus, when studying these cells in

cancer, it is essential to acknowledge their shared pro-tumorigenic roles, as both PMN-MDSCs and neutrophils contribute to tumor progression through overlapping immunosuppressive and tumor-promoting mechanisms.

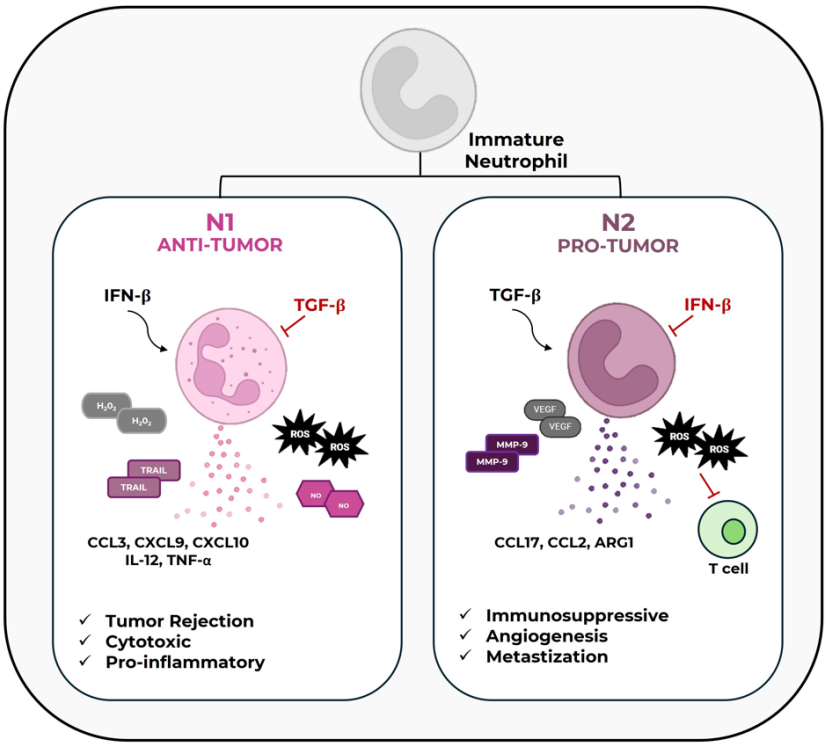


Figure I-10: Tumor-associated neutrophils (TANs) polarization: N1 and N2 subtypes. Different stimuli induce the generation of N1 or N2 TANs. N1 and N2 differ phenotypically and functionally. Figure created with the help of BioRender.

2.4. Cancer Immunoediting

The relationship between the immune system and cancer development has been a complex area of study in recent years. It is now recognized that the immune system plays a dual role in cancer: immune inflammatory cells within tumors can both inhibit and promote tumor growth[27]. The Cancer Immunoediting Hypothesis, first elaborated by Dunn and colleagues[57], explains this dynamic interaction by detailing three sequential phases that immune cells experience during cancer progression: Elimination, Equilibrium, and Escape (Figure I-11). During these phases, the immunogenicity of tumors changes, and cancer cells acquire mechanisms to suppress the immune response, facilitating disease progression[58].

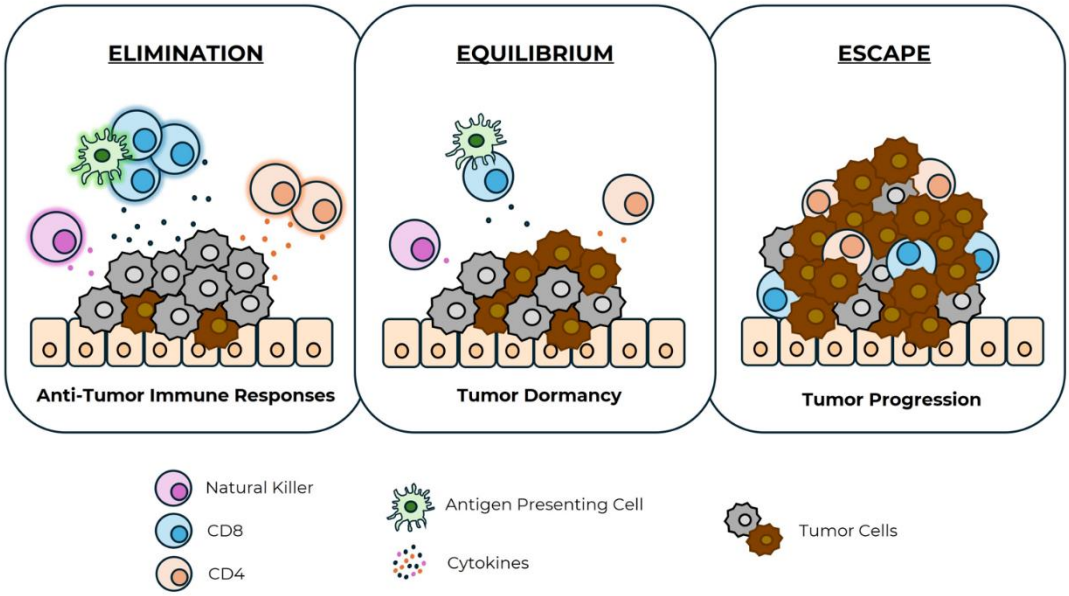


Figure I-11: Cancer Immunoediting. Cancer immunoediting involves three different steps: elimination, equilibrium and escape. Adapted from Dunn *et al*[57].

In the Elimination phase, the immune system identifies and targets cancer cells as threats, initiating a response to destroy them through both innate and adaptive immunity. However, some cancer cells manage to survive this attack, entering a dormant state known as the Equilibrium phase, where the immune system continues to monitor and control the tumor. Eventually, in the Escape phase, cancer cells undergo genetic changes that allow them to evade immune detection. As a result, the immune system's response is diminished or ineffective, enabling the tumor to grow and spread[57,58].

Cancer immunoediting significantly impacts patients' responses to treatments. Therefore, understanding the interactions between the immune system and cancer cells is essential for developing therapies that enhance the immune system's ability to fight cancer more effectively.

2.5. The Cancer Immunity Cycle

Considering the diverse populations of immune cells present in the tumor microenvironment (TME) and their distinct roles, effective immune surveillance and tumor cell elimination require a coordinated interplay of

various cellular players and optimal conditions. This intricate collaboration is essential for mounting a successful immune response against cancer.

The Cancer Immunity Cycle (Figure I-12) describes a sequence of events necessary for an effective anti-cancer immune response, involving the detection and elimination of cancer cells[59,60]:

1) Antigen Release: The cancer immunity cycle begins when cancer cells release antigens, which are unique markers that identify them as foreign invaders to the immune system.
2) Antigen Presentation: Antigen-presenting cells (APCs), such as dendritic cells, engulf the released antigens and process them into smaller peptides. These peptides are then displayed on the surface of APCs, bound to major histocompatibility complex (MHC) molecules.
3) Priming and Activation: Primed APCs migrate to nearby lymph nodes, where they present the cancer-derived peptides to naive T cells. This interaction activates T cells, particularly CD8+ cytotoxic T cells, which recognize the presented antigens as foreign and become primed to attack cancer cells.
4) Trafficking of T Cells: Activated cytotoxic T cells leave the lymph nodes and travel through the bloodstream to reach the tumor site. This involves chemokines creating a gradient that guides T cells and adhesion molecules helping them to stick and pass through blood vessel walls.
5) Infiltration of T Cells: T cells move through the extracellular matrix and enter the tumor tissue. Tumors often create barriers to prevent this, but successful infiltration means T cells have penetrated these barriers. High levels of infiltration generally lead to better immune responses against the tumor.
6) Recognition of Cancer Cells: Within the tumor microenvironment, cytotoxic T cells bind to antigens presented on cancer cells by MHC molecules. This enables T cells to identify these cancer cells as targets for elimination.
7) Tumor Cell Death: Upon recognition of cancer cells, cytotoxic T cells become fully activated and initiate a cascade of events leading to the destruction of cancer cells. This process may involve direct cytotoxicity

mediated by perforin and granzymes, as well as the activation of other immune cells, such as macrophages and natural killer cells, to aid in cancer cell clearance.

Following cancer cell death, new and additional antigens are released, perpetuating the cancer immunity cycle (step 1 again). These antigens can be captured by APCs, initiating subsequent rounds of T cell priming and activation, thereby amplifying the immune response against cancer.

The cancer immunity cycle exemplifies the dynamic interplay between cancer cells and the immune system. Understanding the complex crosstalk and regulatory mechanisms among these cells can provide valuable insights for developing targeted therapies to fight cancer[59,60].

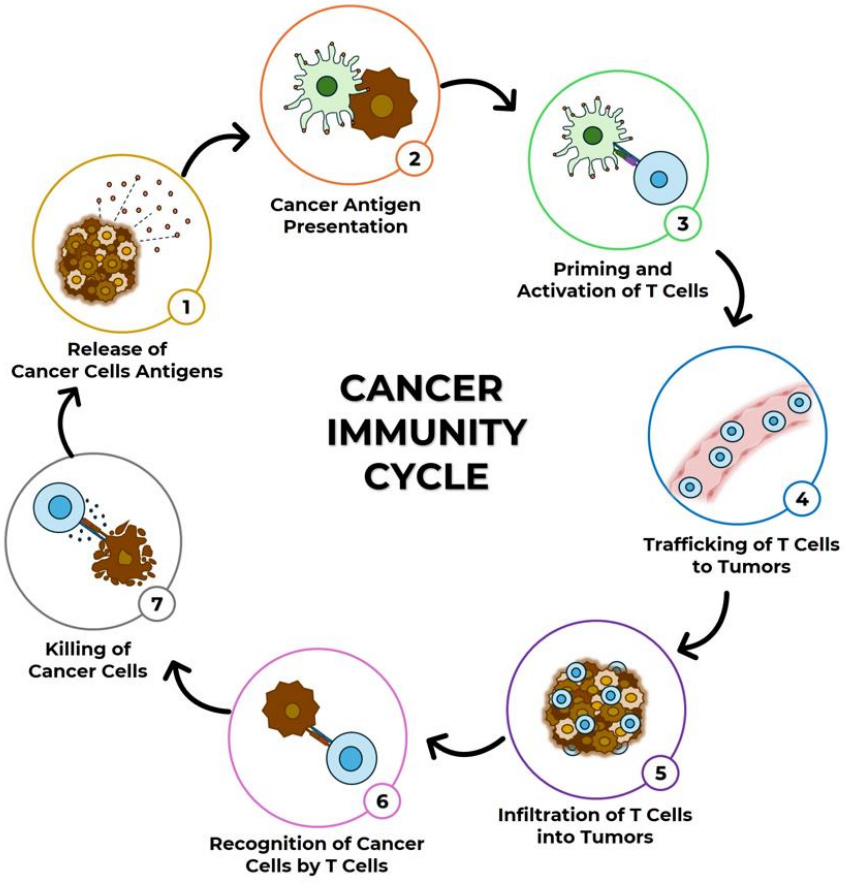


Figure I-12: Cancer Immunity Cycle. This cycle illustrates the sequential steps of how the immune system recognizes and eradicates cancer cells, from the release of cancer cell antigens to the killing of cancer cells by cytotoxic T cells. Adapted from Chen, D, *et al*[59].

2.6. The Cytokine Landscape Within the Tumor Microenvironment

The immune response in the tumor microenvironment (TME), influenced by various immune cell populations, relies not only on direct cell-cell contact but also on the release of cytokines. Cytokines are soluble proteins with molecular weights up to 70 kDa, while chemokines are a subset of small cytokines with molecular weights ranging from 7 to 15 kDa[61]. These molecules are crucial players in the TME, providing essential growth conditions and regulating cellular trafficking and diverse signaling pathways[61,62]. Cytokines include interleukins (IL), interferons (IFN), the tumor necrosis factor (TNF) superfamily, colony-stimulating factors (CSF), chemokines, and growth factors (GF)[63]. Both immune cells and tumor cells release cytokines, creating a complex interplay that influences the immune response. As already mentioned in the sections above, immune cells, such as macrophages, and T cells, secrete cytokines that can either stimulate or suppress immune activity. On the other hand, tumor cells themselves can release cytokines that modulate the immune response in their favor[62] (Figure I-13).

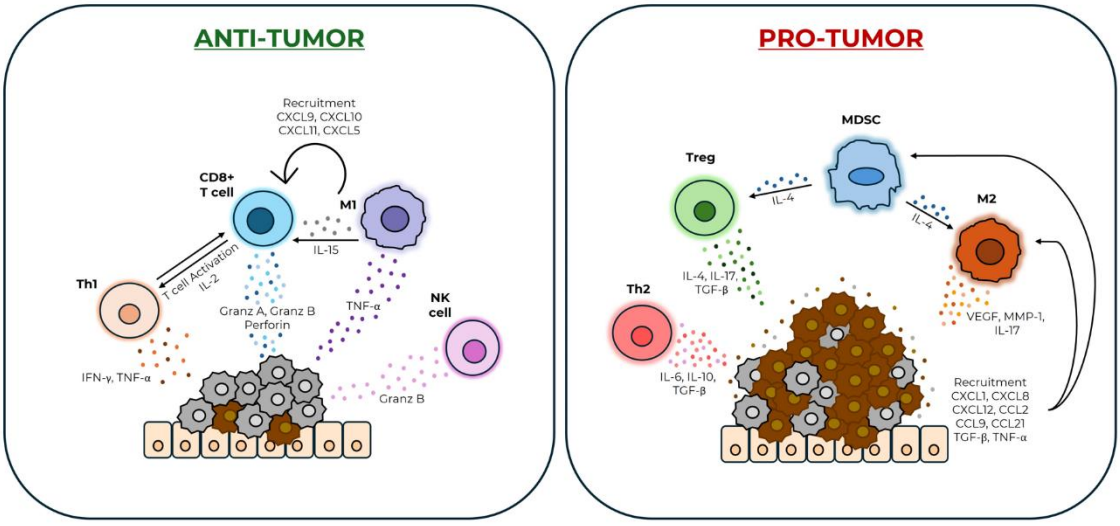


Figure I-13: Anti-tumor and pro-tumor cytokines. Pro-tumor cytokines, such as IL-4, IL-6, IL-10, VEGF and TGF-β, promote tumor growth and survival, while anti-tumor cytokines, including IFN-γ and granzyme B (Granz B), enhance immune responses against tumor cell. Adapted from Abousaway, O, *et al*[64].

2.6.1. The Significance of Cytokines Released by Immune Cells

Cytokines released by immune cells, play a pivotal role in regulating the immune response. They assist as key signaling molecules that coordinate the communication between cells in the immune system. Released by various immune cells, such as macrophages, dendritic cells, T cells, and B cells, cytokines influence cell behavior, modulate immune activity, and maintain homeostasis[65].

One of the primary functions of cytokines is to mediate and regulate inflammation. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), are crucial in initiating and sustaining the inflammatory response to infections and injuries. These cytokines help recruit immune cells to the site of infection or injury, enhance phagocytosis, and promote the clearance of pathogens and damaged tissues[66].

In normal immune activity, cytokines also play a role in the activation and differentiation of immune cells. For instance, interleukin-2 (IL-2) is essential for the proliferation and differentiation of T cells, which are critical for adaptive immunity[67]. Interleukin-4 (IL-4) promotes the differentiation of naive T helper cells into Th2 cells, which are important for humoral immunity and the activation of B cells[68]. Similarly, interferon-gamma (IFN-γ) activates macrophages and enhances their microbial activity, thereby playing a crucial role in the body's defense against intracellular pathogens[69].

Moreover, cytokines are involved in regulating the balance between pro-inflammatory and anti-inflammatory responses. Anti-inflammatory cytokines, such as interleukin-10 (IL-10), help limit excessive inflammation, prevent tissue damage, and maintain normal tissue homeostasis[70].

In the context of cancer, cytokines released by immune cells can have both beneficial and unfavorable effects. Certain cytokines, like IFN-γ and interleukin-12 (IL-12), have anti-tumor properties and can enhance the immune system's ability to recognize and destroy cancer cells[71]. Additionally, Granzyme B works as a potent cytotoxic cytokine employed by cytotoxic T lymphocytes and natural killer cells to promote tumor cell death[72]. However,

some tumors can exploit cytokines to create an immunosuppressive microenvironment that promotes tumor growth and evasion from the immune system. For example, tumor-derived TGF-β can inhibit the function of cytotoxic T cells, thereby allowing cancer cells to escape immune surveillance[73].

The therapeutic potential of cytokines has been used in various clinical settings. Given their critical roles in many immune-mediated diseases, cytokines have been extensively investigated as therapeutic targets. Inhibiting cytokine function through monoclonal antibodies or receptor blockers has shown success in conditions characterized by excessive cytokine production, such as autoimmune and inflammatory diseases. For instance, blocking TNF-α has proven to be an effective treatment strategy for rheumatoid arthritis and Crohn's disease. Despite their therapeutic promise, developing cytokine-based therapeutics presents challenges. The short blood half-lives, pleiotropic effects, and unfavorable tissue distribution of cytokines contribute to their limited therapeutic range. Although cytokine-based drugs were among the earliest immunotherapies developed for advanced cancer treatment, their poor pharmacokinetic properties and systemic side effects have limited their applications. Significant exceptions include the use of interferons for chronic hepatitis or multiple sclerosis, recombinant IL-2 (rIL-2) for renal carcinoma, and colony-stimulating factors for neutropenia. Future advances in cytokine therapies are expected to focus on re-engineering cytokines for targeted delivery to pathological sites and improving trial designs, patient stratification, and precision-medicine-based approaches[74].

2.6.2. The impact of Tumor-Produced Cytokines

Tumor cells secrete various cytokines that significantly shape the TME, promoting tumor growth, survival, and metastasis while suppressing effective immune responses (Figure I-13). For instance, cytokines such as vascular endothelial growth factor (VEGF) promote angiogenesis, providing the tumor with essential nutrients and oxygen. Additionally, tumor cells release immunosuppressive cytokines like transforming growth factor-beta (TGF-β)

and interleukin-10 (IL-10), which inhibit effector T cells and natural killer (NK) cells, preventing effective immune responses against the tumor[75] (Figure I-13).

Indeed, these cytokines not only facilitate tumor cell survival but also recruit and activate regulatory immune cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), thereby enhancing the immunosuppressive environment. This recruitment suppresses the immune response, aiding in tumor evasion and survival. Furthermore, cytokines like interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) induce chronic inflammation within the TME, supporting tumor progression and metastasis. Through NF-kB signaling, cytokines such as IL-1, IL-6, TNF, IL-8, and IL-17 induce cell proliferation and promote tumor growth[76].

IL-6, in particular, plays a significant role in tumor growth, promoting cell proliferation in various cancers, including oral squamous cell carcinoma, prostate cancer, and colon cancer. Other cytokines, including IL-1β, IL-8, IL-17, and IL-11, are extensively involved in tumor development and progression. TNF-α further promotes the secretion of IL-17 and IL-11 by cancer cells, indicating its indirect role in tumor growth[76].

IL-23 is another critical cytokine upregulated in various cancers, including colon, ovarian, lung, breast cancer, and melanoma. It induces IL-17-producing helper T cells, thereby indirectly reducing the cytotoxic function of CTLs by enhancing IL-17-induced Treg activity. IL-23 is associated with tumor incidence and therapy resistance, reducing CTLs infiltration in tumor tissues and contributing to a poorer prognosis.[77]

Inflammation is a key factor in cancer initiation and progression, particularly in metastasis, beginning with epithelial-to-mesenchymal transition (EMT). This process is promoted by TGF-β, IL-1β, TNF-α, and IL-6. Angiogenesis, driven by VEGF, IL-8, and TGF-β, is essential for tumor survival and spread. For instance, cytokines like IL-17 create a metastasis-supportive environment in breast cancer[76]. Moreover, IL-1β, produced in colon, lung cancer and melanoma, is associated with poor prognosis by inducing metalloproteinases for extracellular matrix degradation and VEGF for angiogenesis[78].

The presence of immune cells further complicates the inflammatory state in the TME. Tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), MDSCs, and Tregs produce IL-10, TGF-β, and prostaglandins that suppress NK, T, and B cell activities, allowing cancer cell survival and proliferation. IL-10, in particular, is an immunosuppressive cytokine involved in resistance to apoptosis and promoting angiogenesis[76].

Another important type of inflammation, although not only originated in the TME, is cancer therapy-induced inflammation. In this context, high IL-6 expression correlates with multiple drug resistance in cancer cells, while IL-4, IL-10, and IL-13 contribute to drug resistance by inhibiting apoptotic pathways. TGF-β levels can predict chemotherapy responses, with high levels associated with resistance[76].

In summary, tumor-produced cytokines significantly reshape the TME by promoting angiogenesis, suppressing immune responses, and inducing chronic inflammation. Understanding the roles of these cytokines is crucial for developing targeted therapies to disrupt these processes and enhance the efficacy of cancer treatments.

3. Breast Cancer

Breast cancer (BC) comprehends a diverse group of biologically and molecularly heterogeneous diseases originating from breast tissue. It is the second most prevalent cancer worldwide, surpassed only by lung cancer[1]. Despite significant advancements in early detection and treatment, BC remains a major global public health challenge. Understanding the risk factors and molecular mechanisms underlying this disease is crucial for developing more effective therapeutic strategies.

3.1. Epidemiology

Breast cancer (BC) remains the most frequent type of cancer in women worldwide, with approximately 1 in 7 women expected to receive a diagnosis during their lifetime[79]. Although male BC is rare, accounting for only about 1%

of all diagnoses, BC remains the second most common cancer when considering both sexes, surpassed only by lung cancer[80] (Figure I-14).

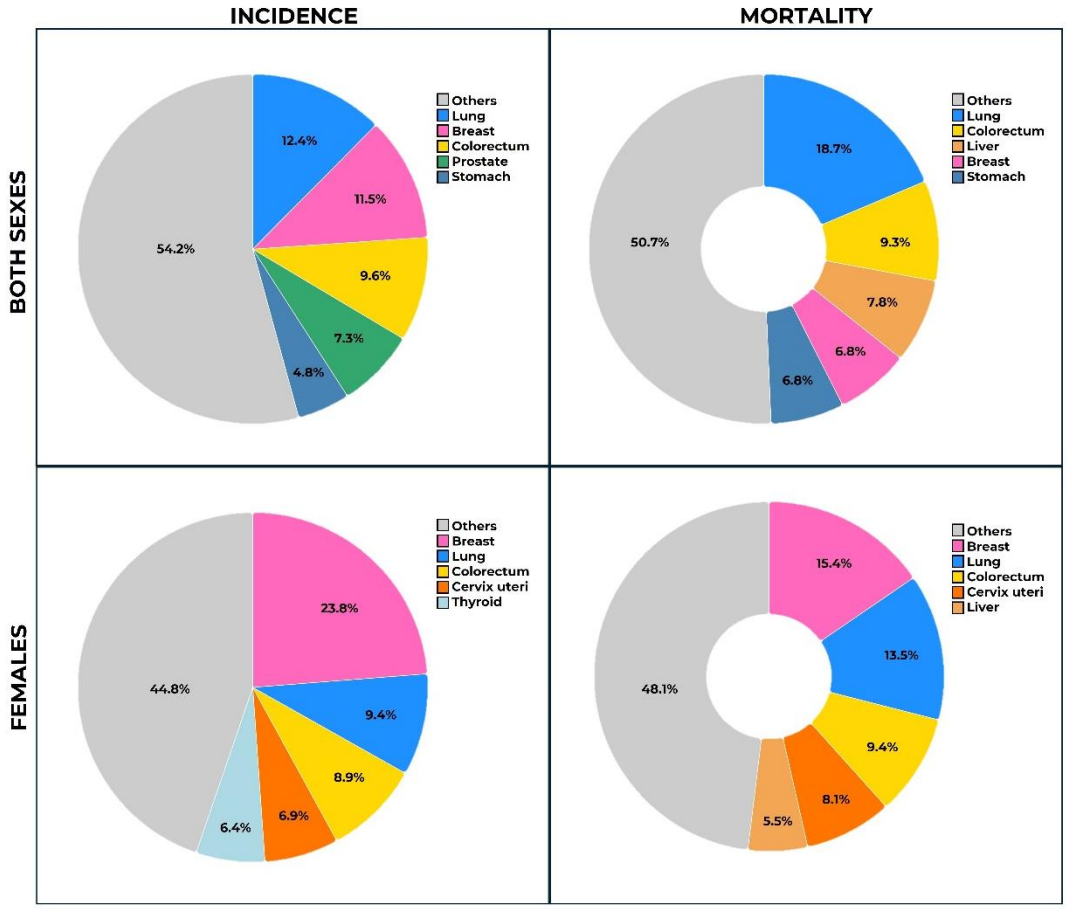


Figure I-14: Breast Cancer: Incidence and Mortality. Breast cancer (BC) accounts for approximately 25% of the cancers diagnosed in women and around 12% in both sexes, worldwide. In terms of mortality, BC represents about 15% of cancer-related deaths in the female population and approximately 7% in both sexes. Adapted from Global Cancer Observatory (Globocan) 2022.

In 2022, 2.3 million women were diagnosed with BC. This disease continues to be the primary cause of cancer-related deaths in women, with more than 660,000 deaths annually[1] (Figure I-14). Metastasis presents a significant challenge for BC patients, as the 5-year overall survival rate decreases considerably once the cancer spreads. Bone, lung, liver, and brain are the four most common metastatic sites, with brain metastasis being the leading cause of death among these patients[81]. Indeed, while BC is curable in approximately 70–80% of patients with early-stage disease, advanced and/or metastatic BC remains incurable[82]. In Portugal, the high prevalence of BC in women is also evident, resulting in more than 1800 deaths annually[83].

BC exhibits significant discrepancies in terms of incidence and mortality rates observed between countries with high and low Human Development Index scores. Incidence rates are higher in high-income countries compared to low-income countries. However, the opposite occurs when concerning the mortality rates. These disparities in incidence and mortality are influenced by differences in risk factors and access to screening and treatment, which are affected by socioeconomic status, legislative policies, and access to medical services[84].

By the year 2040, projections indicate that population growth and ageing will contribute to nearly 3 million new cases of BC and 1 million deaths annually, confirming that current trends will continue[84].

3.2. Classification

Breast cancer (BC) is a heterogeneous disease that can be categorized based on various factors, including the tumor's characteristics, stage, and molecular features. In terms of histological patterns, breast cancers are classified as either pre-invasive (*in situ*) or invasive and are further divided into ductal or lobular types according to the location where the cancer originates within the mammary gland. Ductal carcinoma arises from the milk ducts, while lobular carcinoma originates in the milk-producing lobules[85]. The World Health Organization (WHO) identifies at least 18 different histological types of breast cancer, with invasive BC of no special type (NST), formerly known as invasive ductal carcinoma, being the most common subtype, accounting for 40-80% of cases[86].

BC can be also classified according to the molecular classification, based on the presence or absence of certain receptors on the cancer cells' surface. The PAM50 risk model, introduced by Parker *et al*. in 2009, evaluates the expression of 50 key genes and enables the categorization of BC tumors into four principal intrinsic subtypes: Luminal A, Luminal B, HER2-enriched, and Basal-Like (Figure I-15). Luminal breast cancers, which are estrogen receptor-positive (ER+) and account for approximately 70% of cases in Western populations, are further divided into Luminal A and Luminal B subtypes.

Luminal A tumors express ER and/or progesterone receptor (PR) and lack HER2 expression (Figure I-15). They are characterized by low expression of proliferation-related genes, making them low-grade and slow-growing with a favorable prognosis. Luminal B tumors (Figure I-15) are of higher grade, have a worse prognosis, and may express ER, have low PR expression or be PR-negative, and can be HER2-positive/negative. They show high expression of proliferation-related genes such as MKI67 and AURKA, and lower expression of luminal epithelial markers like PR and FOXA1, but similar levels of ER expression as Luminal A tumors. HER2-enriched breast cancers (Figure I-15) account for 10-15% of cases and are characterized by high HER2 expression with no ER or PR expression. These tumors predominantly express genes related to proliferation, such as ERBB2/HER2 and GRB7, rather than luminal or basal genes. HER2-enriched cancers have historically poor prognoses, but outcomes have improved significantly with the advent of HER2-targeted therapies. Triple-negative breast cancers (TNBC), which lack ER, PR, and HER2 expression, comprise about 20% of BC cases (Figure I-15).

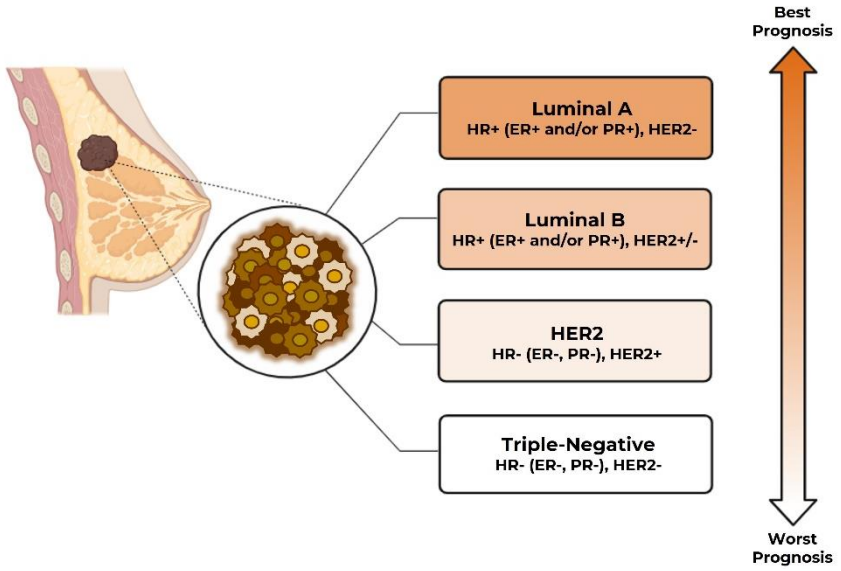


Figure I-15: Molecular classification of Breast Cancer. The four main subtypes are: Luminal A, Luminal B, HER2-enriched, and Triple-Negative, depending on the presence or absence of certain receptors on the surface of cancer cells. Figure created in BioRender.

TNBCs are more common in younger women and African American women and are often associated with BRCA1 mutations. These cancers are typically aggressive and have poorer prognoses. Although the terms basal-like and TNBC are often used as equivalent terms, not all TNBCs are basal-like. Gene

expression profiling has further subdivided TNBC into six subtypes: basal-like (BL1 and BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), luminal androgen receptor (LAR), and an unspecified group (UNS)[86,87].

Additionally, BC can be further classified using the TNM system (Figure I-16), which standardizes the description of the cancer's extent and spread.

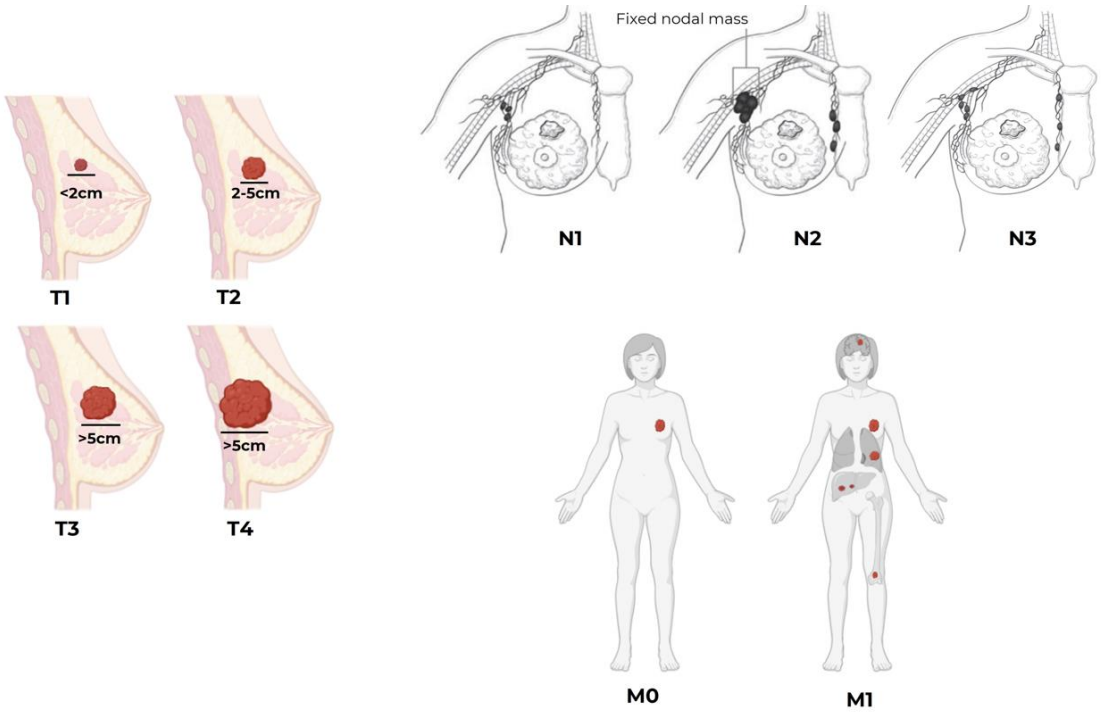


Figure I-16: Breast Cancer TNM staging. TNM staging is used to classify breast tumors according to their size (T), the presence of affected lymph nodes (N), and the presence of distant metastasis (M). Figure elaborated with BioRender. The lymph node images were adapted from Breast Cancer Staging System, in AJCC Cancer Staging Manual[88].

The T (Tumor) component assesses the primary tumor's size and extent within the breast (Figure I-16). This is divided into four categories: T1, where the tumor is 2 cm or less in terms of size; T2, for tumors larger than 2 cm but not exceeding 5 cm; T3, for tumors bigger than 5 cm; and T4, for tumors of any size that have spread into the skin and/or chest wall, including inflammatory tumors. The N (Nodes) component describes the extent of regional lymph node involvement: N0 indicates no affected lymph nodes, N1 denotes the presence of palpable, mobile axillary lymph nodes, N2 indicates the presence of fixed axillary lymph nodes, and N3 represents the involvement of infraclavicular, internal mammary, or supraclavicular lymph nodes (Figure I-

16). The M (Metastasis) component indicates whether the cancer has spread to distant organs or tissues outside the breast and nearby lymph nodes. M0 indicates no distant metastasis, while M1 suggests the presence of distant metastasis[89] (Figure I-16). These TNM components are used combined to determine the overall stage of BC, typically expressed as stages I to IV. Stage I refers to early-stage cancer with a small tumor of up to 2 cm confined to the breast tissue, with no lymph node involvement. Stage II means a moderate-stage cancer that may be in the breast, the nearby lymph nodes, or both. Stage III indicates locally advanced cancer, with a larger tumor that has spread to lymph nodes close to the breast, the skin of the breast, or the chest wall. Stage IV is the most advanced stage, where cancer cells have spread to distant organs or tissues[90]. The complexity and diversity of BC emphasize the importance of its classification in guiding treatment decisions and providing insights into the disease's progression and the patient's prognosis.

3.3. Risk factors

Breast cancer (BC) risk is influenced by various genetic and lifestyle factors (Figure I-17).

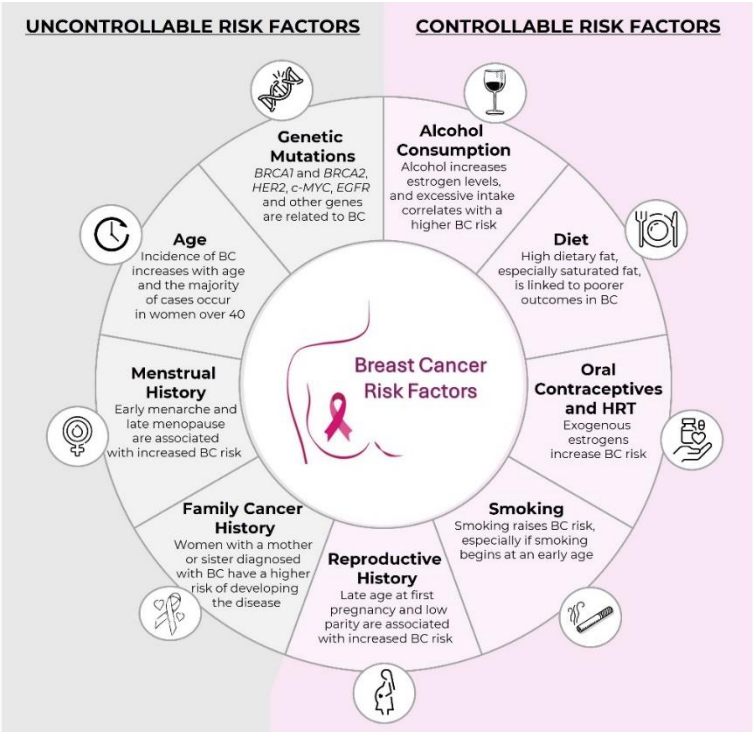


Figure I-17: Risk factors for Breast Cancer. Controllable factors include lifestyle choices such as diet, smoking and alcohol consumption, while uncontrollable factors encompass age, genetics, and family history.

Different genes have been implicated in BC, with mutations and abnormal amplification of oncogenes and tumor suppressor genes playing critical roles in tumor initiation and progression. For instance, *BRCA1* and *BRCA2* are two well-known tumor suppressor genes associated with BC. Located on chromosomes 17q21 and 13q12 respectively, these genes encode important proteins for DNA repair and cell cycle regulation. Mutations in *BRCA1* disrupt cell cycle checkpoints, centrosome duplication, and genetic stability, often leading to apoptosis. *BRCA2* mutations impair DNA repair. Mutations in both genes significantly increase the risk of developing BC, accounting for 20-25% of hereditary BC and 5-10% of all breast cancers. The *HER2* gene, located on chromosome 17q12, and the c-*Myc* gene, located on chromosome 8q24, are also relevant in BC. Overexpression of *HER2* is found in about 20% of primary breast cancers, while overexpression of *c-Myc* is predominantly seen in high-grade, invasive breast carcinomas and is linked to tumor progression. Additionally, *EGFR*, located on chromosome 7p12, is another key player in BC. As a cell surface receptor, *EGFR* is activated by binding to various ligands, triggering downstream pathways that enhance cell proliferation, invasion, and survival. Overexpression of *EGFR* is observed in more than 30% of inflammatory breast cancer cases, a particularly aggressive subtype, and is also common in triple-negative breast cancer (TNBC)[91].

In addition to the genetic factors, age, family history, reproductive factors, estrogen levels, and lifestyle choices are significant risk factors for BC. Age is a critical risk factor, with the incidence of BC increasing with age, and the majority of cases occurring in women over 40 years. A family history of BC considerably raises the risk. Women with first-degree relatives (mother or sister) diagnosed with BC have a higher likelihood of developing the disease. This increased risk is partly due to inherited mutations in genes such as *BRCA1* and *BRCA2*. Reproductive history also impacts BC. Factors such as early menarche, late menopause, late age at first pregnancy, and low parity are associated with increased risk. Delayed menopause increases risk by 3% *per* year, while later menarche decreases risk by 5-10%. Both endogenous and exogenous estrogen levels also influence BC risk. Endogenous estrogen, produced by the ovaries, can be reduced by oophorectomy. Exogenous

estrogens, from oral contraceptives and hormone replacement therapy (HRT), are linked to increased BC risk[91,92].

Finally, modern lifestyle choices, such as alcohol consumption and high-fat diets, contribute to a higher BC risk. Alcohol increases estrogen levels, and excessive intake correlates with a higher risk. High dietary fat, especially saturated fat, is linked to poorer outcomes in BC patients. Smoking, particularly when combined with alcohol consumption, also raises BC risk, especially if smoking begins at an early age[91,92].

Understanding these risk factors (Figure I-17) can aid in the prevention and early detection of BC, potentially improving patients' outcomes.

3.4. Survival

Based on data from the Surveillance, Epidemiology, and End Results (SEER) database of the National Cancer Institute, the 5-year relative survival rates for breast cancer (BC) are influenced by factors such as stage of diagnosis, cancer subtype, and demographic variables like age, gender, and ethnicity. The SEER database considers data from the years 2014-2020, and the 5-year relative survival rate refers to the proportion of people likely to be alive five years after a BC diagnosis, excluding deaths from other causes[93].

When considering all races, ages, and stages of diagnosis, the 5-year relative survival rate is 91.2% for females and 84.1% for males. However, this rate changes depending on the stage at which the cancer is diagnosed[93]:

Table I-1: The 5-year relative survival rate by stage.

Stage	5-year relative survival rate
Localized - Invasive cancer confined to the breast.	Female: 99.6%; Male: 96.3%
Regional - Invasive cancer that has spread to nearby parts of the body, such as the chest wall or lymph nodes.	Female: 86.7%; Male: 85.7%

Stage	5-year relative survival rate
Distant - Invasive cancer that has spread to other parts of the body, such as the lungs, liver, bones, and brain.	Female: 31.9%; Male: 28.3%

Age at diagnosis also plays a crucial role in survival rates. Younger patients generally have better outcomes, while older patients may have lower survival rates as demonstrated in the table below[93]:

Table I-2: Impact of age at diagnosis on 5-year relative survival rate.

Age	Stage	5-year relative survival rate
<50 years	Localized	Female: 98.8%; Male: 99.0%
	Regional	Female: 88.1%; Male: 86.9%
	Distant	Female: 43.3%; Male: 25.9%
50-64 years	Localized	Female: 98.8%; Male: 94.4%
	Regional	Female: 88.1%; Male: 85.0%
	Distant	Female: 33.2%; Male: 28.7%
65+ years	Localized	Female: 100.0%; Male: 96.4%
	Regional	Female: 83.7%; Male: 85.9%
	Distant	Female: 25.3%; Male: 29.5%

Ethnicity is another important factor affecting survival in BC patients. Survival rates can vary among different ethnic groups due to factors such as access to healthcare, genetic differences, socioeconomic status, and cultural attitudes towards medical care. Below, table I-3, explains the influence of ethnicity on the 5-year survival rate of BC[93]:

Table I-3: The 5-year relative survival rate in breast cancer by ethnicity.

Ethnicities	Stage	5-year relative survival rate
Hispanic (any race)	Localized	Female: 97.2%; Male: 98.4%
	Regional	Female: 85.2%; Male: 76.2%
	Distant	Female: 32.0%; Male: 19.4%
Non-Hispanic American Indian or Alaska Native	Localized	Female: 98.3%; Male: NA
	Regional	Female: 82.2%; Male: NA
	Distant	Female: 35.4%; Male: NA
Non-Hispanic Asian or Pacific Islander	Localized	Female: 98.3%; Male: 97.3%
	Regional	Female: 88.8%; Male: 81.3%
	Distant	Female: 39.3%; Male: 41.6%
Non-Hispanic Black	Localized	Female: 97.2%; Male: 90.9%
	Regional	Female: 79.0%; Male: 80.6%
	Distant	Female: 23.7%; Male: 23.4%
Non-Hispanic White	Localized	Female: 100.0%; Male: 95.8%
	Regional	Female: 88.5%; Male: 88.1%
	Distant	Female: 33.1%; Male: 28.5%

The subtype of BC significantly influences survival rates as well. In the table below are the 5-year relative survival rates for female BC by subtype, considering all ages and races[93]:

Table I-4: The 5-year relative survival rate for female breast cancer by subtype.

Cancer Subtype	Stage	5-year relative survival rate
HR+/HER2-	Localized	100.0%
	Regional	90.5%
	Distant	35.4%

Cancer Subtype	Stage	5-year relative survival rate
HR-/HER2-	Localized	92.0%
	Regional	66.8%
	Distant	14.3%
HR+/HER2+	Localized	99.3%
	Regional	90.4%
	Distant	45.8%
HR-/HER2+	Localized	97.3%
	Regional	84.2%
	Distant	39.7%

To effectively reduce BC mortality and mitigate the overall impact of the disease, global efforts and public health measures are essential. These should focus on prevention, early diagnosis, screening, equitable access to care, and advancements in targeted therapies. Such comprehensive strategies are crucial for improving survival rates and enhancing the quality of life for BC patients.

3.5. Current Treatments in Breast Cancer

The treatment of breast cancer (BC) involves a multidisciplinary approach that considers tumor load and molecular markers. For non-metastatic BC, surgery-based treatments are standard, often accompanied by preoperative systemic chemotherapy to reduce tumor volume and facilitate breast conservation. In metastatic BC, systemic treatment is preferred[94]. Advances in endocrine therapy, targeted therapy, and immunotherapy have expanded treatment options for both metastatic and non-metastatic BC. Additionally, innovative therapies, such as gene therapy, vaccines, and adoptive cell therapies are also under investigation. Actually, the main treatments used for BC include surgery, chemotherapy, radiotherapy, endocrine therapy, targeted therapy,

and for some cases of TNBC, PARP inhibitors could be used, specifically when *BRCA* is mutated, and immunotherapy (Figure I-18), as explained below.

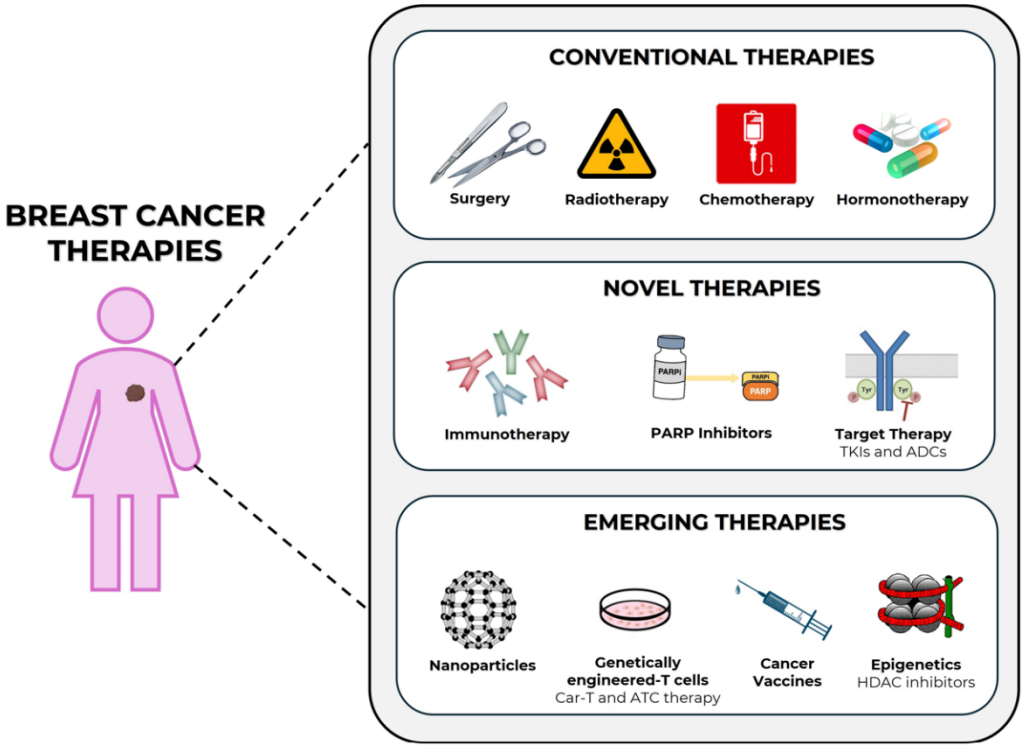


Figure I-18: Overview of breast cancer (BC) therapies. Currently available breast cancer therapies and potential strategies to enhance clinical outcomes for BC patients.

- Surgery: Breast surgery is a well-established local treatment for early invasive BC. The most common surgical approaches are total excision of the breast (mastectomy), often followed by breast reconstruction, or breast-conserving surgery (lumpectomy). Studies indicate that mastectomy and lumpectomy plus irradiation are equivalent in terms of relapse-free and overall survival (OS)[95].

- Radiotherapy: High-energy radiation is applied to the whole breast or a portion of the breast (after breast-conserving surgery), the chest wall (after mastectomy), and regional lymph nodes. Postmastectomy radiation to the chest wall in patients with positive lymph nodes is linked to a lower risk of recurrence and BC mortality compared to those with negative lymph nodes. Radiation can also be incorporated after mastectomy for patients at higher risk for recurrence or can be

administered in parallel with other therapies like anti-HER2 therapy or endocrine therapy[95].

- Chemotherapy: Chemotherapy is a treatment for BC that plays a significant role in both early and advanced stages of the disease. It can be categorized into two types: neoadjuvant chemotherapy (NACT) and adjuvant chemotherapy (AC)[94,95]. NACT is administered before surgical intervention. Its primary goals are to downstage tumors and axillary lymph nodes, making breast-conserving surgery possible and converting inoperable BC into resectable. This approach is particularly beneficial for patients with large tumors, nodal involvement, low hormone receptor expression, and aggressive subtypes such as triple-negative breast cancer (TNBC) and HER2-positive BC[94]. AC, on the other hand, is given after surgical removal of the tumor to eliminate any remaining cancer cells and reduce the risk of recurrence[95]. The optimal chemotherapy regimen typically includes a combination of taxanes and anthracyclines. Anthracyclines are DNA intercalating agents that inhibit cellular replication, with common examples including doxorubicin and epirubicin[95]. Cyclophosphamide, frequently used in combination chemotherapy regimens, forms DNA crosslinks, leading to cell apoptosis. Taxanes, such as docetaxel and paclitaxel, disrupt cellular cytoskeletal properties, causing defects in mitosis and inhibiting cell division. Despite some controversy, anthracyclines are considered essential for high-risk patients. Standard chemotherapy regimens often comprise cyclophosphamide and doxorubicin for four cycles, followed by paclitaxel for four cycles. Another regimen might include a combination of cyclophosphamide, doxorubicin, and either weekly paclitaxel for 12 weeks or docetaxel every three weeks for four cycles[95]. Chemotherapy regimens can be tailored according to the molecular subtype of BC. For hormone receptor-positive (HR+) BC, patients should receive endocrine therapy after completing chemotherapy. HER2-positive BC patients benefit from combining trastuzumab with chemotherapy. For TNBC, the frontline therapy includes a combination of taxanes and anthracyclines[95]. Adjuvant chemotherapy is typically recommended to start 3–4 weeks post-

surgery, as delays beyond 91 days from surgery to the start of this therapy have been associated with worse survival outcomes, especially in TNBC patients[94].

- Endocrine Therapy: Endocrine therapy is a standard adjuvant treatment for hormone receptor (HR)-positive BC, defined by estrogen receptor (ER) or progesterone receptor (PR) staining of ≥1%[94]. This therapy is typically administered over 5–10 years and is important in reducing recurrence and improving survival rates[95]. For premenopausal patients, tamoxifen, an estrogen receptor modulator approved by the US Food and Drug Administration (FDA) in 1977, is widely used and significantly reduces the risk of recurrence. Studies have shown that extending tamoxifen therapy to 10 years further decreases BC mortality compared to a 5-year regimen[95]. Additionally, in high-risk premenopausal patients, augmenting ovarian suppression drugs with either an aromatase inhibitor or tamoxifen can amplify disease-free survival (DFS)[94]. Aromatase inhibitors are essential in endocrine therapy, particularly as aromatase expression is heightened in breast tumors, correlating with elevated estrogen levels. Thus, aromatase inhibitors act by blocking aromatase enzyme activity, thereby impeding estrogen synthesis[95]. For metastatic BC, the combination of cyclin-dependent kinase (CDK) inhibitors with aromatase inhibitors has shown improved DFS, making it a viable option for treating endocrine-resistant metastatic BC[94].

- Targeted Therapy: Targeted therapy has revolutionized the approach to treat HER2-positive BC. This treatment comprises various modalities, including antibodies targeting specific HER2 epitopes, tyrosine kinase inhibitors (TKIs), and, more recently, antibody-drug conjugates (ADCs). Trastuzumab (Herceptin), the pioneer anti-HER2 targeted therapy, gained FDA approval in 1998[95]. Since then, trastuzumab has demonstrated significant benefits, particularly when combined with anthracycline/taxane-based adjuvant therapy[94]. Later, other anti-HER2 drugs like pertuzumab have emerged, showing promising results in clinical trials. For instance, combining pertuzumab with trastuzumab and chemotherapy has enhanced overall survival (OS) in metastatic

HER2+ BC patients compared to trastuzumab and chemotherapy alone. Pertuzumab's efficacy extends also to early-stage HER2+ BC[95]. Additionally, in the management of metastatic HER2-positive BC, early initiation of anti-HER2 therapy is recommended, with docetaxel plus trastuzumab and pertuzumab standing as the frontline standard[94]. Despite the developments made with anti-HER2 antibodies, only a fraction of HER2+ BC patients benefit from this treatment approach. One explanation for this limited efficacy lies in structural alterations of HER2 that impede antibody binding. To address resistance issues, TKIs such as lapatinib have been developed. These small molecules disrupt HER2 downstream signaling by competing with ATP at the receptor's catalytic domain, inhibiting tyrosine phosphorylation. Lapatinib has demonstrated more benefits than chemotherapy alone in metastatic BC, and as a neoadjuvant treatment, lapatinib plus trastuzumab combined with chemotherapy has shown to be more efficient than chemotherapy combined only with lapatinib or trastuzumab as standalone. However, challenges persist with TKIs, prompting the emergence of ADCs therapies. Trastuzumab-emtansine (T-DM1) represents a significant advancement in this domain. T-DM1 combines the targeting abilities of trastuzumab with the cytotoxic effects of DM1, a derivative of maytansine. By binding to HER2 and releasing DM1 metabolites into the cytoplasm, T-DM1 exerts potent anti-tumor effects, improving OS and DFS in HER2+ metastatic BC patients compared to alternative treatments[95].

- PARP Inhibitors: PARP (poly-(ADP-ribose) polymerase) inhibitors have been used to treat BRCA-mutated BC. *BRCA1* and *BRCA2* genes are crucial for DNA repair, and mutations in these genes significantly increase the risk of breast and ovarian cancers. PARP proteins also play a role in DNA repair, and inhibiting PARP in BRCA-mutated cells leads to cell death due to accumulated DNA damage. Several PARP inhibitors are currently in clinical use, including olaparib, talazoparib, and rucaparib. Indeed, olaparib, the first FDA-approved PARP inhibitor for BRCA-mutated BC, has shown significant benefits in clinical trials and is now part of standard clinical practice. Specifically, a phase III trial

demonstrated that olaparib had better efficacy and tolerability than standard chemotherapy for BRCA-mutated HER2-negative BC. This PARP inhibitor has also been tested in combination with chemotherapy, showing promising results. For instance, combining olaparib with paclitaxel in TNBC patients achieved an overall response rate (ORR) of 37%. Similar success was seen in studies combining olaparib with cisplatin or carboplatin in BRCA-mutated BC patients[95].

- Immunotherapy: Immunotherapy has revolutionized the treatment landscape for BC, particularly for TNBC[95,96]. Effectively, immunotherapy harnesses the body's immune system to combat cancer by stimulating or enhancing its natural defenses. The immune system represents a crucial key in designing immunotherapies because tumor cells often escape immune surveillance by manipulating T-cell activity and inhibiting immune responses[95]. Indeed, the rationale behind immunotherapy lies in the ability to target specific molecules or pathways that tumors exploit to evade immune detection and destruction. In BC, immunotherapy has demonstrated potential, particularly in subtypes expressing elevated levels of immune checkpoint proteins like programmed cell death protein receptor (PD-1), its ligand PD-L1, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4)[96]. These biomarkers are critical in understanding immune evasion mechanisms and guiding effective treatment strategies. For instance, atezolizumab, an anti-PD-L1 antibody, demonstrated safety and efficacy in clinical trials for metastatic TNBC, significantly improving pathological complete response (pCR). Durvalumab, another anti-PD-L1 antibody, was tested in combination with an anthracycline taxane-based neoadjuvant therapy, increasing pCR rates, especially in patients pretreated with durvalumab monotherapy before chemotherapy[95]. Avelumab, an anti-PD-L1 antibody, demonstrated an acceptable safety profile and clinical activity, particularly in tumors expressing PD-L1 in locally advanced or metastatic BC[95,96]. Pembrolizumab, an anti-PD-1 antibody, was tested in diverse clinical trials[95]. The KEYNOTE-522 trial demonstrated that pembrolizumab plus chemotherapy significantly improved pCR rates in early TNBC

compared to placebo plus chemotherapy[94–98]. Currently, immunotherapy is already used in clinical practice, namely through the use of immune checkpoint inhibitors (ICIs) (Figure I-19). Recent estimates indicate that approximately 43% of cancer patients in the United States are eligible for ICIs therapy, though only 12.5% of these patients respond to the treatment[99]. Pembrolizumab and atezolizumab are two FDA-approved drugs used in clinical routine as first-line treatments for PD-L1 positive metastatic TNBC[94,97,98]. Also, in Portugal, pembrolizumab received INFARMED approval in the summer of 2023 for adults with recurrent unresectable or metastatic TNBC whose tumors express PD-L1[100]. Ongoing research and clinical trials continue to explore the full potential of these therapies, both as monotherapy and in combination with other treatments, especially for other BC subtypes, to improve outcomes for BC patients[97].

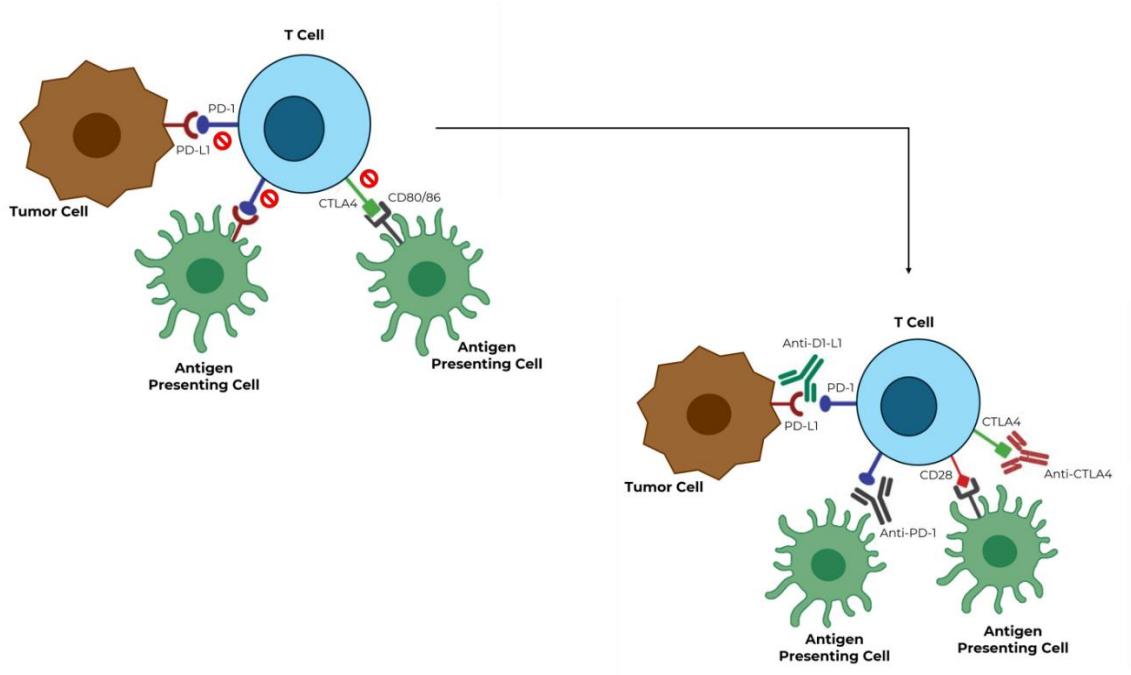


Figure I-19: Immune checkpoint blockade therapy. PD-1 is typically found on T cells, particularly cytotoxic T cells, and binds to its ligand PD-L1, which can be expressed on either tumor cells or antigen-presenting cells like dendritic cells. CTLA-4 can also be expressed on T cells and binds to CD80/CD86 on antigen-presenting cells. These immune checkpoints, when engaged by their ligands, give an inhibitory signal to T cells, dampening their function. Immune checkpoint blockade therapy involves antibodies targeting PD-1, PD-L1, or CTLA-4, which restrict these inhibitory signals, thereby activating T cells to exert their anti-tumor activity. Figure created in BioRender.

3.6. Emerging Therapies for Breast Cancer

Despite advancements in treatment, breast cancer (BC) remains a disease with a high mortality rate among women worldwide, attributed to its heterogeneous nature and variable effectiveness of current treatments[95]. Because of this, several emerging treatments for BC are being investigated (Figure I-18). Gene therapy is one promising approach involving the delivery of genetic material to target cells to modify gene expression. Strategies include gene editing, targeting transcription factors, microRNA, and DNA or RNA vaccination. Clinical trials have demonstrated that genetic prodrug activation therapy targeting the *HER2* gene promoter is safe and can effectively target gene expression. Other studies exploring the use of microRNA in anti-cancer therapy have shown promising results in inhibiting BC cell proliferation[94]. Regarding BC vaccines, this technology aims to make the targeted antigen recognizable by the immune system to induce a therapeutic effect. These vaccines can be categorized based on their platforms and formulations, including peptide-based, DNA-based, tumor cell antigen-based, and carbohydrate-based vaccines[101]. Early trials have shown good tolerance and detectable immune responses, but significant clinical benefits in phase 3 trials are still lacking[95,97]. However, combining vaccines with immune checkpoint blockade to overcome cancer tolerance is an active area of investigation[102]. Preclinical studies indicate that tumor vaccines can upregulate inhibitory receptors on T cells, making this combination a promising strategy that may enhance and prolong the immune response. Additionally, some HER2-derived peptide vaccines may work synergistically with anti-HER2 monoclonal antibodies[97].

CAR-T cell therapy and adoptive T cell therapy (ATC) (Figure I-20) are also innovative immunotherapy approaches tested for BC therapy, harnessing the body's immune system to target and destroy cancer cells more effectively. CAR-T cell therapy involves modifying patients' T cells to express chimeric antigen receptors (CARs) designed to recognize and attack specific cancer cells. This therapy has shown remarkable success in hematologic malignancies and is now being explored for solid tumors like BC. Researchers have identified several potential targets for CAR-T therapy in BC, including HER2, EGFR, and MUC1. Preclinical studies show significant anti-tumor effects,

but clinical application faces challenges such as insufficient T cell trafficking and infiltration into tumors, an immunosuppressive tumor environment, and potential toxicities[103]. On the other hand, ATC involves extracting, expanding, and activating a patient's T cells outside the body before reinfusing them to target cancer cells (Figure I-20). This method is evolving and being tested in clinical trials both as a standalone treatment and in combination with other immunotherapies. A recent case highlighted the potential of ATC, where autologous T lymphocytes engineered to target neo-antigens were successfully used in a patient with chemo-refractory metastatic BC, especially when combined with inhibitory checkpoint blockade[104].

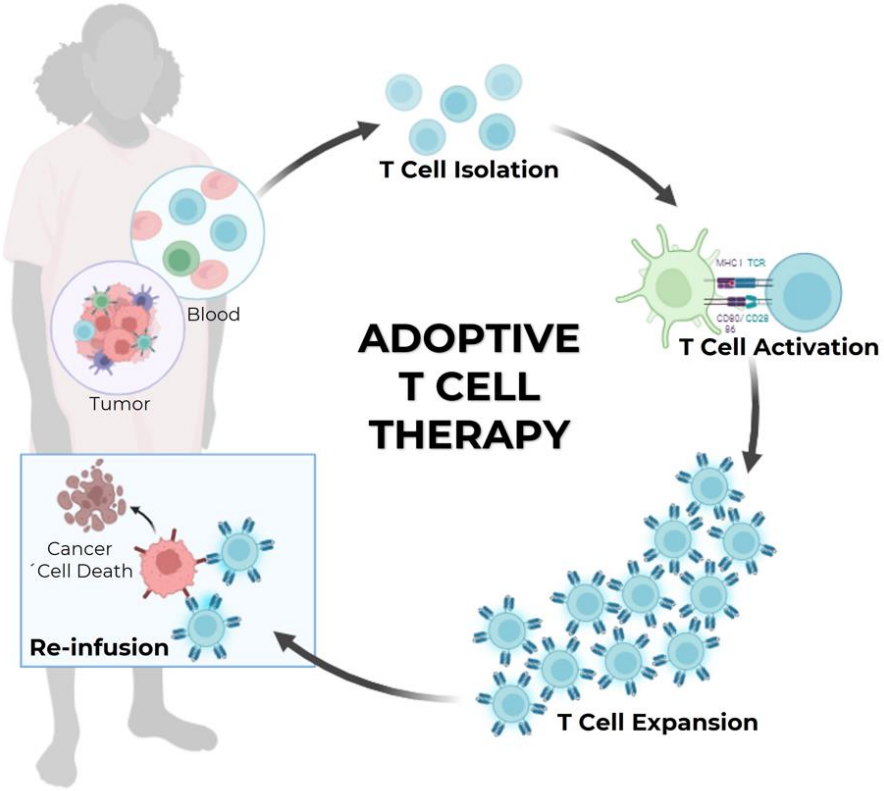


Figure I-20: The rationale of adoptive T cell therapy (ATC). ATC aims to harness cytotoxic T cells sourced from either tumor-infiltrating lymphocytes or peripheral blood. These cells are cultured and expanded outside the body before being reintroduced into the patient. Figure created in BioRender.

Also, nanoparticles represent a promising advancement in BC treatment by addressing limitations associated with traditional antineoplastic drugs, such as systemic toxicity and poor bioavailability. These selective drug-delivery

systems enhance the efficacy and safety of cancer therapies by delivering drugs directly to malignant cells, minimizing adverse effects on healthy tissues, and improving the overall quality of life for patients. Nanotechnology involves the manipulation of cellular and molecular components to create particles ranging from 1 to 100 nanometers. Nanoparticles provide several advantageous properties, including prolonged elimination times, increased contact time at the drug site, and reduced drug resistance. They can penetrate the dense extracellular matrix of tumors and evade the immune system, releasing their drug in response to specific stimuli within the tumor microenvironment. Some nanoparticulate-based chemotherapeutics, like doxorubicin hydrochloride and nanoparticle albumin-bound paclitaxel, have already been approved by the US FDA. Several other formulations, such as liposomes and polymeric nanoparticles, are in clinical trials. Combining nanoparticles with other therapeutic modalities, such as chemotherapy, radiation, and immunotherapy, shows potential for enhanced treatment efficacy. For example, nanoparticles can deliver a combination of drugs simultaneously for a synergistic effect or deliver gene therapy agents, such as siRNA or CRISPR-Cas9 components, to modify the genetic profile of cancer cells, potentially providing a more durable and long-lasting treatment response[105].

Finally, epigenetics, particularly the modulation of histones, represents a significant innovative strategy in BC treatment. Histone deacetylases (HDACs) are enzymes that remove acetyl groups from histone proteins, leading to a condensed chromatin structure and suppressed gene expression. Inhibiting HDACs can result in a more relaxed chromatin state, allowing for the activation of tumor suppressor genes and other critical regulatory genes involved in cancer progression. Indeed, HDAC inhibitors (HDACi) are emerging as promising therapeutic agents in BC treatment due to their ability to alter gene expression and enhance the efficacy of other treatments, such as immunotherapy. Currently, five HDACi have received FDA approval for cancer therapy: vorinostat, belinostat, panobinostat, pracinostat, and romidepsin. These inhibitors have shown clinical benefits, particularly in hematological malignancies, but their efficacy in solid tumors, including BC, is still under investigation[106].

3.7. Biomarkers in Breast Cancer

In recent decades, breast cancer (BC) treatment has advanced significantly also due to the discovery of specific biomarkers that enable more personalized therapies for different BC subtypes. These biomarkers, which include serological, histological, and genetic indicators, play crucial roles in BC care. They are essential for initial diagnosis, detecting relapses during follow-up, guiding targeted therapies, and providing critical information throughout the disease course, particularly for aggressive tumor subtypes[107]. There are two types of biomarkers: prognostic and predictive. Prognostic biomarkers predict patient clinical outcomes objectively and independently of treatment, based on patient or tumor characteristics. Predictive biomarkers, on the other hand, indicate how likely a patient is to respond to a specific therapy, reflecting tumor sensitivity or resistance to that treatment[108].

Among prognostic biomarkers, Ki-67 reflects cell proliferation rates and is associated with poorer outcomes, especially in hormone receptor-positive cases[108,109]. Lymph node involvement signals a higher risk of recurrence and worse survival, while tumor size and grade also correlate with prognosis, with larger and higher-grade tumors showing more aggressive behavior[109,110]. Gene expression profiles, such as Oncotype DX and MammaPrint, help stratify recurrence risk and guide chemotherapy use. Markers like uPA/PAI-1 are linked to tumor invasion and poorer outcomes[110], while immune markers, including tumor-infiltrating lymphocytes (TILs), lymphocyte-to-monocyte ratio (LMR), platelet-to-lymphocyte ratio (PLR) and neutrophil-to-lymphocyte ratio (NLR), also influence prognosis[111–113].

In terms of predictive biomarkers, hormone receptors (ER and PR) are key in predicting response to endocrine therapy, while HER2 is vital for guiding targeted treatments in HER2-positive cases. PD-L1 is another important marker, used to direct immune checkpoint inhibitor therapies[108–110,114].

Other investigational biomarkers include microRNAs, Cyclin D1, circulating tumor cells (CTCs), and serum markers such as carcinoembryonic antigen (CEA) and cancer antigens (CA) 15-3, all of which show promise in improving BC diagnosis and treatment, though further clinical validation is needed[107–110,114].

3.8. The Challenges and Future Landscape of Breast Cancer

Significant advancements in medical research, technology, and treatment options have marked the evolving landscape of breast cancer (BC). However, numerous challenges persist. One of the main difficulties lies in the heterogeneity of tumors among patients. The distinct molecular and genetic profiles of each tumor demand tailored therapies[115]. In addition to treatment complexities, the access to innovative therapies remains a significant barrier, particularly in low-income countries. Disparities in healthcare access mean that many individuals cannot benefit from early detection, advanced treatments, or comprehensive supportive care services. Effectively, in 2017, Newman L. highlighted that while advancements in BC treatments have improved overall survival rates, significant disparities persist among different socioeconomic groups and racial/ethnic minorities. Factors such as income, education, healthcare access, and insurance critically impact early detection rates, treatment options, and survival outcomes. These socioeconomic disparities often lead to delays in diagnosis, suboptimal treatments, and poorer prognoses for disadvantaged populations. Newman L. also emphasized that biological factors, including tumor biology, genetic mutations, and BC subtypes, influence treatment responses and outcomes. Integrating biological insights with socioeconomic considerations is crucial for developing more effective personalized medicine strategies and equitable healthcare delivery. This includes improving access to screening programs, enhancing patient education, promoting health policy reforms, and fostering research to understand and mitigate disparities in BC treatment outcomes[116].

However, despite global disparities, the new era has seen the rise of innovative omics technologies, allowing researchers to examine data from various biological layers, such as the genome, epigenome, transcriptome, proteome, and metabolome. These technologies, including bulk and single-cell omic approaches, provide a comprehensive understanding of tumor behavior, enabling molecular classification of cancers and personalized treatment strategies. The application of multi-omics can help decipher the molecular complexity of BC, classify different tumor types and subtypes, and predict survival outcomes[115]. Nevertheless, the vast amount of data generated by multi-omics analysis presents significant challenges in data management

and interpretation. Developing integrated systems to handle and interpret this data is fundamental, especially for complex and aggressive cases like triple-negative breast cancer (TNBC). Artificial intelligence (AI) has shown promise in analyzing multi-modal data sets, identifying gene signatures, and enhancing the precision of BC management. AI imaging is also a rapidly evolving field in BC research and diagnosis. AI-assisted imaging provides more accurate and efficient diagnostic models, potentially replacing invasive biopsies. Techniques like radiomics, which extract high-throughput data from medical images, offer non-invasive insights into tumor features and heterogeneity. AI algorithms can improve diagnostic accuracy, identify biomarkers, and enhance imaging exams by reducing false positives and negatives. However, the high cost of these new technologies and treatments can limit accessibility, creating further disparities in care[101,115].

Thus, while new technologies and ongoing investments in BC research are valuable tools, attending to the challenges of the BC landscape requires a multifaceted approach. This includes improving access to care, effectively integrating new technologies, and ensuring the equitable distribution of advancements in BC treatment. By addressing these issues, the future landscape of BC care can become more inclusive, personalized, and effective, ultimately improving outcomes for BC patients worldwide[101].

4. 3D *in vitro* Models to Understand Tumor-Immune Interactions

The tumor microenvironment (TME) is multifaceted and dynamic, including not just the tumor cells, but also several supporting cell types such as activated fibroblasts, blood vessels, infiltrating immune cells, and the extracellular matrix. This complex system is regulated by cellular interactions, hormonal influences, and inflammatory responses, all of which are crucial for understanding tumorigenesis. Traditionally, cancer therapies target tumor cells directly, however, the essential role of the immune system in cancer progression has highlighted the importance of targeting other components within the TME[117]. Considering this, three-dimensional (3D) *in vitro* models are emerging as suitable platforms to study tumor-immune interactions. Indeed, despite two-dimensional (2D) co-culture systems with exogenous immune

cells being commonly used in tumor immunology research due to their simplicity and cost-effectiveness, they fail to replicate the 3D architecture and complexity of *in vivo* tumors. These limitations include an incapability to mimic the spatial and temporal dynamics of the TME and the loss of genetic and phenotypic heterogeneity along the time. Similarly, humanized mouse models have been helpful in studying human tumor-immune interactions but also present weaknesses, such as limited simulation of the human immune system and high technical and financial barriers[118–120]. Effectively, unlike traditional 2D cultures, 3D models preserve the complexity and heterogeneity of tumors, enabling a more realistic investigation of TME and tumor biology. These models can be categorized by their production methods and organization as organ-slice cultures, derived from sections of tumor tissues; multi-layered cell cultures, consisting of adherent cells that grow in layers post-confluence and still as spherical models, where tumor cells grow in spheres[121]. Indeed, 3D models could integrate multiple cell types and more accurately mimic the TME. This capability allows for more detailed studies of the interactions between tumor cells and the immune system, providing insights that are critical for developing effective therapies, in specific, immunotherapies. Moreover, 3D models can facilitate high-throughput drug screening and personalized medicine approaches by using patient-derived cells to create more accurate models of individual tumors[122]. Actually, the best-known 3D *in vitro* models include organoids, air-liquid interface cultures, spheroids, microfluidic devices, and 3D bioprinting, with each presenting pros and cons (Table I-5)[117].

Organoids are 3D structures cultivated from stem or progenitor cells that replicate the cellular diversity and organizational structure of specific organs. Unlike traditional 2D cell lines, organoids maintain the genotypic and phenotypic characteristics of the original tissue, including the heterogeneity of tumor cells, which is essential for studying cancer biology and developing therapies. There are two primary methods for constructing organoids: scaffold-dependent and scaffold-free. Scaffold-dependent methods involve using physical supports, such as matrigel or synthetic hydrogels, to provide a structure for cell growth and self-assembly into 3D forms. In contrast, scaffold-free methods, including the hanging drop method, magnetic levitation, and

rotary cell culture, rely on external forces like gravity or magnetic fields to facilitate the aggregation and formation of 3D structures without an extracellular matrix[117]. Tumor organoids are particularly interesting for cancer research because they can be generated from patient-derived samples, preserving the histopathological, genetic, and phenotypic features of the "parent" tumor[117,118,122]. In these cases, the organoids are called patient-derived tumor organoids (PDTOs) and represent an alternative to patient-derived tumor xenografts where patient tumor tissue is implanted into an animal model. PDTOs from colon, pancreatic, lung, breast, and other cancers have been successfully cultured, showing high success rates and maintaining long-term viability and characteristics of the "parent" tumors. PDTOs allow also functional tests, such as drug sensitivity and prediction of treatment-related side effects[122]. In tumor immunology research, organoids can be also co-cultured with immune cells to study interactions and test immunotherapeutic approaches. One common method involves directly adding immune cells to the organoid culture medium, where they can secrete cytokines and, if sufficiently migratory, infiltrate the organoid structure. Alternatively, organoids can be separated from the matrix and cultured in suspension with immune cells to facilitate direct contact[117].

Table I-5: Comparison of 3D in vitro models. Each 3D *in vitro* model has pros and cons for studying tumor-immune interactions.

Model	Pros	Cons
Immune cell co-culture model (spheroids and organoids)	✓ Flexible technique ✓ Co-culture with immune cells	⌦ The co-culture was performed with immune cells that are in a different state compared to those in the original TME
ALI culture model	✓ Better mimic the TME	⌦ Only suitable for short-term studies
3D-Microfluidic culture model	✓ Require a small amount of tumor tissue ✓ Cost-effective	⌦ Requiring special equipment ⌦ High cost ⌦ Limited by the volume of the device
3D-bioprinting model	✓ Models with precise architecture and composition ✓ High reproducibility	⌦ Requiring special equipment

More recently, the air-liquid interface (ALI) culture system upsurges as an innovative model for culturing tumor organoids. In this method, tumor tissues are mechanically cut into small fragments and mixed with collagen gel. This mixture is layered on top of pre-solidified collagen gel within a permeable transwell insert. The insert is placed in a cell culture dish, with culture medium added between the dish and the insert, forming a double-dish culture system. This setup allows the upper part of the collagen and tumor tissue mixture to be exposed to air, while the culture medium infiltrates the bottom through micropores, creating an air-liquid interaction model. The ALI culture system is particularly advantageous for studying respiratory, gastrointestinal, or surface tumors, as the air exposure better mimics the *in vivo* tumor environment. This method enhances the oxygen supply, supporting normal tumor cell growth to form tumoroids that retain the pathological and genetic features of the original tumor[117,118,122].

Besides organoids, another common 3D model for studying tumor biology is spheroids. Several techniques exist for spheroid formation, both scaffold-free and scaffold-based[118]. Scaffold-free methods, such as the hanging drop, rely on strong cell-to-cell interactions to promote aggregation and deposition. Scaffold-based approaches use hydrogels or matrix to provide a structure for cell growth[118]. Tumor spheroids created in a scaffold-free environment where cells aggregate in suspension or in a non-adherence surface are less complex than organoids but convenient for co-culturing with immune cells[117]. For example, Tristan Courau *et al.* cultured tumor cells into spheroids and added T cells and NK cells from peripheral blood, allowing for direct contact and infiltration, which enabled the study of immune cell interactions[117]. In addition, in this thesis, we also used spheroids as a 3D platform to test tumor-immune cell interactions. The spheroids are also advantageous for high-throughput drug screening due to their ability to mimic key features of tumors. In fact, they form micro-sized aggregates that recapitulate the internal structure and morphology of solid tumors, making them suitable for testing anti-cancer drugs[118]. Depending on their origin and preparation methods, spheroids can be classified into four types: multicellular tumor spheroids (MCTSs), tumorospheres, tissue-derived tumorospheres, and organotypic multicellular spheroids. MCTSs, which are created from primary cells or cell lines, are the

most studied and widely used for modeling various cancers, including breast, cervical, colon, lung, pancreatic, and prostate cancers[118].

Additionally, advances in bioengineering, have led to the development of more complex *in vitro* 3D models that offer potential as preclinical platforms for cancer research. These models include microfluidics and 3D bioprinting.

Microfluidics is a technique for manipulating the flow of one fluid in micro volumes within microscopic channels[117]. This technique has advanced the ability to mimic natural biophysical and chemical conditions *in vitro*. The goal of microfluidics is to create platforms, known as organ-on-chip devices, that model the physiological functions of tissues and organs. These devices consist of networks of microfluidic channels allowing continuous cell culture perfusion. The main advantage of microfluidics is the ability to design complex 3D culture systems where various parameters, such as mechanical forces, tissue interface orientation, cell types, and chemical gradients, can be independently controlled, optimizing cell survival and function. Moreover, microfluidic devices utilize microscale volumes, making them cost-effective compared to other 3D culture methods. They also enable high-content and high-throughput screening with increased controllability. Also, microfluidic devices are employed to analyze the effects of growth factors or drugs in a biomimetic microenvironment. Moreover, this technology has already demonstrated the capability to be used in research lines that involve lymphocyte infiltration, migration, cytotoxicity, and mechanisms of cancer resistance and immune suppression. However, microfluidics have limitations that include the specialized skills required to fabricate the chips and the potential issues like air bubbles, which can disrupt laminar flow and affect cell distribution[120].

3D bioprinting is an emerging technology that enables the creation of *in vitro* 3D structural models using biological units (cells, proteins, DNA, etc.) and biological materials. This approach allows for the fabrication of models with precise architecture, composition, and high reproducibility, addressing the limitations of conventional organoids formed by stem cell proliferation, differentiation, and self-assembly. 3D bioprinting can control the number and type of cells, as well as the microenvironment, by simultaneously printing

multiple cell components, extracellular matrixes, and growth factors. Indeed, 3D bioprinting models allow for 4D manipulation - adding time as a fourth dimension - critical for studying the dynamics of growth factors, drugs, or metastatic processes[120–122].

In brief, the use of various 3D *in vitro* models has significantly advanced tumor immunology research. These models are expected to continue evolving, providing increasingly comprehensive simulations of the tumor microenvironment.

5. Immunomodulation for Successful Anti-Cancer Therapies

Cancer is a complex and heterogeneous disease that requires integrated therapeutic approaches[123]. Meanwhile, the immune system plays a critical role in defending against pathogenic infections and maintaining homeostasis by mounting a balanced response to threats. However, an excessive immune response can lead to a self-perpetuating cycle with severe consequences, including chronic inflammatory conditions and autoimmune diseases. Thus, moderating the immune response is essential, as evidenced by the increasing use of immunomodulation in clinical settings[124].

Indeed, immunomodulation, which involves the strategic manipulation of the immune system, is emerging as an approach to enhance the effectiveness of anti-cancer therapies. The immune system is a complex network of cells, tissues, and organs that protect the body from harmful agents, including cancer cells[124]. Immunomodulation seeks to modify the immune response to either amplify its activity or suppress it, when necessary, thereby improving the body's ability to combat cancer[123,125]. Advances in understanding these networks have enabled more targeted treatments in oncology, such as enhancing T cells' ability to recognize tumor cells and inactivating specific proinflammatory factors with monoclonal antibodies[124].

Today, therapeutic methods or drugs known as immune modulators are used to promote immunomodulation. These agents vary in mechanisms, indications, and side effects, necessitating tailored selection based on individual patients. Key types of immune modulators include immune

checkpoint inhibitors, adoptive cellular therapy, cytokines, tumor vaccines, immune cell therapies, small molecules, and immune adjuvants[123].

As explained earlier in this introduction, immune checkpoint inhibitors enhance the immune system's ability to attack tumors by blocking inhibitory signals between tumor cells and immune cells. Prominent immune checkpoints targeted in cancer therapy include CTLA4, PD-1, and PD-L1, which have shown efficacy in various cancers by restoring immune cell activation and cytotoxic functions. These agents primarily enhance immune responses, reduce immunosuppressive factors, and adjust immune balance. [123,125–127].

Another promising approach in immunomodulation is the adoptive transfer of genetically modified lymphocytes for treating hematological malignancies. This method has produced impressive results in patients with advanced-stage cancers previously considered untreatable, and it is expected to become a standard treatment for more patients. The objective of adoptive T-cell therapy is to create a potent immune-mediated anti-tumor response through the *ex vivo* manipulation of T cells. This can be achieved by selecting and expanding tumor-infiltrating lymphocytes (TILs) or by introducing a synthetic T-cell receptor (sTCR) or a chimeric antigen receptor (CAR) into T cells via gene transfer[126].

Furthermore, small molecule-based immunomodulators represent a promising alternative in cancer therapy development. Immunotherapy has traditionally relied on antibody-based approaches due to their high specificity and efficacy. However, these therapies have significant limitations, including poor tissue penetration, long half-lives, lack of oral bioavailability, and immunogenicity that can lead to adverse effects. In response to these challenges, researchers are increasingly turning to small molecules as potential immunotherapeutic agents. Small molecules generally have better pharmacokinetic properties, including reasonable oral availability, improved tissue penetration, and the ability to target intracellular molecules. They can often be more easily modified to optimize their pharmacokinetics and pharmacodynamics, potentially leading to more effective and less toxic treatments. Additionally, small molecules are typically less expensive to

produce than antibodies and can complement antibody-based therapies, offering synergistic effects when used in combination[127].

Also, cytokines are protein molecules that can be used to bolster immune cell activity and promote immune responses. Common cytokines used in cancer therapies include interferons (such as interferon-α and interferon-γ) and interleukins (such as IL-2 and IL-12). These agents activate immune cells, increase tumor-specific T cells, and enhance cytotoxicity against tumor cells[125].

Moreover, tumor vaccines, which stimulate specific immune responses to recognize and eliminate tumor cells, are another form of immunomodulators. These vaccines may contain tumor-associated antigens, whole tumor cells, or their products, aiming to prime the immune system against cancer cells. This approach holds promise in preventing recurrence and achieving long-term remission[123,125,126].

Research is also exploring the role of the microbiome in regulating immune responses and its potential in enhancing immunotherapies[123,128]. However, it is important to note that, independently of their nature, immunomodulators can cause immune-related toxic reactions, such as autoimmune diseases, necessitating careful monitoring and management[123]. The therapeutic efficacy of immunomodulators also varies among patients, with some exhibiting resistance due to tumor immune escape mechanisms. Developing new immunomodulators is critical for enhancing cancer therapy efficacy and reducing side effects. Gene editing technologies, such as CRISPR-Cas9, offer new possibilities for modifying immune cells to better target cancer[123]. Also, techniques like gene testing and genetic sequencing enable the development of precise treatment plans, enhancing the efficacy of targeted therapies and tailoring treatments based on individual patient characteristics, such as genetic background and immune status[123].

Combining immunomodulators with other treatments, such as chemotherapy, radiation therapy, and surgery, also represents a comprehensive strategy to enhance treatment efficacy. Immunomodulators can mitigate the side effects of chemotherapy and radiation, improving patient tolerance and outcomes. In addition, immunomodulators hold

potential in cancer prevention and early diagnosis. By enhancing immune surveillance, they can help clear pre-cancerous lesions and detect early-stage cancers, reducing the development of malignancies and improving early detection rates[123].

Thus, immunomodulation offers a promising approach to cancer therapy. While challenges remain, ongoing research and technological advancements continue to refine these therapies, aiming for more precise, effective, and personalized cancer treatments. Effectively, in the recent years, the use of immunotherapy has gained significant importance in cancer research and clinical oncology. However, despite this progress, not all patients benefit equally from immunotherapy. Some achieve long-term remission, while others relapse or fail to respond. Additionally, the efficacy of immunotherapy varies across different tumor types. Therefore, it is crucial to identify patients likely to benefit from these therapies and develop improved therapeutic strategies[128].

Keeping this in mind, this thesis delves into the therapeutic potential of HLA-DR-expressing CTLs and formulates strategies to enhance their presence in breast cancer (BC) patients. We anticipate that this immunomodulation approach will provide more tailored therapeutic options for BC, as better explained in this thesis's subsequent section.

6. Unmet Needs, Thesis Rationale and Aims

Breast cancer (BC) is the most prevalent cancer among women globally and ranks as the second most common cancer overall[1]. Despite significant advances in BC treatment, including the development of various chemotherapeutic and immunotherapeutic agents, unmet needs remain, particularly in predicting and improving patient response to therapies[129]. Indeed, current clinical practices do not fully address the variability in patient responses to treatment, leading to suboptimal outcomes for BC patients. Conventional BC treatments and newer modalities like immunotherapy frequently confront challenges such as the development of resistance mechanisms.[95,130]. Neoadjuvant chemotherapy (NACT), widely utilized for high-

risk, locally advanced, or inoperable BC tumors, exemplifies these challenges, with fewer than 50% of patients achieving complete tumor eradication. This underscores the urgent need for more accurate and effective treatments for chemotherapy-resistant tumors[131]. Furthermore, while immune checkpoint inhibitors (ICIs) targeting PD-1/PD-L1 have transformed the management of advanced or metastatic PD-L1 positive triple-negative breast cancer (TNBC)[132], their efficacy varies significantly across patients and BC subtypes[133]. This highlights the necessity to expand the scope of immunotherapy to encompass all BC subtypes, particularly in early-stage disease[97]. Moreover, CAR-T cell therapy, although successful in hematologic cancers, has demonstrated limited effectiveness and feasibility in treating solid tumors like BC, lacking FDA-approved therapies.[134,135] Adoptive T cell therapy (ATC) also holds promise but faces barriers such as high costs, technical complexities, and potential toxicities associated with excessive cytokine release[136].

Building on the concept that HLA-DR-expressing cytotoxic T lymphocytes (CTLs) play a crucial role in the tumor microenvironment by aiding in the elimination of cancer cells and exhibiting potent anti-tumor properties, we previously proposed that increasing the presence of CTLs with elevated HLA-DR expression could revolutionize therapeutic approaches for BC[137]. Thus, we now undertake a deeper exploration of the therapeutic potential of HLA-DR-expressing CTLs and the development of strategies to augment their frequency during T lymphocytes *ex vivo* expansion protocols. This approach seeks to enhance anti-tumor efficacy and refine BC therapies, contributing to enhance the efficacy of personalized treatments.

So, given the gaps in the field outlined above and following the rationale described, this thesis aims to exploit HLA-DR expression in CTLs to enhance anti-tumor immunity, anticipating the development of more effective T cell-based therapies for BC. Namely, we intended to investigate the mechanisms underlying HLA-DR-mediated CTLs' superior activation and cytotoxicity and find strategies to increase the frequency of HLA-DR-expressing CTLs during the preparation of those cellular therapies. Ultimately, our goal is to improve treatment outcomes for BC patients.

More specifically, the work described in this thesis is structured into several aims:

- **Aim 1: To validate the predictive and therapeutic value of HLA-DR-expressing CTLs, substantiating their extraordinary anti-tumor abilities.** This aim encompasses both *in vivo* and *ex vivo* experiments. In our previous work, we identified HLA-DR levels in CTLs, assessed in biopsies, as a highly sensitive, specific, and independent biomarker for predicting the response of BC to NACT. We also developed a predictive probability of response algorithm and demonstrated a positive correlation between HLA-DR levels in tumor-infiltrating CTLs and HLA-DR levels in systemic CTLs. In this study, we utilized a new cohort of BC patients undergoing NACT, according to clinically established criteria, to validate our predictive model and corroborate the therapeutic value of HLA-DR-expressing CTLs. For this part, we established collaborations with hospitals to obtain biopsies and blood samples from BC patients. Additionally, to further demonstrate the anti-tumor effect of HLA-DR-expressing CTLs, we conducted *in vivo,* and *ex vivo* experiments. In our *in vitro* studies, we stimulated peripheral blood mononuclear cells (PBMCs) isolated from BC patients' blood and co-cultured them into spheroids of the BC cell line MDA-MB-231. Furthermore, in collaboration with Fior Lab (Champalimaud Foundation), we utilized xenografted zebrafish models bearing human BC tumors. These models were injected with PBMCs from BC patients with a high frequency of CTLs expressing high levels of HLA-DR, compared to PBMCs from donors with a low frequency of these cells. These experiments aimed to demonstrate the robust anti-tumor capabilities of HLA-DR-expressing CTLs.

- **Aim 2: To investigate the role of HLA-DR in CTLs and the interplay between CTLs and CD4+ T Cells.** This segment explores the significance of HLA-DR in CTLs' anti-cancer responses and its potential therapeutic implications. To achieve this, we conducted experiments investigating the activation and cytotoxic profiles of stimulated CTLs when HLA-DR was inhibited using a blocking antibody. Additionally, to

understand the interplay between CTLs and CD4+ T cells, we conducted experiments with PBMCs depleted of CD4+ T cells. The results described in this section were achieved by flow cytometry analysis and aimed to provide deeper insights into the mechanisms underlying CTLs-mediated anti-tumor responses and inform for the development of more effective immunotherapeutic strategies.

- **Aim 3: To optimize T cell expansion protocols to enhance HLA-DR expression and cytotoxic capabilities of CTLs.** Cellular therapy involving the expansion of T cells has emerged as a promising avenue to fight cancer, harnessing the body's own immune system to target and eliminate cancer cells[138,139]. In this context, we explored the dynamics of T cell expansion with a specific focus on the analysis of HLA-DR-expressing CTLs.

- **Aim 4: To identify strategies to augment CTLs' HLA-DR expression and enhance their cytotoxicity against BC cells.** CTLs play a pivotal role in the immune response against cancer cells, and enhancing their cytotoxic capacity is crucial for effective cancer immunotherapy. Here, we utilized our established 3D co-culture model as a screening platform[140] to evaluate potential immune-based agents aimed at augmenting HLA-DR expression on CTLs and consequently enhancing their cytotoxicity against BC cells. This part of the research builds on our previous findings that HLA-DR-expressing CTLs exhibited more effective anti-tumor properties. Additionally, in this section, we explored the potential of metabolic modulation, specifically the manipulation of the glycolysis pathway to influence HLA-DR expression of CTLs and improve their functionality.

- **Aim 5: To explore the influence of anti-PD-1 treatment on HLA-DR expression in CTLs.** This part of the work employs bioinformatic analyses, performed in collaboration with LabCHO from Universidade Federal do Paraná, Brazil, to elucidate how anti-PD-1 treatment might influence HLA-DR expression in CTLs. Through *in silico* analysis of single-cell RNA sequencing (scRNA-seq) data from BC patients, we

analyzed gene expression data from CTLs to explore the potential regulatory networks involving the *HLA-DR* and *PD-1* genes.

To conclude, in the **Chapter IV**, we will summarize the major findings of this work, discuss its contributions to the field, and identify avenues for future research, acknowledging the limitations of the current work.

CHAPTER II

Materials and Methods

1. Human Samples

This study used immune cells isolated from the buffy coats of healthy donors, provided by the Instituto Português do Sangue e da Transplantação (IPST). Additionally, samples were used from a cohort of 234 patients diagnosed with breast cancer (BC) sourced from Hospital CUF Descobertas, Hospital CUF Tejo, Instituto Português de Oncologia de Lisboa Francisco Gentil, Hospital de Vila Franca de Xira and Hospital Prof. Doutor Fernando Fonseca. Various types of samples, namely biopsies, whole blood, and buffy coats, were collected, and processed according to the methods described below. As detailed in Table II-1, biopsies were obtained from 195 BC patients selected for neoadjuvant chemotherapy (NACT). Blood samples were collected from 14 patients with advanced or metastatic BC and 25 patients prior to NACT. Additionally, PBMCs were isolated from the buffy coats of 32 healthy donors.

Table II-1: Overview of participant characteristics enrolled in the study. Median values for age and body mass index (BMI) are included, along with key tumor characteristics such as dimensions and Ki67 levels (related to tumor proliferation rate). Additionally, other clinical data including breast cancer subtype, node status, and grade are summarized.

	Patients			**Healthy Donors**
	Neoadjuvant Breast Cancer		**Advanced Breast Cancer**	
Number of subjects	195	25	14	32
Sample type	Biopsies	Blood	Blood	PBMCs
Age	Median: 57 (Range: 29 - 90)	Median: 51 (Range: 34 - 76)	Median: 58 (range: 47 - 84)	range: 18 – 65
Body Mass Index (BMI)	Median: 26.45 (range: 19 - 47)	Median: 26.91 (range: 21 – 39)	Median: 26.06 (range: 18 - 36)	NA
Subtype:				
• **ER+ (PR –/+)**	48.72%	40.00%	71.42%	NA
• **HER2+**	37.95%	35.00%	14.29%	NA
• **TNBC**	13.33%	25.00%	14.29%	NA

	Patients			Healthy Donors
	Neoadjuvant Breast Cancer		Advanced Breast Cancer	
Tumor dimension (mm)	Median: 32.91 (range: 6 - 200)	Median: 38.53 (range: 6 - 110)	Median: 57.92 (range: 2.5 - 160)	NA
Ki67	Median: 35 (range: 1 - 97)	Median: 58 (range: 10 - 98)	Median: 40 (range: 19 - 90)	NA
Axillary lymph node invasion status	Negative – 62.57% Positive – 37.43%	Negative – 57.89% Positive – 42.11%	Negative – 50.00% Positive – 50.00%	NA
Grade	G1 - 20.29% G2 - 51.49% G3 - 28.22%	G1 – 8.00% G2 – 20.00% G3 – 44.00% Not know – 28.00%	G1 – 0.00% G2 – 35.72% G3 - 28.57% Not know – 35.71%	NA
Treatment:				
• **NACT**	100%	100%	35.71%	NA
• **Other treatments**	NA	NA	64.29%	

*NA: non-applicable.

1.1. Biopsies

Biopsies obtained before any treatment were immediately placed in saline solution and subsequently transferred to Transfix (Cytomark), a stabilizing solution that preserves cellular antigens and fixes cells. Within 24 hours of collection, the samples were processed using a series of steps to ensure optimal cell viability and preparation for immunophenotyping by flow cytometry.

First, the biopsies underwent mechanical tissue dissociation using a BD Medicon (BD Biosciences), which facilitated the breakdown of tissue into a single-cell suspension. This suspension was then carefully filtered through a 30 µm mesh (Sysmex) to remove any remaining tissue fragments and ensure a uniform cell size distribution. Following filtration, the cell suspension was centrifuged at 300g for 5 minutes to pellet the cells. The supernatant was

discarded, and the cell pellet was resuspended in 1x Phosphate-Buffered Saline (PBS) to maintain the cells in an isotonic environment suitable for further analysis. This prepared cellular suspension was then used for immunophenotyping by flow cytometry, allowing for detailed characterization of the cell populations present in the biopsy samples.

1.2. Whole Blood

Approximately 9 mL of whole blood were collected in Vacutainer tubes with ethylenediaminetetraacetic acid (EDTA, Vacutest). An aliquot was promptly prepared for immunophenotyping by flow cytometry to ensure a timely and accurate analysis of cell populations. To obtain the plasma fraction, the whole blood was centrifuged at 2000 rpm for 20 minutes. The plasma was then frozen at -80°C until further use. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood by carefully layering the blood on top of a ficoll solution (Sigma Aldrich) at a 3:5 ratio (ficoll:blood). This mixture was centrifuged at 2000 rpm for 20 minutes without brake. After centrifugation, the blood components were separated (Figure II-1), and the PBMCs layer was carefully isolated using a pasteur pipette into a new falcon tube. The cells were then washed with 1X PBS to remove any remaining ficoll and other impurities, by centrifuging at 1100 rpm for 5 minutes. The number of PBMCs was counted using a Neubauer chamber and then cryopreserved in a solution containing 90% fetal bovine serum (FBS, Biowest) and 10% dimethyl sulfoxide (DMSO, Sigma Aldrich). This cryopreservation step is crucial for maintaining cell viability and functionality for subsequent use in 3D co-culture systems and other experimental applications.

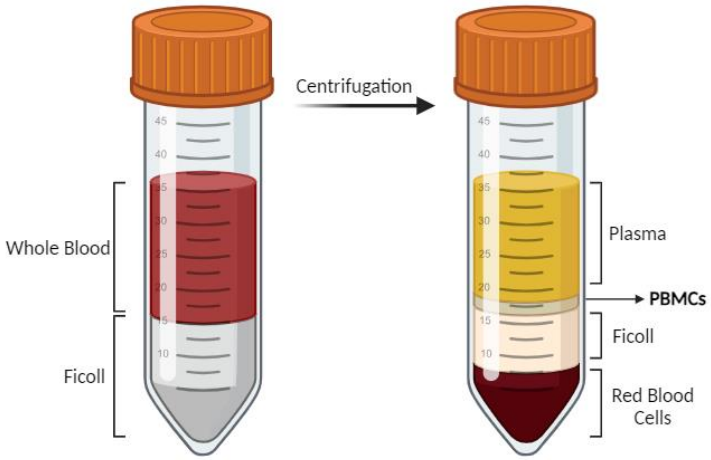


Figure II-1: Diagram illustrating the fractionation of whole blood after centrifugation. The process results in the formation of four distinct layers: the top layer consists of plasma, the superior middle layer contains peripheral blood mononuclear cells (PBMCs), the inferior middle layer is composed of ficoll, and the bottom layer comprises red blood cells. Figure created in BioRender.

1.3. Peripheral blood mononuclear cells (PBMCs) obtained from buffy coats

Buffy coats from 32 healthy donors provided by the Instituto Português do Sangue e da Transplantação were used. Each buffy coat was divided into three 50 mL falcon tubes (25 mL each), diluted in a 1:2 ratio with PBS 1X, and centrifuged at 2500 rpm for 10 minutes without brake. Post-centrifugation, a leukocyte-enriched layer was formed and carefully transferred to a single falcon tube. The volume was adjusted to 20 mL with 1X PBS. This leukocyte suspension was then layered on top of 12 mL of ficoll (Sigma Aldrich) using a pasteur pipette and centrifuged at 2500 rpm for 20 minutes without brake. Then, a distinct layer of PBMCs was visible and transferred to a new falcon tube using a pasteur pipette, followed by two washes with PBS 1X in which the PBMCs were centrifuged: first at 1400 rpm for 10 minutes with the brake applied, and for the second wash, at 800 rpm for 5 minutes also with the brake applied. The number of PBMCs was counted and cryopreserved at -80°C until further use.

2. Ethics Statement

This study was approved by the Ethics Committees of Hospital CUF Descobertas, Hospital CUF Tejo, Instituto Português de Oncologia de Lisboa Francisco Gentil, Hospital de Vila Franca de Xira and Hospital Prof. Doutor Fernando Fonseca. Approval was also obtained from Instituto Português do Sangue e da Transplantação (IPST) and NOVA Medical School. Participants aged 18 years or older were recruited voluntarily and provided informed consent after understanding the nature of the study (Appendix I). Written informed consent was obtained from all participants (Appendix II).

Blood and biopsies were collected as part of routine clinical procedures, without affecting the participants' diagnosis or treatment. Sample processing was conducted exclusively at NOVA Medical School in accordance with the principles outlined in the Declaration of Helsinki. The study also adhered to national legislation governing the scientific use of human biological samples (Law n. 12/2005 and Decree-Law n. 131/2014). Clinical data were managed in compliance with both EU and Portuguese legislation, anonymized, and securely stored in accordance with the EU's Data Protection Directive (Directive 95/46/EC) and Portuguese Legislation (Law n. 67/98).

3. Breast Cancer Cell Lines

In vitro experiments were carried out primarily using the triple negative breast cancer (TNBC)-derived cell line MDA-MB-231. Additionally, some experiments were conducted with other BC cell lines, covering different BC subtypes. Specifically, the BC cell lines BT-474 (HER2+ subtype), HCC1806 (TNBC subtype), Hs578T (TNBC subtype) and MCF-7 (ER+ subtype) were used. The Hs578T, MCF-7, and MDA-MB-231 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS (Biowest) and 1% Penicillin/Streptomycin (GE Healthcare) to support optimal growth. For the BT-474 and HCC1806 cell lines, RPMI 1640 (Gibco) supplemented with 10% FBS and 1% Penicillin/Streptomycin was used. Insulin (Sigma Aldrich) at 10µg/mL was included in the culture media for BT-474, Hs578T and MCF-7 cells to enhance proliferation. The cells were grown in monolayer cultures in

T75 flasks under controlled humidified conditions at 37°C with 5% CO_2. The cultures grew until they achieved 80-90% confluency. Upon reaching the desired confluency, cells were passaged by detachment using TrypLE (Gibco), a gentle enzymatic dissociation reagent that aids in cell harvesting while maintaining cell viability and integrity.

4. Establishment of 3D co-cultures

A 3D co-culture involving BC cell lines and PBMCs on agarose-coated plates was implemented, following a protocol we have previously established[140]. This protocol involves a scaffold-free 3D culture technique with low adherence. To create low adherence conditions, 1.5% agarose (Invitrogen) in MilliQ H_2O was autoclaved, then placed in a 96-well plate (50 µL per well) and allowed to cool for approximately 20 minutes. Subsequently, 1000 BC cells in 200 µL of supplemented DMEM were added to the agarose-coated wells. The following day, 100 µL of the medium was replaced with fresh medium. After the first day, medium changes were conducted every two days (Figure II-2).

After six days (for MDA-MB-231 BC cell line), spheroids were fully formed. Then, 100 µL of medium was removed and replaced with 100 µL of a PBMCs suspension (containing 3000 cells) in supplemented RPMI 1640, achieving a 3:1 ratio of PBMCs to BC cells. PBMCs, obtained as described above in this chapter (sections 1.2 and 1.3), were added unstimulated or previously stimulated. Indeed, before co-culture, PBMCs were stimulated through TCR engagement using mouse anti-human CD3 (5 µg/mL), anti-CD28 (1 µg/mL), and rat anti-mouse IgG1 (5 µg/mL) as the crosslinking antibody (Biolegend) for 48 hours. Additionally, interleukin-2 (IL-2) at 100 UI/mL and interleukin-12 (IL-12) at 20 ng/mL (PeproTech) were added to the PBMCs monoculture during the last 24 hours of the stimulation protocol.

After 24 hours of co-culture with the BC cell lines, PBMCs successfully infiltrated the cancer spheroid structure (Figure II-2). To confirm PBMCs infiltration into the spheroid, in a few experiments, PBMCs were stained with 5 µM CellTracker™ Orange CMTMR (Invitrogen) for 30 minutes in

unsupplemented RPMI at 37°C, followed by replacement with fresh supplemented RPMI. Tumor cells were stained with 5 µM CellTrace™ CFSE (Invitrogen). Briefly, cells in suspension in PBS 1X were stained for 20 minutes at 37°C, followed by incubation in the supplemented medium at 37°C for 5 minutes. Cells were then centrifuged at 1100 rpm for 5 minutes and resuspended in supplemented DMEM. PBMCs' infiltration into the spheroid was assessed 24 hours later using a Zeiss Axiovert 40 microscope.

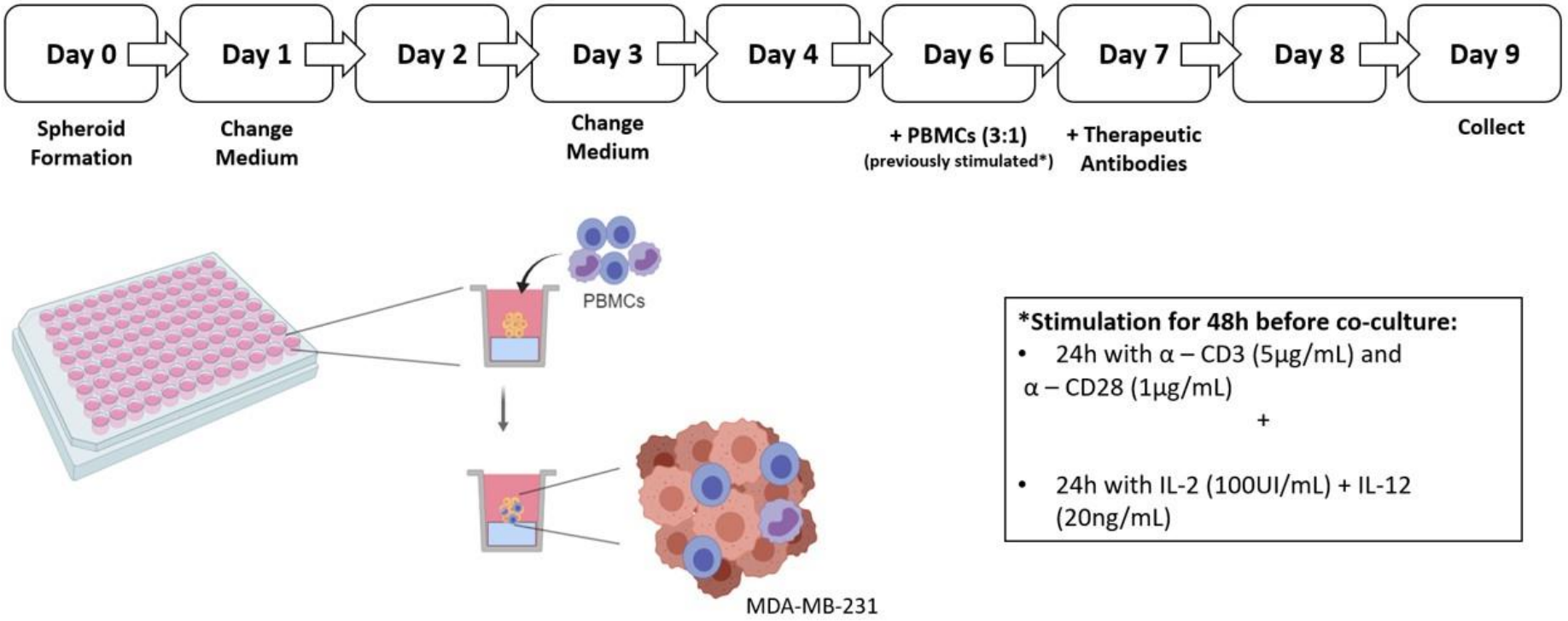


Figure II-2: A 3D co-culture model involving breast cancer (BC) cell line and peripheral blood mononuclear cells (PBMCs) on agarose-coated plates. The scaffold-free, low-adherence culture was established by coating wells with 1.5% agarose. BC cells were seeded into the agarose layer, and spheroids formed after six days. PBMCs, either unstimulated or pre-stimulated, were then added to the spheroids at a 3:1 PBMC to BC cell ratio. After 24 hours, PBMCs successfully infiltrated the spheroid structure. In some experiments, therapeutic antibodies were introduced post-infiltration. After 48 hours, spheroids were dissociated and analyzed for cancer cell viability using flow cytometry. Figure created with the help of BioRender.

In some experiments, therapeutic antibodies were introduced into the 3D co-culture once PBMCs had infiltrated the spheroid (Figure II-2). Subsequently, after another 48 hours, spheroids were removed from the plate, dissociated by pipetting, and stained with Fixable Viability Dye (BD Biosciences) to evaluate the percentage of viable cancer cells by flow cytometry. A pan-leukocyte marker, anti-CD45 (clone HI30, Biolegend), was also used to distinguish between tumor and immune cells. The gating strategy applied to evaluate the viability of cancer cells is described in Figure II-3.

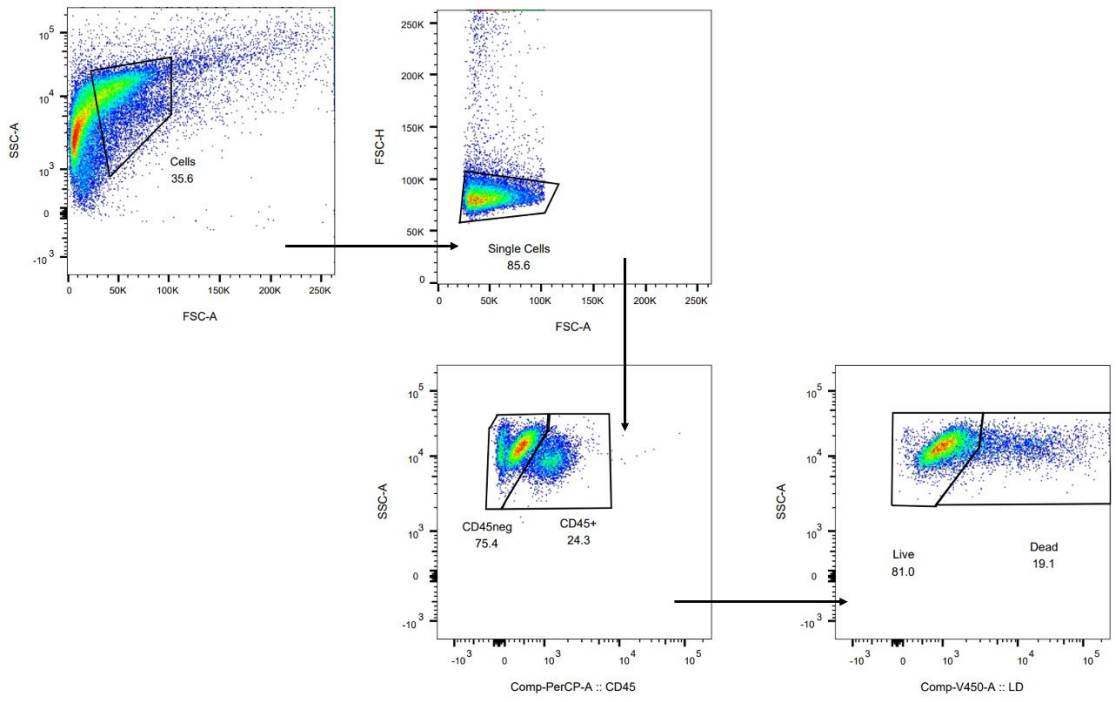


Figure II-3: Gating Strategy for Evaluating Cancer Cell Viability in 3D Co-Cultures. After co-culturing breast cancer (BC) cell line spheroids with peripheral blood mononuclear cells (PBMCs), spheroids were dissociated and analyzed via flow cytometry. Cells were stained with Fixable Viability Dye to assess the viability of cancer cells. Anti-CD45 was used as a pan-leukocyte marker to distinguish tumor cells from immune cells. The figure details the gating strategy used to evaluate the percentage of viable cancer cells.

5. Flow cytometry

5.1. Staining

For flow cytometry analysis, biopsies, blood samples, or BC cells-PBMCs 3D structures were stained with a cocktail of monoclonal mouse anti-human conjugated antibodies (mAbs) after being processed to a single cell suspension. In the case of blood samples, the protocol included an additional step of red blood cell lysis using RBC lysis buffer (Biolegend) in the dark for 20 minutes at 4°C. Briefly, the staining protocol involved incubating cells with extracellular mAbs for 15 minutes at room temperature, followed by washing with 1 mL of PBS 1X and centrifugation at 300g for 5 minutes. Whenever intracellular markers were analyzed, cells were fixed and permeabilized with Fix/Perm kit (eBioscience) for 30 minutes at room temperature. Intracellular mAbs were added for 30 minutes, followed by a wash step with 1 mL of PBS 1X and centrifugation at 300g for 5 minutes.

The mAbs employed included: anti-CD3-PercP (clone HIT3a), anti-CD3-APC (clone UCHT1), anti-CD25-PE (clone BC96), anti-CD4-FITC (clone OKT4), anti-CD8-PE (clone HIT8a), anti-CD8-PacificBlue (clone HIT8a), anti-CD15-PE (HI98), anti-HLA-DR-APC (clone L243), anti-Granzyme B-FITC (clone QA16A02), anti-CD127-PE-Cy7 (A019D5), anti-CD69-PercP (clone FN50), anti-CD45-PercP (clone HI30), all from Biolegend. Cell viability was determined with BD Horizon™ Fixable Viability Stain 450 (BD Biosciences) incubated with the cell suspension for 20 minutes in the dark at 4°C.

Data acquisition was conducted using BD Fluorescence Activated Cell Sorting (FACS) Canto II with FACSDiva Software v8 (BD Biosciences), and the results were analyzed using FlowJo software v10.

5.2. Gating Strategy

Using the mAbs previously listed, the immunophenotyping applied to blood samples and biopsies involved the analysis of different immune populations. These populations were defined as follows: cytotoxic T lymphocytes (CD3+/CD8+), helper T lymphocytes (CD3+/CD4+), regulatory T lymphocytes (CD4+/CD25^{high}/CD127^{low}), and neutrophils (CD15+). The gate strategy applied is presented in Figure II-4.

For other immune markers analyzed within T cells, namely HLA-DR, CD25, CD69, IFN-γ, and Granzyme B, data are generally presented as a percentage of the positive population concerning the single cells' gate or to the positive population for a given marker within the T cells.

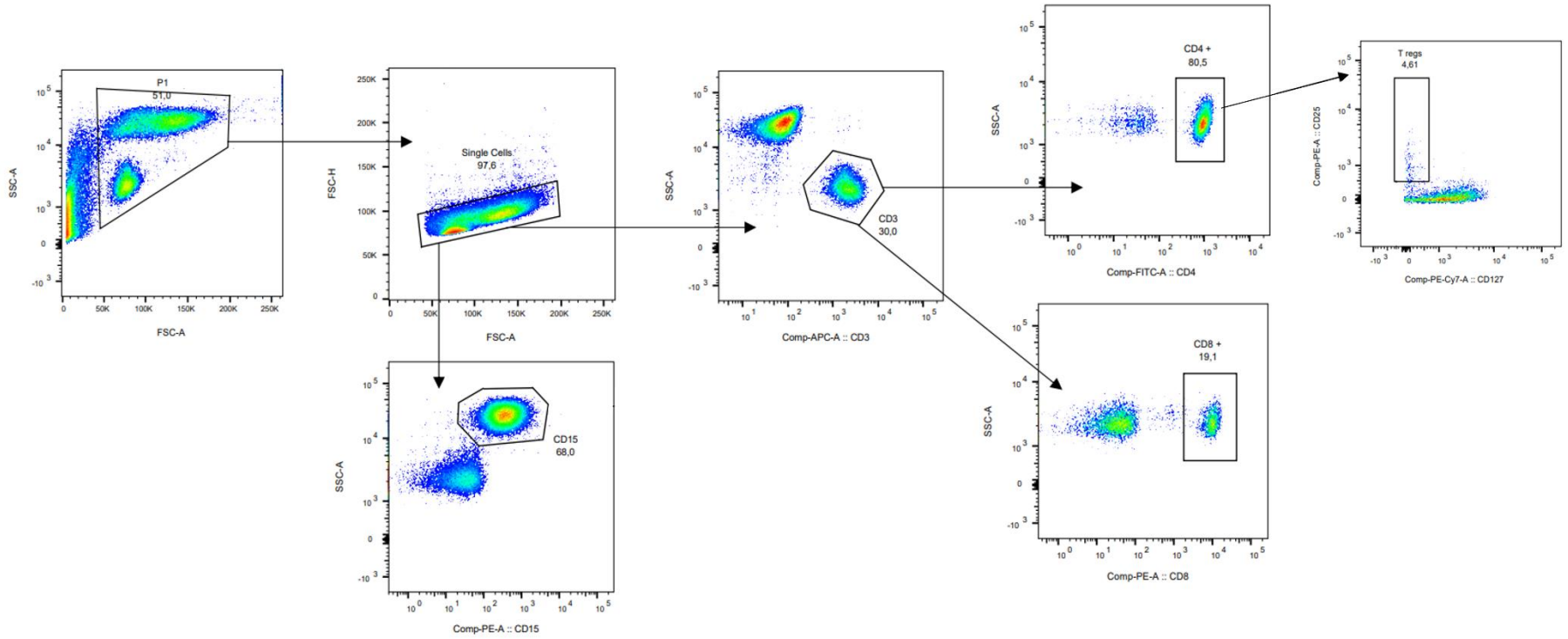


Figure II-4: Gating strategy for immunophenotyping of blood and biopsy samples using monoclonal antibodies (mAbs). The analysis identifies distinct immune cell populations, including cytotoxic T lymphocytes (CD3+/CD8+), helper T lymphocytes (CD3+/CD4+), regulatory T lymphocytes (CD4+/CD25high/CD127low), and neutrophils (CD15+).

6. Fluorescence-activated cell sorting (FACS)

Fluorescence-activated cell sorting (FACS) was performed using the FACS Aria III system (BD Biosciences) for two distinct experimental purposes: isolating CD25+/HLA-DR- and CD25+/HLA-DR+ cells to assess the role of HLA-DR in enhancing CTLs-mediated tumor cell elimination; and depleting CD4+ T cells in PBMCs fractions to evaluate the role of HLA-DR in antigen presentation.

To deplete CD4+ T cells, PBMCs were initially stained with anti-CD45-PercP (clone HI30) and anti-CD4-FITC (clone OKT4) antibodies. The cells were incubated with the antibodies for 20 minutes in the dark at room temperature, followed by a wash step with PBS 1X. Subsequently, the cells were resuspended in PBS 1X and filtered through a 0.2 µm mesh (Sysmex). The cells were then sorted into two distinct populations: CD45+/CD4+ and CD45+/CD4- (CD4 negative). The gating strategy used for this sorting process is detailed in Figure II-5.

In a separate set of experiments, PBMCs were also used. These cells were sorted to isolate specific T cell subpopulations based on the expression of CD25 and HLA-DR. The staining procedure was similar to the one described above but with anti-CD25-PE (clone BC96) and anti-HLA-DR-APC (clone L243) antibodies. This enabled the separation of cells into CD25+/HLA-DR- and CD25+/HLA-DR+ populations. The gating strategy utilized in these experiments is also described in Figure II-6.

In both experimental setups, cell debris, aggregates, and dead cells were removed. The cells were sorted with maximum stringency to prevent cross-contamination between cell populations. The FACS process achieved an efficiency of over 90%, ensuring highly purified cell populations for downstream analyses.

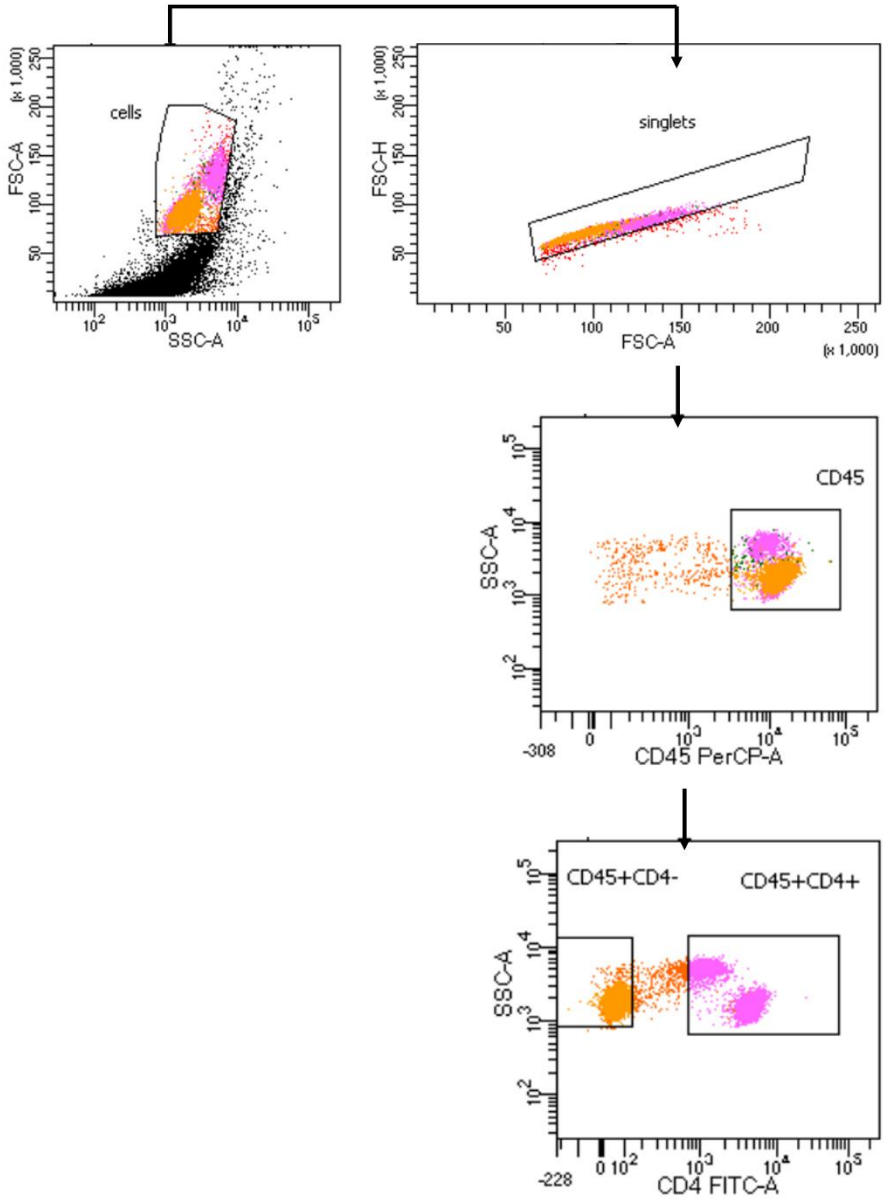


Figure II-5: Fluorescence-activated cell sorting (FACS) gating strategy for isolating peripheral mononuclear cells (PBMCs) with and without CD4+ T cells. Peripheral blood mononuclear cells (PBMCs) were stained with anti-CD45-PercP and anti-CD4-FITC antibodies. The figure details the gating process used to separate and isolate CD45+/CD4+ and CD45+/CD4- cell populations.

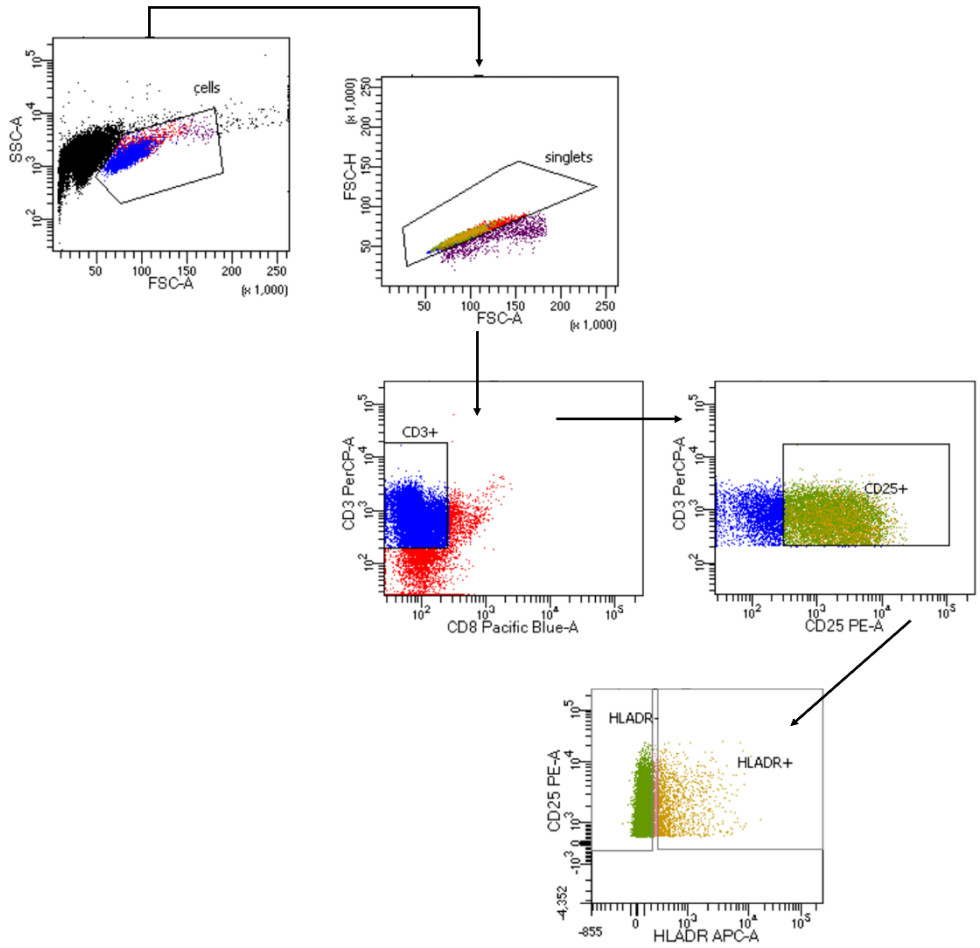


Figure II-6: Gating strategy for fluorescence-activated cell sorting (FACS) to isolate CD25+/HLA-DR- and CD25+/HLA-DR+ subpopulations from PBMCs. *Cells were first stained with anti-CD3, followed by staining with anti-CD25-PE and anti-HLA-DR-APC antibodies. This approach enabled* the separation of these specific subpopulations.

7. Breast Cancer Zebrafish Xenograft Experiments

This part of the work was carried out in collaboration with Rita Fior and Cátia Rebelo de Almeida from the Fior Lab at the Champalimaud Foundation.

7.1. Animal Care and Handling

In vivo experiments were performed in zebrafish model (*Danio rerio*) *Tg(fli1:GFP)*, which were maintained and handled in accordance with European Animal Welfare Legislation and Champalimaud Fish Platform Program.

7.2. Cell Culture

Human breast cancer cell line Hs578T was cultured in filtered Dulbecco's Modified Eagle Medium (DMEM) High Glucose (Biowest), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma Aldrich), 1% Penicillin-Streptomycin (P/S) (10,000 U/mL) (Hyclone), and insulin at a concentration of 10 µg/mL. Cells were maintained in an incubator (inCu Safe) with a humidified atmosphere containing 5% CO_2 at 37°C.

To facilitate subsequent visualization of the Hs578T cancer cells, we used Hs578T cell line expressing TdTomato fluorescent protein via lentiviral mediated transduction. Cells were transduced using various dilutions of lentiviral vectors. Following transduction, the cells were expanded, and transduction efficiency was assessed by flow cytometry (BD LSRFortessa™ X-20 cell analyzer - Biosciences). Cells were then sorted (BD FACSAria Fusion) to create a biobank for experiments.

7.3. Hs578T TdTomato and PBMCs processing for zebrafish injection

Frozen PBMCs were thawed at 37°C for 10 minutes. RPMI medium supplemented with 10% FBS was added to the PBMCs at a ratio of three times the volume of the cell suspension. The cells were then centrifuged for 7 minutes at 1700 rpm. After centrifugation, the PBMCs were resuspended in CellTracker™ Deep Red (Thermo Scientific) at a 1:1000 dilution from the stock solution and incubated at 37°C for 10 minutes, followed by an additional 15 minutes incubation at 4°C. The cells were then centrifuged for 7 minutes at

580g, and resuspended in RPMI medium with 10% FBS to a final concentration of 0.50×10^6 cells/µL.

Cells at 70-80% confluence cultured in a T-175 flask were washed with DPBS 1X buffer and dissociated with EDTA 1mM (diluted in DPBS 1X), followed by mechanical detachment with cell scrapers. Cells were centrifuged for 5 minutes at 300g and resuspended in RPMI + 10% FBS to a final concentration of 0.50×10^6cells/µL.

Hs578T TdTomato cells were injected alone or mixed with PBMCs at a ratio of 1:1.

7.4. Zebrafish Xenograft Assay

Zebrafish larvae were anesthetized with Tricaine 1X, and fluorescently labelled cancer cells were microinjected into the perivitelline space (PVS) at 48 hours post fertilization (hpf). The injection procedure followed a previously published protocol[141]. After injection, the xenografts were maintained in embryonic medium (E3) at 34°C. At 24 hours post injection, successfully injected zebrafish xenografts were sorted into classes according to their tumor size, being the E3 media renewed daily, as well as the removal of dead xenografts. At the end of the assay, zebrafish xenografts were sacrificed and fixed in 4% (v/v) formaldehyde (FA) (Thermo Scientific) overnight at 4°C.

On the day after fixation, FA was removed, and the xenografts were permeabilized with PBS-Triton 0.1%. Nuclei were counterstained with DAPI at a final concentration of 0.05 mg/mL and incubated overnight at 4°C. On the next day, DAPI was removed through sequential washes with PBS-Triton 0.1%, and xenografts were mounted between two coverslips using MOWIOL mounting medium. The figure II-7 provides a comprehensive overview of the procedure from injection to visualization.

7.5. Imaging and Quantification

All images were acquired using a Zeiss LSM 710 fluorescence confocal microscope. Tumor size quantification was performed as described in Fior *et al*. 2017[142].

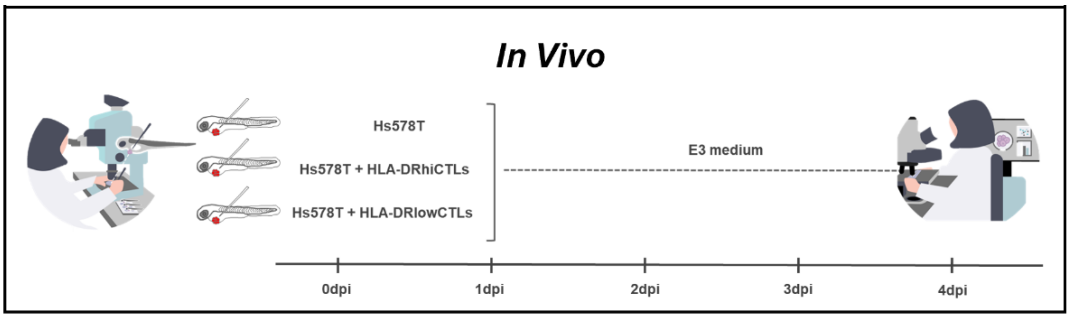


Figure II-7: Schematic representation of the zebrafish xenograft assay methodology. Zebrafish xenograft models were injected with a breast cancer (BC) cell line, Hs578T, alone or in combination with immune cells. The immune cells used were either high HLA-DR cytotoxic T lymphocytes (HLA-DRhiCTLs) or low HLA-DR CTLs. Four days post-injection, the cytotoxic activity of the immune cells was assessed by measuring tumor size in the zebrafish using confocal microscopy.

8. HLA-DR blocking Assay

To comprehensively assess the impact of HLA-DR blockade on the activation and cytotoxic functions of T lymphocytes, and to elucidate the role of HLA-DR in modulating T cell responses and its implications for immune regulation, an HLA-DR blocking assay was designed. In this experiment, the blockade of HLA-DR was investigated using PBMCs obtained from healthy donors and processed as described above in this chapter (section 1.3). These PBMCs were cultured for 72 hours under three distinct conditions: unstimulated, stimulated, and stimulated in the presence of an HLA-DR-blocking antibody (Figure II-8). The TCR stimulation protocol followed the method previously outlined in this chapter (section 4). For HLA-DR blockade, 10 µg/mL of purified anti-human HLA-DR antibody (clone L243, Biolegend) were used.

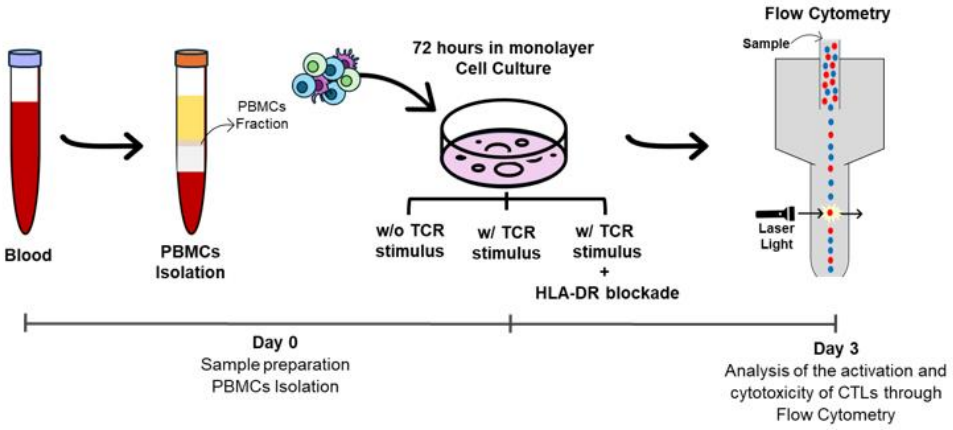


Figure II-8: Experimental setup for assessing the impact of HLA-DR blockade on T lymphocyte activation and cytotoxicity. PBMCs from healthy donors were cultured for 72 hours under three conditions: unstimulated, stimulated, and stimulated with the addition of an HLA-DR-blocking antibody.

Following the 72-hour culture period, cells were harvested and labeled with specific monoclonal antibodies to evaluate the activation status of cytotoxic T lymphocytes (CTLs), employing the gating strategy detailed in Figure II-9. The antibodies utilized included anti-CD25 (clone BC96, Biolegend), anti-CD69 (clone FN50, Biolegend), and anti-Granzyme B (clone QA16A02, Biolegend). Anti-CD69 serves as an early activation marker, indicating initial T cell activation, while anti-CD25 identifies activated T cells, typically upregulated 12-24 hours post-activation. Anti-Granzyme B was used to detect the presence of this serine protease, critical for cytotoxic granule function in T cells, indicating their capacity to induce apoptosis in target cells. To measure intracellular levels of Granzyme B, Brefeldin A (BioLegend) was added during the last 4 hours of culture to inhibit its extracellular transport, ensuring an accurate assessment of Granzyme B within the cells.

In control experiments, Purified Mouse IgG1, κ Isotype Control Antibody (clone MOPC-21, Biolegend) was applied to confirm that observed effects on activation and cytotoxicity were specifically induced by HLA-DR blocking.

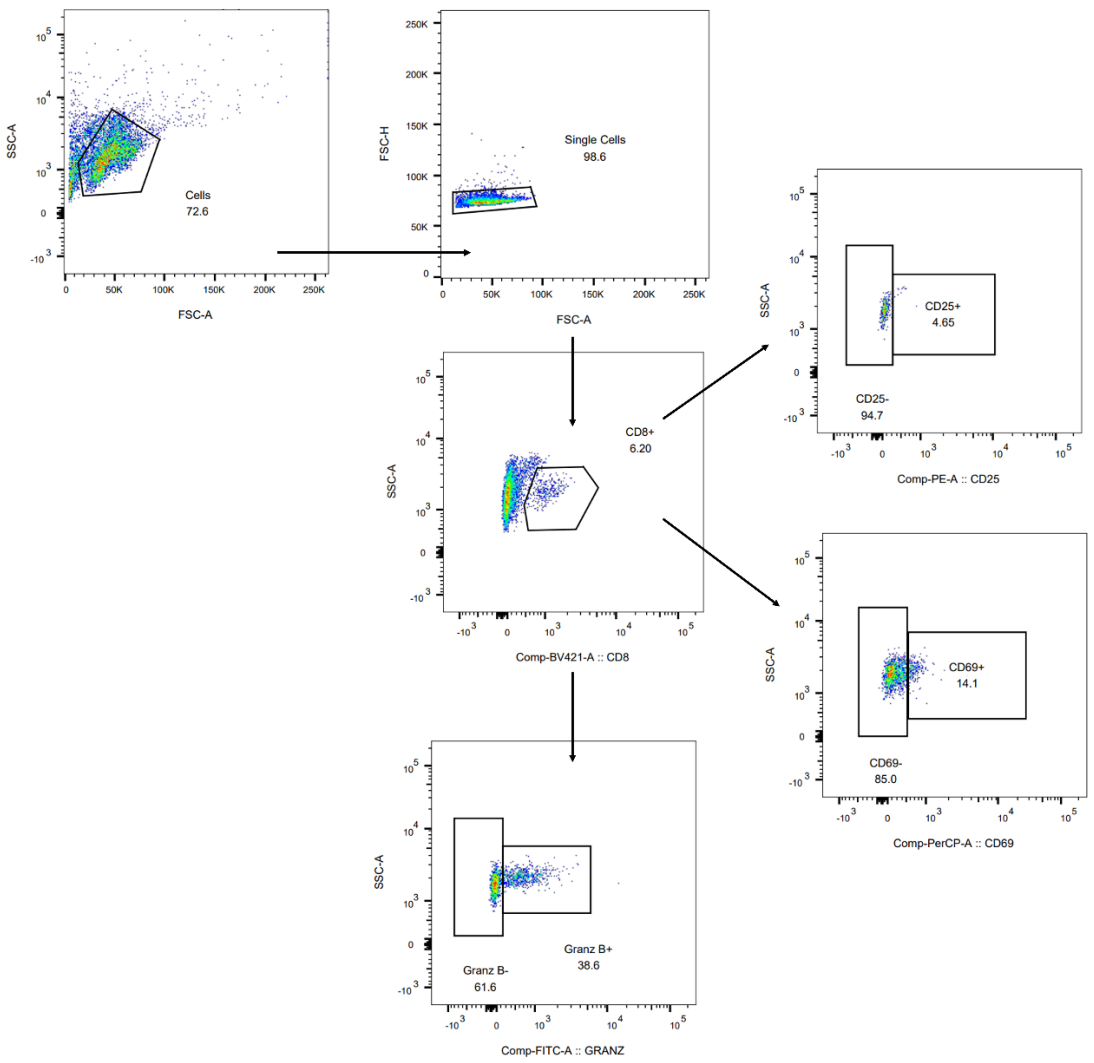


Figure II-9: Gating strategy for CD8+ T Cell activation and cytotoxic function post HLA-DR Blockade. This figure illustrates the flow cytometry gating strategy used to evaluate CD8+ T cell activation (CD69, CD25) and cytotoxic potential (Granzyme B) after a 72-hour culture of PBMCs under unstimulated, stimulated, and HLA-DR-blocked conditions.

9. ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) was conducted to quantify IFN-γ levels in cell culture supernatants. Supernatants were previously collected, centrifuged at 2000 rpm for 10 minutes to remove cell debris, and stored at -20°C. The IFN-γ ELISA kit from Biolegend, based on the sandwich method (see Figure II-10), was employed following the manufacturer's instructions.

Initially, a 96-well plate was coated overnight at 4°C with the capture antibody diluted in coating buffer (1 liter of dH2O with 8.4g NaHCO3 and 3.56g Na2CO3, pH 9.5). The plate was subsequently washed four times with PBS containing 0.05% Tween-20 (wash buffer). To prevent nonspecific binding, the plate was blocked for 1 hour with PBS 1X containing 1% BSA, followed by another four washes.

Next, 100 µL of the standards and supernatant samples were added to each well and incubated for 2 hours with shaking. After incubation, the plate underwent four additional wash cycles. The detection antibody was then added, and the plate was incubated for 1 hour with shaking, followed by another round of washing.

Avidin-HRP (horseradish peroxidase) was added and incubated for 30 minutes with shaking. The plate was then washed five times with 1-minute intervals between washes. Subsequently, TMB substrate (Biolegend) was added to each well and incubated in the dark for 30 minutes. The reaction was stopped with the addition of the stop solution (Biolegend).

Finally, the absorbance of each well was measured at 450 nm and 570 nm using a Tecan Infinite F200 PRO microplate reader. The concentration of IFN-γ in the samples was determined based on the standard curve generated from the standards with known concentrations, taking into account the difference in absorbance between 450 nm and 570 nm.

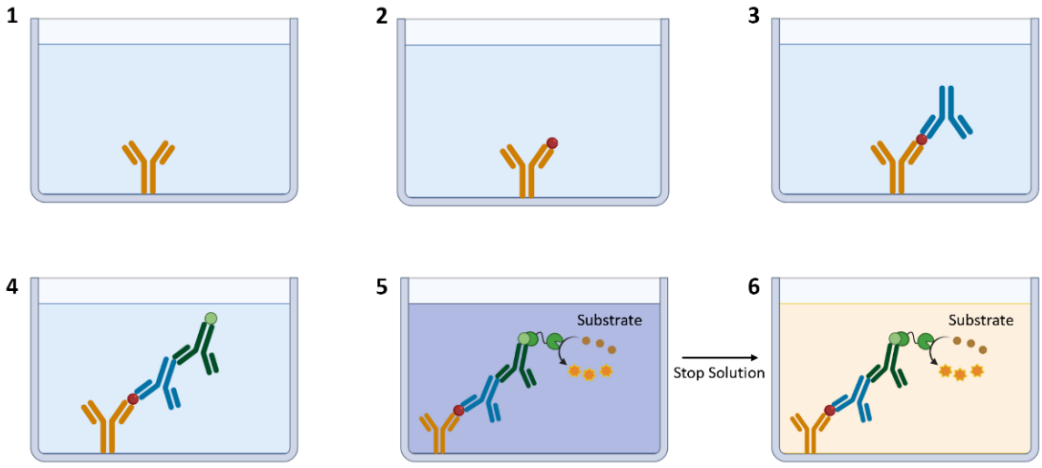


Figure II-10: Overview of the Sandwich ELISA procedure. The steps are as follows: 1) Coating the plate with capture antibody; 2) Incubation with the specific antigen; 3) Incubation with detection antibody; 4) Addition of Avidin/Streptavidin; 5) Addition of substrate; 6) Addition of stop solution. Figure created with BioRender.

10. Expansion of HLA-DR+ cytotoxic T lymphocytes

Recent efforts have been dedicated to refining the *ex vivo* expansion of T cells for therapeutic applications. In this thesis, we investigated the dynamics of T cell expansion with a specific emphasis on HLA-DR expression.

For that, PBMCs were initially seeded into 24-well plates at a density of $1x10^6$ cells/mL in 1 mL of RPMI 1640 Medium (Gibco) supplemented with 10% FBS (Biowest) and 1% Penicillin/Streptomycin (GE Healthcare). To activate and expand T lymphocytes, a combination of stimulatory antibodies was added on day 0 namely 5 µg/mL of mouse anti-human CD3, 1 µg/mL of mouse anti-human CD28, and 5 µg/mL of rat anti-mouse IgG1 as the crosslinking antibody (Biolegend). After 24 hours of incubation, various interleukin cocktails were introduced into the culture. Throughout the 14 days culture period, cell viability and growth were monitored by counting the cells at seven time points (days 0, 3, 5, 7, 9, 11, and 14) using trypan blue exclusion dye (GE Healthcare). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. To ensure optimal cell proliferation, half of the culture medium was refreshed every 2-3 days with fresh medium containing interleukins (PeproTech), with IL-2 consistently at 100 UI/mL, and IL-7, IL-12, and IL-15 at concentrations of 20 ng/mL. Upon reaching confluence, cultures were split into new wells to maintain cell health (Figure II-11).

At each specified time point, the total cell numbers for each condition were calculated by summing counts from all wells under that condition. Additionally, the frequency of HLA-DR+ cytotoxic T lymphocytes was assessed using flow cytometry at corresponding time points to track HLA-DR expression dynamics during T cell expansion. The gating strategy utilized in these experiments is described in Figure II-12.

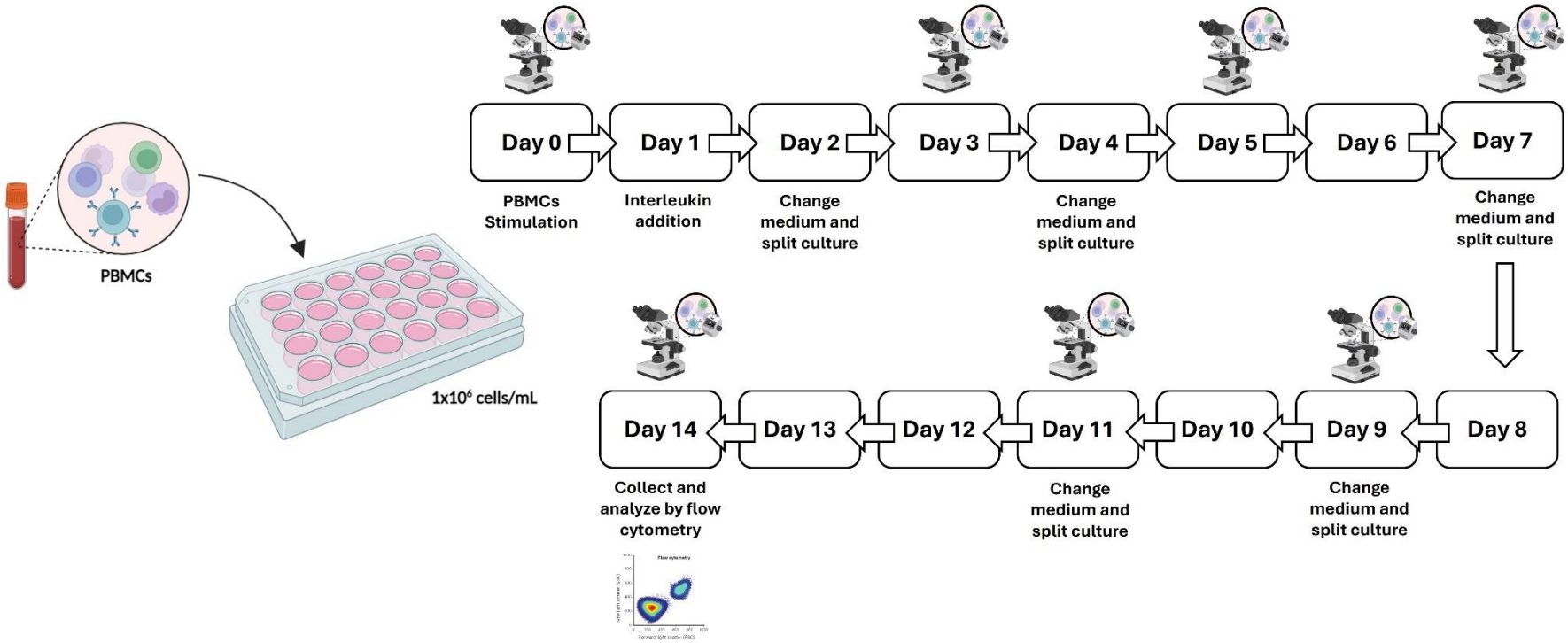


Figure II-11: Schematic representation of the peripheral blood mononuclear cells (PBMCs) expansion protocol. PBMCs were seeded at an initial density of $1x10^6$ cells/mL and stimulated on day 0 with anti-CD3, anti-CD28, and crosslinking antibodies to activate T lymphocytes. The culture was supplemented with a periodic addition of interleukins (IL-2, IL-7, IL-12, and IL-15) to support T cell proliferation. Cell viability and growth were monitored and evaluated at seven specific time points (days 0, 3, 5, 7, 9, 11, and 14) using trypan blue exclusion dye. The figure depicts the timeline of cell culture, activation, interleukin supplementation, and cell counting intervals.

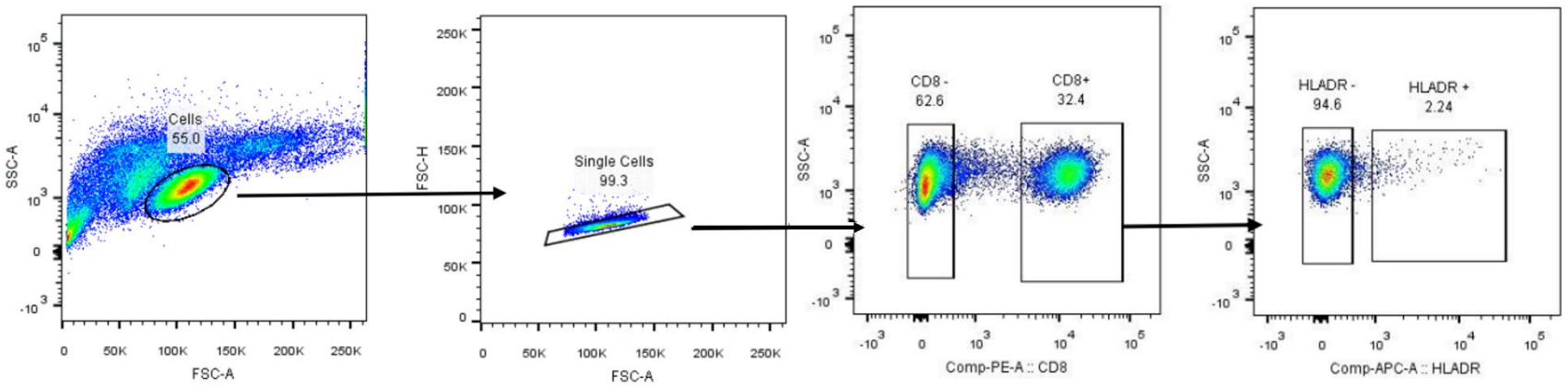


Figure II-12: Gating strategy used for the analysis of HLA-DR expression in cytotoxic T lymphocytes (CTLs). The frequency of HLA-DR+ CTLs was assessed at various time points during cell expansion protocol.

11. Drug Screening

Cytotoxic T lymphocytes (CTLs) are key players in the immune defense against cancer cells, highlighting the need to boost their cytotoxic capacity for more effective cancer immunotherapy. Here, we utilized our established 3D co-culture model[140] (see section 4) as a screening platform to evaluate agents that enhance HLA-DR expression in CTLs, thereby boosting their cytotoxicity against breast cancer (BC) cells. Briefly, the BC cell lines BT-474, HCC1806, Hs578T, MCF-7, and MDA-MB-231 were challenged with PBMCs, that had been pre-treated with TCR stimulation plus interleukins 2 and 12, as previously detailed in section 4. Additionally, the following candidates were tested: Nivolumab (Anti-PD-1, Bristol-Myers Squibb), Bevacizumab (Anti-VEGF, Roche), gently provided by the Champalimaud Foundation, 4-1BB (Anti-137, clone 4B4-1, Biolegend), and OX40 (Anti-134, clone Ber-ACT35, Biolegend).
All the compounds were tested at a concentration of 50 µg/mL. The effect of the compounds on the viability of the cancer cells was assessed by flow cytometry, as referred above (section 4).

12. Metabolic Modulation with Sodium Oxamate

To investigate the impact of metabolic modulation on cytotoxic T lymphocytes (CTLs) functionality, we used sodium oxamate (Sigma-Aldrich) in PBMCs cultures. Sodium oxamate was added to the PBMCs cultures at final concentrations of 20 mM, 50 mM, and 100 mM. PBMCs were cultured in RPMI 1640 medium (Gibco) in 24-well plates at a density of $1x10^6$ cells/mL. The cultures were treated with the specified concentrations of sodium oxamate for 72 hours. A control group of PBMCs was treated with the vehicle (PBS 1x) under the same conditions.

After the 72-hour incubation period, the culture supernatants were collected for lactate quantification. Lactate levels were measured using a commercial enzymatic lactate detection kit (L-Lactate Assay Kit, Sigma-Aldrich) according to the manufacturer's instructions.

13. Bioinformatic Analysis

To investigate potential regulatory networks involving the *HLA-DR* and *PD1* genes, we utilized scRNA-seq data from patients diagnosed with BC, focusing on the expression of CD8+ T lymphocytes. These data were extracted from the TIGER database (http://tiger.canceromics.org/#/), which serves as a repository for such information. This work was conducted in collaboration with Carolina Mathias and Suelen Baal from LabCHO at the Federal University of Paraná, Brazil. Our strategy for identifying genes correlated with *HLA-DR* and *PD1* was divided into three stages: (a) search for genes positively/negatively correlated with the *HLA-DR* gene; (b) search for genes positively/negatively correlated with the *PD1* gene; and (c) identification of commonly correlated genes.

We adopted a correlation value cutoff of $|r| > 0.5$ with a significance level of $p<0.05$ as our search criteria. This stringent threshold ensured the robustness and reliability of the identified correlations. To narrow down the search for target genes shared between *HLA-DR* and *PD1*, we considered only genes detected in the largest datasets, specifically including those that showed significant correlations in at least three different datasets. This approach enhanced the validity of our findings by focusing on consistently observed gene correlations across multiple datasets.

Data analysis was conducted using R, a statistical computing software, version 4.4.0. During the analysis, we did not find any significant correlations for genes negatively correlated with either *HLA-DR* or *PD1*. Consequently, the subsequent analysis focused solely on positively correlated genes.

14. Statistical Analysis

Plots were generated and statistical analyses were performed using GraphPad Prism version 8. Comparisons between samples were conducted using appropriate statistical tests, including the nonparametric Mann-Whitney test, paired t-test, one-way ANOVA, or two-way ANOVA with multiple comparisons, depending on the experimental design and data distribution.

All experiments were carried out with at least three independent biological replicates. For experiments involving PBMCs, each biological replicate was obtained from a different donor to account for inter-donor variability. Statistical significance was determined with p-values less than 0.05 considered significant. The levels of significance were denoted as follows: one symbol (*) for $p<0.05$, two symbols (**) for $p<0.01$, three symbols (***) for $p<0.001$, and four symbols (****) for $p<0.0001$. Error bars represent the standard deviation (SD) of the mean.

Detailed statistical information, including exact p-values and sample sizes, can be found in the legends of the respective figures.

CHAPTER III

Results

1. The predictive and therapeutic value of HLA-DR-expressing CTLs

Breast cancer (BC) remains the most frequent type of cancer in women worldwide, with rising incidence and mortality rates highlighting the urgent need for effective interventions[79,143,144]. The heterogeneity of BC, categorized into four subtypes, namely luminal A, luminal B, HER2+, and triple-negative breast cancer (TNBC), complicates treatment and demands personalized approaches[87,129].

The field of BC treatment is rapidly evolving, with a pressing need to explore novel strategies that can enhance the efficacy of existing therapies. Despite advancements in surgery, chemotherapy, radiation, and, more recently, immunotherapy, significant limitations remain. Issues such as incomplete tumor eradication, resistance mechanisms, and variable treatment responses highlight the critical need for interventions that can improve the effectiveness of current therapeutic modalities[95,130].

Neoadjuvant chemotherapy (NACT) remains a widely accepted strategy for high-risk, locally advanced, or inoperable BC tumors. However, the success rate of NACT is suboptimal, with less than 50% of patients achieving a pathological complete response. This underscores the urgent need to predict patients' response to NACT for informed clinical decision-making.

Immunotherapy, namely with immune checkpoint inhibitors, has emerged as a promising approach for some BC patients, but its success rate is also limited.

In the realm of cancer immunology, the ability to predict patient responses to chemotherapy and immunotherapy remains a critical challenge, and accurate biomarkers are essential for personalizing treatment regimens and improving patients' outcomes. Our team has previously identified HLA-DR, a major histocompatibility complex class II molecule, expressed on cytotoxic T lymphocytes (CTLs), as a significant predictor of therapeutic response in BC patients. Specifically, high levels of HLA-DR in CTLs have been associated with enhanced anti-tumor responses and improved outcomes following NACT[137,145].

In this study, we further investigated the predictive value of HLA-DR-expressing CTLs by applying our previously developed predictive probability model[137] to a new cohort of BC patients selected for NACT based on clinically

established criteria. Our objective was to confirm the accuracy and reliability of this model in predicting NACT responses, thereby advancing the precision of treatment strategies for BC patients.

Beyond the predictive value of HLA-DR expression in CTLs, this study also explores the functional impact of these cells through both *ex vivo* and *in vivo* models. By examining the role of HLA-DR-expressing CTLs, we aim to demonstrate their potential not only as biomarker, but also as a functional enhancer of anti-tumor immunity.

1.1. Validation of our predictive probability model of response to NACT based on the HLA-DR expression in CTLs

Neoadjuvant chemotherapy (NACT) is the standard treatment for patients with locally advanced breast cancer (BC). However, its effectiveness is reduced in patients with chemotherapy-resistant tumors. Given this challenge, and the limited alternative therapeutic options for BC patients with poor or modest responses to conventional treatments, our team has proposed and validated the HLA-DR level in CTLs as a reliable biomarker to predict the likelihood of tumor remission following NACT. Additionally, we have developed a predictive probability model aimed at integrating this biomarker into routine clinical practice.

Then, we continued to analyze HLA-DR levels in CTLs within BC patient biopsies, to predict the likelihood of response to NACT, by applying our model.

This new cohort of patients included 69 patients, categorized as responders or non-responders based on their radiological and pathological outcomes. Responders were defined as patients achieving a pathological complete response (pCR) or a partial response with less than 10% of the initial tumor remaining and no axillary lymph node involvement. Non-responders included patients with more than 50% of the initial tumor mass remaining post-treatment, those who developed metastasis, or those who experienced early relapse after NACT.

Within the new cohort, 24 patients proceeded with NACT treatment, while 45 did not. Among patients who underwent NACT, 10 were lost to follow-up,

primarily due to transfers to other hospital units during treatment. Of the remaining 14 patients with available follow-up data, all achieved either a pCR (n=10) or a partial response (n=4), which aligns closely with the predictions of our model (10 out of 14 were correctly predicted) (Figure III-1).

Therefore, despite missing the follow-up clinical data for several patients, our findings in this new cohort of BC patients demonstrate that our predictive model accurately predicts most responses to NACT (Figure III-1). These results corroborate the model's utility in identifying which BC patients are most likely to benefit from NACT.

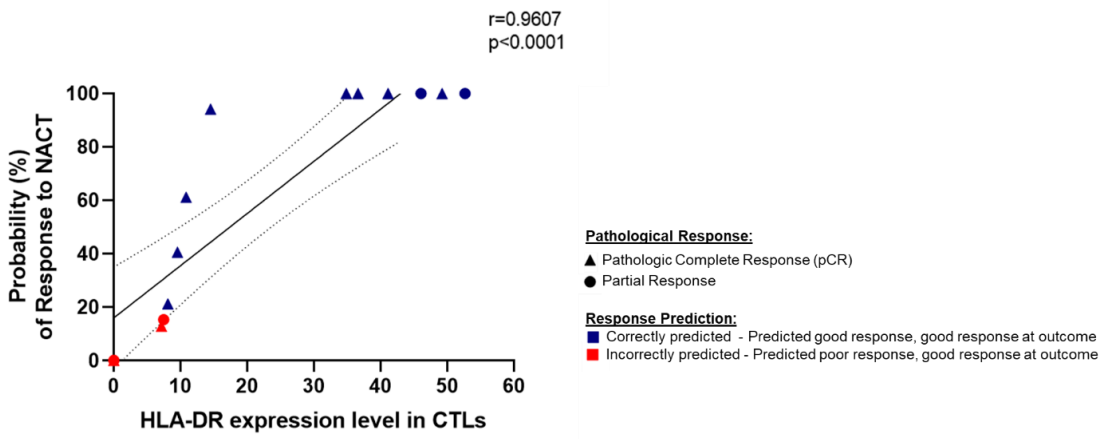
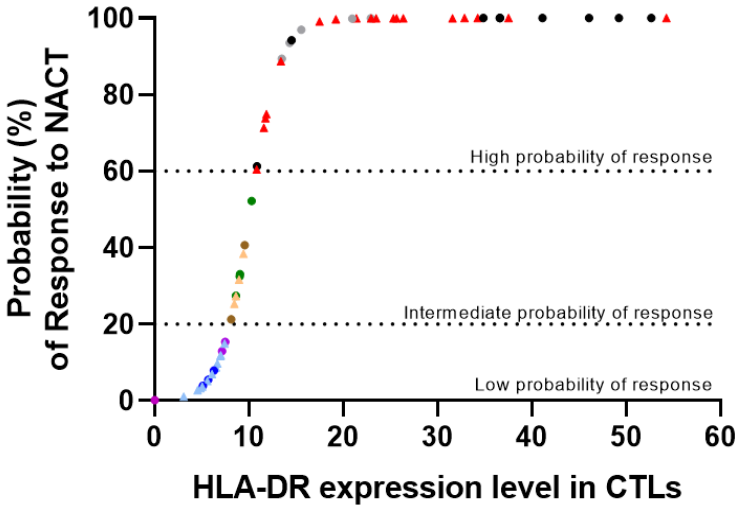


Figure III-1: Validation of the predictive probability model of response to neoadjuvant chemotherapy (NACT) based on the HLA-DR level in cytotoxic T lymphocytes (CTLs). Validation of our predictive probability model of BC patients' response to NACT, based on the HLA-DR level in infiltrating CTLs, assessed in patients' biopsies[137]. This validation was conducted in a cohort of 14 patients who underwent NACT. All patients responded to the treatment, with their responses categorized as either pathologic complete response (triangles) or partial response (circles). Our model accurately predicted the response to NACT for the majority of these patients (blue dots), with only four patients' responses not correctly predicted (red dots). These results confirm the model's utility in predicting which BC patients will benefit from NACT, with a Spearman correlation of r=0.9607 ($p<0.0001$, n=14).

Moreover, our analysis revealed that at least 22 out of 45 patients who were not selected for NACT treatment had an intermediate to high probability of responding to this treatment (Figure III-2). This underscores the importance of considering HLA-DR levels in CTLs for making informed clinical decisions in BC treatment, as it could improve patient outcomes by identifying those more likely to benefit from NACT.



Treatment:

○ Neoadjuvant Chemotherapy (NACT)
△ No Neoadjuvant Chemotherapy

Response Prediction:

- Predicted good response to NACT, treated with NACT, good outcome
- Predicted good response to NACT, untreated with NACT
- Predicted good response to NACT, treated with NACT, response unknown
- Predicted intermediate response to NACT, untreated with NACT
- Predicted intermediate response to NACT, treated with NACT, response unknown
- Predicted intermediate response to NACT, treated with NACT, good outcome
- Predicted poor response to NACT, untreated with NACT
- Predicted poor response to NACT, treated with NACT, response unknown
- Predicted poor response to NACT, treated with NACT, good outcome

Figure III-2: Prediction of response to neoadjuvant chemotherapy (NACT) within breast cancer (BC) patients. Predicted response probabilities to NACT, based on HLA-DR levels in infiltrating CTLs assessed from BC patients' biopsies. The predictive probability model was applied to all BC patients, regardless of whether they received NACT (circles, n=24) or another chemotherapy regimen (triangles, n=45). The distribution is categorized into low, intermediate, and high probabilities of response to NACT. According to the model, at least 22 patients who did not undergo NACT had an intermediate to high probability of responding favorably to it (red and coral circles). These findings underscore the clinical value of incorporating HLA-DR level assessments into treatment planning, as this approach could help to identify patients more likely to benefit from NACT, thereby improving overall treatment outcomes.

1.2. Corroboration of the HLA-DR-expressing CTLs increased anti-tumor effect through *ex vivo* assays

Our previous findings revealed a positive correlation between HLA-DR levels in tumor-infiltrating CTLs and HLA-DR levels in systemic CTLs[145]. This correlation suggests that BC patients with high levels of HLA-DR-expressing CTLs in their blood are also more likely to respond favorably to NACT. Additionally, we verified that these particular circulating CTLs exhibit robust anti-tumor capabilities, effectively eliminating cancer cells, while blood CTLs with low HLA-DR levels exhibit reduced anti-tumor activity. This emphasizes the critical role of HLA-DR in facilitating effective immune responses against BC[145].

Based on these findings, we further explored the composition and functionality of immune cells from BC patients' blood, undergoing NACT. Using a flow cytometry multi-panel (see chapter II, section 5), we conducted a phenotypic characterization of various immune populations, including T lymphocytes (subdivided into T helper cells (Th), cytotoxic T cells (CTLs), and regulatory T cells (Tregs)) and neutrophils (Figure III-3). Blood samples were collected and analyzed from 25 patients between November 2019 and February 2021, prior to BC treatment, sourced from our collaborating hospitals. We also assessed the activation state of the CTLs population by analyzing the expression of established T cell activation markers, CD69 and HLA-DR (Figure III-4). CD69 is an early, transient activation marker, while HLA-DR appears later in the activation process and remains on the cell surface throughout activation[36].

Our results showed no statistically significant differences in the percentages of Th, CTLs, Tregs, and neutrophils between NACT responders and non-responders (Figure III-3). Moreover, there were no significant differences in CTLs activation between NACT responders and non-responders when analyzing the activation marker CD69 (Figure III-4A). However, when exploring HLA-DR-positive cells within the CTLs population, we observed a positive correlation between HLA-DR expression and NACT response, with NACT responders exhibiting a higher percentage of HLA-DR+ cells within the CTLs population (Figure III-4B). These findings reinforce the importance of

HLA-DR-expressing CTLs, including the systemic ones, in predicting the response to NACT, corroborating our previous results.

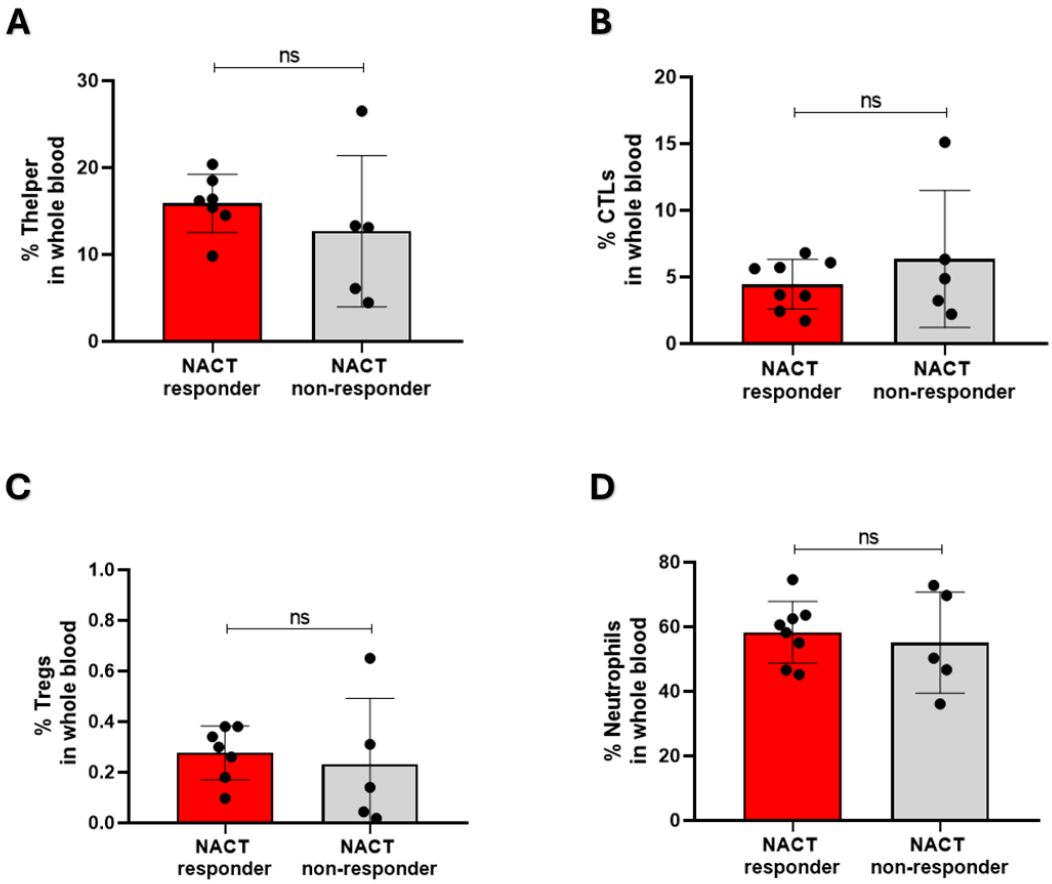


Figure III-3: Analysis of immune cell populations in the blood of breast cancer (BC) patients undergoing neoadjuvant chemotherapy (NACT). Flow cytometry was used to analyze **(A)** T helper cells, **(B)** cytotoxic T lymphocytes (CTLs), **(C)** regulatory T cells (Tregs), and **(D)** neutrophils, all analyzed in whole blood samples. No significant differences were observed in the percentages of these immune cell populations between NACT responders and non-responders. Data are represented as mean ± SD, *ns= non-statistical.*

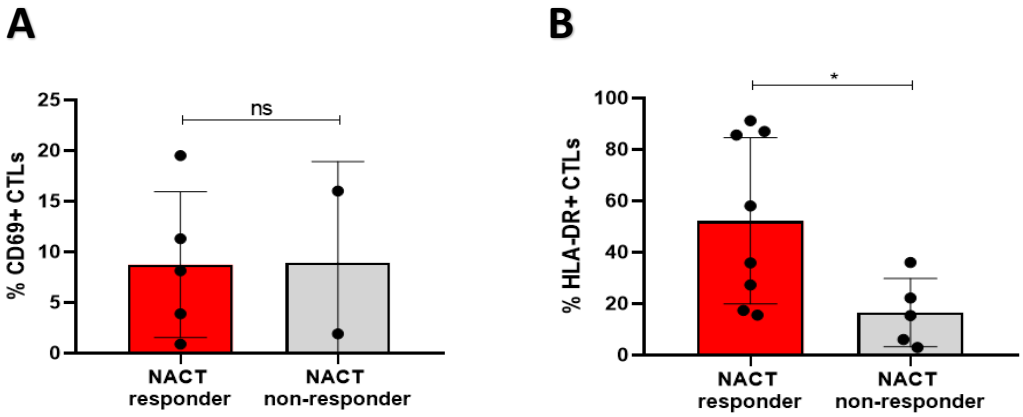


Figure III-4: Activation status of cytotoxic T lymphocytes (CTLs) in the blood of breast cancer patients undergoing neoadjuvant chemotherapy (NACT). **(A)** Analysis of CD69 expression, an early activation marker, revealed no significant differences in CTLs activation between NACT responders and non-responders. **(B)** A higher percentage of HLA-DR^{+} cells within the CTLs population was positively correlated with a favorable NACT response, underscoring the importance of HLA-DR expression in predicting treatment outcomes. Data are represented as mean ± SD, **p < 0.05, ns= non-statistical.*

Thus, given our previous report of a positive correlation between HLA-DR levels in tumor-infiltrating CTLs and systemic CTLs[145], along with our current findings that high levels of HLA-DR in blood CTLs are consistently associated with a favorable NACT response, we pursued to validate the functional significance of HLA-DR-expressing CTLs in BC patients. Our aim was to enhance the understanding of how HLA-DR levels correlate with CTLs-mediated tumor cytotoxicity.

To achieve this, we conducted *ex vivo* experiments to investigate the impact of HLA-DR expression on CTLs functionality. We isolated peripheral blood mononuclear cells (PBMCs) from BC patients and co-cultured them with spheroids of the BC cell line MDA-MB-231 (Figure III-5) using a 3D co-culture protocol previously developed by our team[140]. This approach allowed us to closely mimic the tumor microenvironment and observe the interactions between immune cells and tumor cells. Blood samples for these functional experiments were obtained from 14 BC patients between February 2022 and September 2023. The PBMCs were obtained through the protocol described in chapter II, section 1.

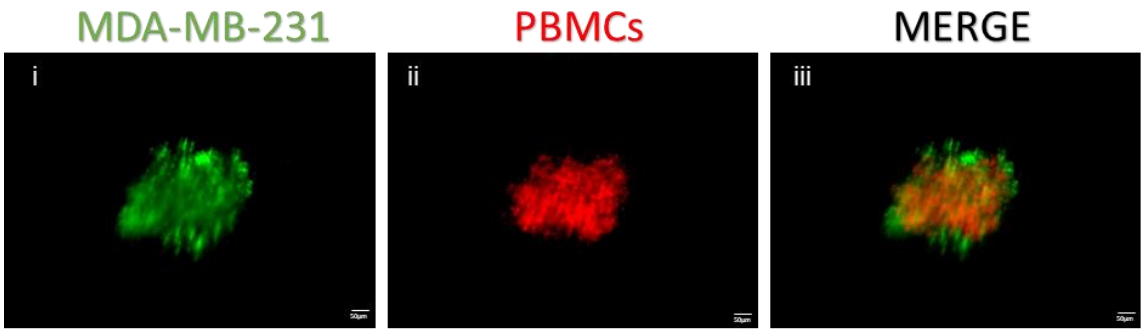


Figure III-5: Peripheral blood mononuclear cells (PBMCs) successfully infiltrate 3D spheroids of the MDA-MB-231 breast cancer cell line. The MDA-MB-231 spheroids were stained with 5 µM CellTrace™ CFSE (i, left panel), while PBMCs were stained with 5 µM CellTracker™ Orange CMTMR (ii, middle panel). The merged image (iii, right panel) shows the co-localization of PBMCs within the spheroids on day 7 of culture, 24 hours after PBMCs were introduced. Scale bar: 50 µm.

Our results demonstrated a significant increase in tumor cell mortality correlated with the frequency of HLA-DR-expressing CTLs within the PBMCs (Figure III-6). This establishes a positive correlation between the percentage of HLA-DR-expressing CTLs in the blood and the ability of PBMCs to kill tumor cells (Spearman r=0.5429, p=0.0479, Figure III-6).

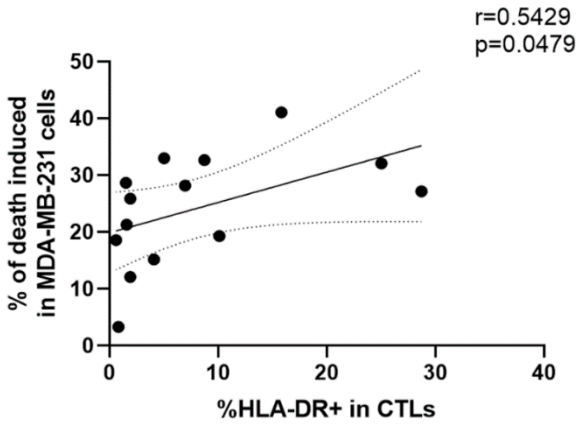


Figure III-6: HLA-DR expression on cytotoxic T lymphocytes (CTLs) is crucial for effective breast cancer (BC) cell elimination. A positive correlation was observed between the percentage of CTLs expressing HLA-DR in the blood of BC patients and the efficacy of their PBMCs in eliminating tumor cells (Spearman r = 0.5429, p = 0.0479, n = 14).

After observing that HLA-DR-expressing CTLs are required for tumor cell killing, we aimed to confirm that the enhanced cytotoxicity of these cells is indeed due to HLA-DR expression and not merely a result of general T cell activation. Since HLA-DR is a recognized marker of T cell activation, distinguishing its role from other activation effects was crucial. To address this, we used CD25-positive sorted cells, as CD25 is another well-established T cell activation marker. By comparing the cytotoxic effects of CD25+CTLs with and without HLA-DR expression, we isolated the specific contribution of HLA-DR to CTLs-mediated tumor cell killing. Notably, we observed that adding sorted HLA-DR-expressing CD25+CTLs to the BC spheroids resulted in enhanced cytotoxicity compared to adding CD25+CTLs without HLA-DR expression (p=0.0323, Figure III-7), underscoring HLA-DR's crucial role in boosting CTLs-mediated tumor cell elimination.

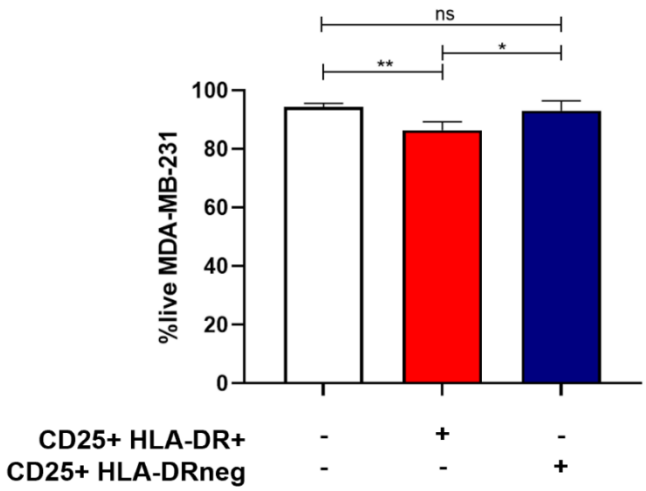


Figure III-7: Breast cancer (BC) cells' elimination requires HLA-DR expression on cytotoxic T lymphocytes (CTLs). Viability of MDA-MB-231 breast cancer cells (white bars, n=9) was significantly reduced following the addition of sorted $CD25^{+}HLA\text{-}DR^{+}$ CTLs to the spheroids (red bar, p = 0.0031, n = 9). In contrast, the addition of $CD25^{+}HLA\text{-}DRneg$ CTLs did not result in a significant decrease in cell viability (blue bar, ns, n = 9). Data are represented as mean ± SD, **p < 0.05, **p < 0.01, ns= non-statistical.*

Collectively, these results highlight the essential role of HLA-DR in mediating effective immune responses against BC and reinforce the utility of evaluating HLA-DR levels in CTLs pre-NACT, as it could serve as a valuable biomarker for predicting patients' response to this treatment. Therefore, strategies aimed at

targeting and enhancing HLA-DR expression on CTLs may offer a promising approach to improve treatment outcomes in BC.

1.3. *in vivo* demonstration of the anti-tumor efficacy of HLA-DR-expressing CTLs

Building on our previous findings showing the substantial role of HLA-DR-expressing CTLs in anti-tumor activity, we pursued to further validate these results using an *in vivo* model. While our *ex vivo* experiments highlighted the correlation between HLA-DR expression and increased tumor cell cytotoxicity, the zebrafish model offers a valuable opportunity to assess these effects in a living organism. Zebrafish are increasingly valued in cancer research for their transparency and the ability to monitor tumor progression and immune interactions in real time[146]. By introducing HLA-DR-expressing CTLs into zebrafish xenograft models, we aimed to confirm and extend our previous findings, offering deeper insights into how HLA-DR mediates effective anti-tumor responses. This part of the work was performed with the help of Cátia Rebelo de Almeida and Rita Fior from the Fior Lab at Champalimaud Foundation.

These *in vivo* findings corroborate the results from our *ex vivo* studies. Specifically, zebrafish xenografted with human BC tumors (from the Hs578T cell line) and injected with PBMCs from BC patients with a high frequency of HLA-DR-expressing CTLs demonstrated a significant reduction ($p < 0.001$) in tumor size compared to those injected with PBMCs from donors with a lower frequency of these cells (Figure III-8A and B).

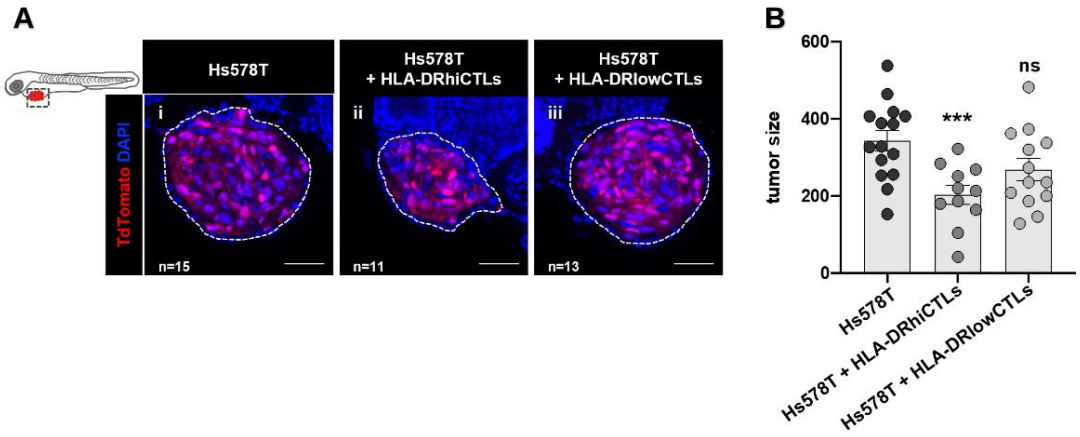


Figure III-8: Breast Cancer cells' elimination requires HLA-DR expression on CTLs. **(A)** Confocal microscopy images showing a reduction in tumor size when xenografted zebrafish models were injected with BC patients' PBMCs enriched in high frequency of HLA-DR-expressing CTLs (HLA-DRhiCTLs) compared to those injected with PBMCs whose CTLs exhibited low HLA-DR levels (HLA-DRlowCTLs). **(B)** Analysis of similar images to the one represented in A demonstrated that PBMCs from patients with increased frequency of HLA-DRhiCTLs exhibited a significant capacity to reduce tumor size ($p<0.001$, n=11), while PBMCs from patients with a higher frequency of HLA-DRlowCTLs did not affect tumor size (ns, n=13). Data are represented as mean ± SD, ****p < 0.001, ns= non-statistical*.

These results further emphasize the importance of incorporating HLA-DR-expressing CTLs in the development of effective therapeutic strategies. Enhancing the frequency of HLA-DR-expressing CTLs within PBMCs holds the potential to significantly boost anti-tumor immunity and improve therapeutic outcomes for BC patients.

2. The role of HLA-DR in CTLs and the interplay between CTLs and CD4+ T Cells

Breast cancer (BC) treatment remains a challenging field, necessitating ongoing exploration of innovative strategies to improve patient outcomes[147]. While conventional treatments have significantly advanced and immunotherapies have emerged as a promising frontier, the pursuit of more effective therapies continues, underscoring the need for deeper insights into immune mechanisms that can be exploited for therapeutic benefit[133]. In this context, cytotoxic T lymphocytes (CTLs) are crucial, as they play a vital role in identifying and destroying cancer cells. However, the effectiveness of CTLs responses is highly dependent on their activation status and functional capacity, which are influenced by various molecular signals and interactions[31].

A key molecule in this context is HLA-DR, a class II major histocompatibility complex (MHC) protein traditionally known for its role in presenting antigens to CD4+ T helper cells[148,149]. Intriguingly, recent research, including our previous studies, has demonstrated that HLA-DR is also expressed in CTLs and plays a vital role in their anti-tumor activity[137,145,150]. This revelation has significant implications for understanding CTLs biology and developing improved therapeutic strategies.

So, this section aims to explore the significance of HLA-DR in CTLs activation and function, and its potential therapeutic implications for BC treatment. By delving into the mechanisms by which HLA-DR influences CTLs activity, we hope to uncover novel strategies to boost immune responses against tumors, ultimately improving patient outcomes.

2.1. Analysis of the impact of HLA-DR inhibition on CTLs activation

To elucidate the role of HLA-DR in the activation of cytotoxic T lymphocytes (CTLs), we conducted experiments using a blocking antibody to inhibit HLA-DR. By measuring the activation markers CD25 and CD69, we aimed to determine how the HLA-DR blockade affects CTLs activation status, following stimulation. Understanding this relationship is crucial for developing strategies to optimize CTLs activation for enhanced anti-tumor responses.

In these experiments, we utilized PBMCs obtained from healthy donors. The cells were cultured for 72 hours under different experimental conditions: without stimulation, with stimulation, and with stimulation in the presence of an HLA-DR-blocking antibody. After a culture period of 72 hours, cells were harvested and labeled with anti-CD25 and anti-CD69, to assess the activation status of CTLs.

Notably, the results demonstrated that blocking HLA-DR led to a reduction in CTLs activation levels, evidenced by a decreased frequency of CD25+ and CD69+ cells within this population (Figure III-9). This finding highlights the importance of HLA-DR in CTLs activation.

However, it is important to note that since total PBMCs were used in our experiments, the blockade may affect HLA-DR expression on other cells besides CTLs. Therefore, the observed results should be interpreted as reflecting this general blockade.

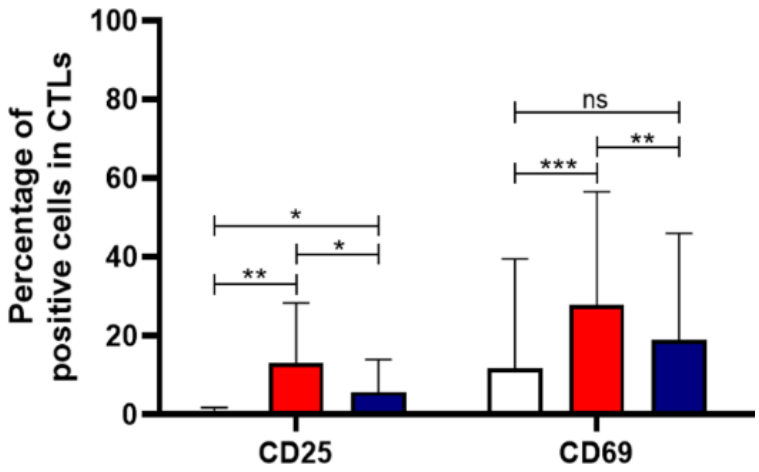


Figure III-9: HLA-DR is essential for effective CTLs activation and immune response. The activation of CTLs following stimulation was confirmed by assessing the percentage of CD69+ and CD25+ within this population. Stimulation of PBMCs increases CTLs activation (red bars), compared to non-stimulatory conditions (white bars). HLA-DR blockade (blue bars) reduces the activation levels of CTLs, shown by decreased percentages of CTLs expressing CD25 (n=19, p=0.0262) and CD69 (n=19, p=0.0050). Data are represented as mean ± SD, **p < 0.05, **p < 0.01, ***p < 0.001, ns= non-statistical.*

2.2. Evaluation of the effect of HLA-DR blockade on CTLs cytotoxicity

We further investigated the impact of HLA-DR blockade on the CTLs' cytotoxic function, focusing specifically on the expression of Granzyme B and IFN-γ. Granzyme B is a key molecule involved in the cytotoxic activity of CTLs[151], while IFN-γ plays a crucial role in immune responses[152].

For these experiments, we followed a similar protocol as previously described. Briefly, PBMCs from healthy donors were cultured for 72 hours under three different conditions: without stimulation, with stimulation, and with stimulation plus an HLA-DR-blocking antibody. Following the culture period, cells were harvested and labeled with antibodies against Granzyme B and IFN-γ to assess their expression levels.

Our results demonstrated that blocking HLA-DR led to a decline in CTLs cytotoxic capacity, evidenced by a decreased frequency of cells expressing Granzyme B (Figure III-10). This finding underscores the pivotal role of HLA-DR in maintaining the cytotoxic function of CTLs. However, as previously noted, since total PBMCs were used in our experiments, the observed results should be interpreted as reflecting this general blockade.

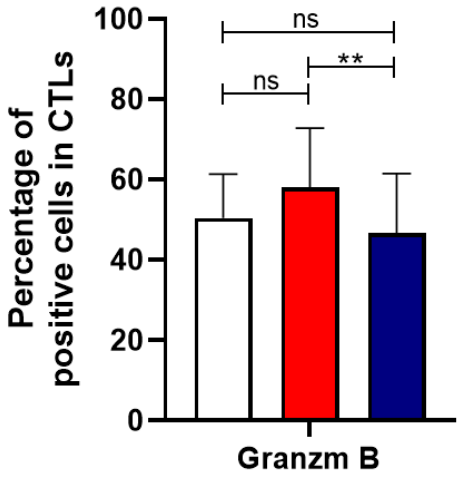


Figure III-10: HLA-DR is essential for effective CTLs cytotoxicity. The cytotoxic capacity of CTLs following stimulation was confirmed by assessing the percentage of Granzyme B+ (Granzm B) cells within this population. Stimulation of PBMCs increases CTLs cytotoxicity (red bars), compared to non-stimulatory conditions (white bars). HLA-DR blockade (blue bars) reduces the cytotoxic levels of CTLs, shown by decreased percentages of CTLs expressing Granzm B (n=14, p=0.0029). Data are represented as mean ± SD, ***p < 0.01, ns= non-statistical.*

Curiously, contrary to our expectations, we observed an increased frequency of cells expressing IFN-γ when HLA-DR was blocked (Figure III-11), assessed by flow cytometry analysis. This increase was corroborated by ELISA analysis of IFN-γ in the culture supernatants, which showed elevated IFN-γ production when HLA-DR was blocked (Figure III-12).

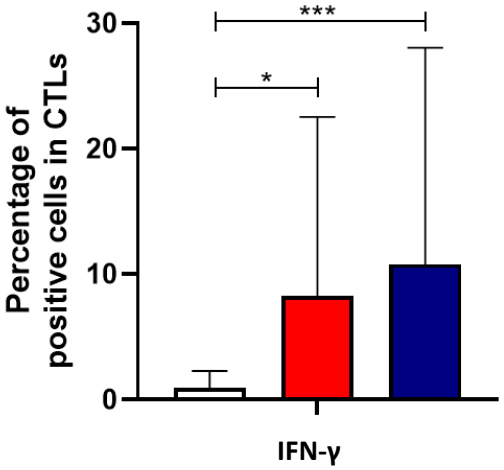


Figure III-11: HLA-DR blockade increases the percentage of CTLs expressing IFN-γ. The cytotoxic capacity of CTLs following stimulation was confirmed by assessing the percentage of Interferon Gamma+ (IFN-γ) cells within this population. Stimulation of PBMCs increases CTLs cytotoxicity (red bar), compared to non-stimulatory conditions (white bar). HLA-DR blockade (blue bar) tends to slightly increase the percentage of CTLs expressing IFN-γ (n=17, p=0.0005). Data are represented as mean ± SD, **p < 0.05, ***p < 0.001*.

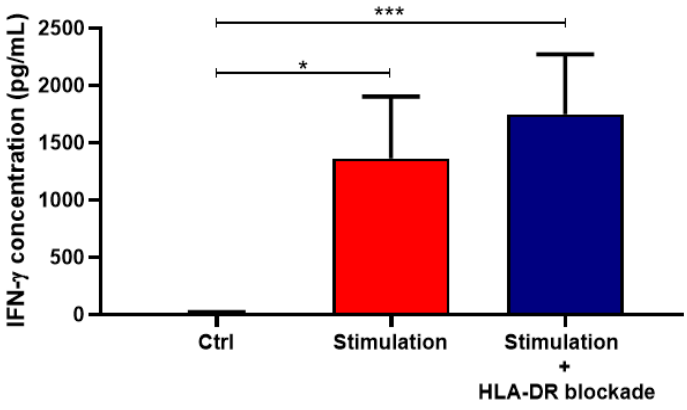


Figure III-12: ELISA analysis indicates that HLA-DR blockade enhances IFN-γ production. The concentration of IFN-γ is higher following PBMC stimulation (red bar) compared to non-stimulatory conditions (Ctrl; white bar). HLA-DR blockade (blue bar) also shows a slight increase in IFN-γ production (n=7, p=0.0009). Data are represented as mean ± SD, **p < 0.05, ***p < 0.001*.

The observed increase in IFN-γ production, contrary to our initial expectations, can be justified by several hypotheses. One possibility is that HLA-DR blockade disrupts a normal regulatory feedback mechanism, leading to a compensatory increase in IFN-γ production as a stress or immune response. Another hypothesis is that the absence of HLA-DR-mediated signaling alters the cytokine environment, promoting a shift towards an inflammatory phenotype that enhances IFN-γ expression. For example, HLA-DR could influence the production or signaling of other cytokines, such as IL-12 or IL-18, which are known to enhance IFN-γ production[153]. Alternatively, the increase in IFN-γ could indicate a more complex interplay between HLA-DR and other immune pathways, including metabolic pathways within CTLs. Blocking HLA-DR could lead to metabolic reprogramming that favors increased IFN-γ production through mechanisms that are yet to be fully understood.

Moreover, it is important to note that to ensure the specificity of our results, we included isotype controls in some experiments. We observed that the addition of an isotype control, at the same concentration as the HLA-DR blocking antibody, did not affect the T cell response to stimulation or alter the results under blockade conditions, either in terms of activation or cytotoxicity. This was confirmed by observing the frequency of cells expressing the markers CD69, CD25, and Granzyme B (Figure III-13). These findings suggest that the described effects are specific to the anti-HLA-DR antibody and are not attributable to nonspecific reactions or experimental artifacts.

Overall, the results suggest a complex role of HLA-DR in regulating CTLs functions and immune responses. They also highlight the therapeutic potential of targeting HLA-DR in CTLs to enhance anti-tumor immunity.

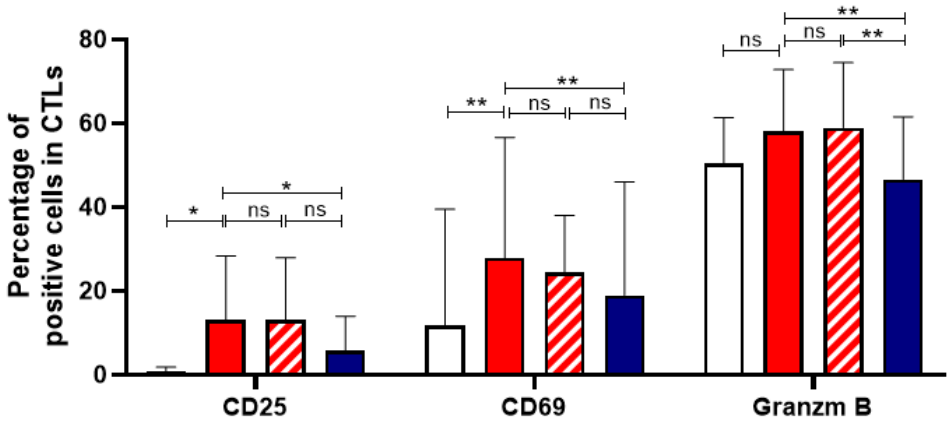


Figure III-13: ***Isotype control assessment confirms the specificity of anti-HLA-DR antibody effects on T cell activation and cytotoxicity.*** The activation of CTLs following stimulation was confirmed by assessing the percentage of CD69+, CD25+ and Granzyme B+ (Granzm B) cells within this population. Stimulation of PBMCs increases CTLs activation (red bars), compared to non-stimulatory conditions (white bars). HLA-DR blockade (blue bars) reduces the activation levels of CTLs, shown by decreased percentages of CTLs expressing CD25 (n=19, p=0.0457), CD69 (n=19, p=0.0092) and Granzm B (n=14, p=0.0053). The addition of the isotype control (red striped bars) did not affect the expression levels of these markers, demonstrating that the observed effects are specific to the anti-HLA-DR antibody and are not attributable to nonspecific binding. For the isotype control conditions (red striped bars), the sample size was n=12 for CD25 and granzyme B, and n=5 for CD69. Data are represented as mean ± SD, **p < 0.05, **p < 0.01, ns= non-statistical.*

2.3. Investigation of CD4+ T cells' role in CTLs activation

Given that HLA-DR is an antigen-presenting molecule that interacts with CD4+ T helper cells[154], we explored the role of CD4+ T cells in modulating CTLs activation. By comparing the activation of CTLs in the presence and absence of CD4+ T cells, we aimed to understand the collaborative dynamics between these two cell types.

To further elucidate this interaction, we conducted experiments with isolated PBMCs, specifically depleting CD4+ T cells. Two PBMCs' populations were stimulated: one containing CD4+ T cells and another without CD4+ T cells. Notably, stimulation of PBMCs lacking CD4+ T cells resulted in no significant changes in CTLs activation, as indicated by the unaltered percentage of cells expressing several activation markers (grey bars), compared to conditions where CD4+ T cells were present (red bars, $p < 0.05$, Figure III-14). This

observation underscores the crucial role of CD4+ T cells in promoting effective CTLs activation.

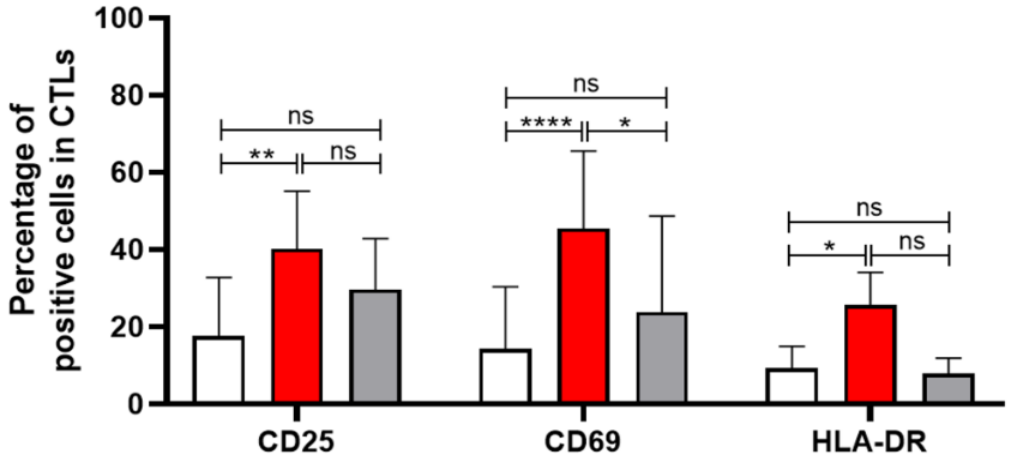


Figure III-14: CD4+T cells are essential for effective CTLs activation and immune response. PBMCs, depleted (grey bars) or not depleted (red bars) of CD4+ T cells, were stimulated, and the CTLs activation levels were assessed. Following stimulation, the frequency of CTLs expressing the activation markers was heightened compared to the basal state (white bars), in the condition with CD4+ T cells (red bars, n=9, $p < 0.05$). Nonetheless, stimulatory conditions barely affected the percentage of positive cells for the activation markers when CD4+ T cells were depleted (grey bars, n=6). Data are represented as mean ± SD, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, *ns= non-statistical*.

Thus, we hypothesized that HLA-DR-mediated antigen presentation not only primes CTLs for efficient recognition and elimination of target cells but also reinforces CTLs activation through interactions with CD4+ T cells. These CD4+ T cells release cytokines, ensuring a robust and sustained CTLs' immune response against tumors. Disrupting the interaction between CTLs and CD4+ T cells, either by blocking HLA-DR or depleting CD4+ T cells likely impairs anti-tumor immune responses by interfering with the cascade of events essential for effective CTLs activation.

These results shed light on the necessity of HLA-DR expression in both CTLs and CD4+ T cells for an efficient anti-cancer response. Understanding this interplay is crucial for the efficacy of T lymphocyte-mediated treatments and highlights potential areas for therapeutic intervention to enhance anti-tumor immunity.

3. T cell expansion to enhance HLA-DR-expressing CTLs

The pursuit of advanced therapeutic strategies for cancer treatment continues to drive research in immunotherapy, particularly in the realm of cellular therapy[155]. Cytotoxic T lymphocytes (CTLs) are at the vanguard of this approach, exploiting the body's immune system to identify and eliminate cancer cells[156]. Recent advances in T cell therapy have underscored the necessity of refining *ex vivo* expansion techniques to maximize the efficacy of CTLs in clinical applications[157].

Adoptive T cell transfer, a promising strategy for treating various malignancies including breast cancer (BC), relies heavily on generating large numbers of functionally potent CTLs. The expansion of T cells *ex vivo* involves stimulating them with specific signals that promote their proliferation and enhance their cytotoxic properties[158]. By increasing the number of T cells available for transfer and improving their ability to target malignant cells, these protocols aim to bolster the overall effectiveness of cancer treatments.

In this context, the therapeutic potential of CTLs can be significantly enhanced through optimized *ex vivo* expansion protocols. These protocols should be designed not only to increase CTLs quantity but also to improve their functionality for adoptive T cell transfer.

Given our findings, guaranteeing HLA-DR expression in CTLs is likely a promising approach for this optimization. In fact, in previous sections, we demonstrated that HLA-DR expression plays a crucial role in CTLs effective function. Understanding how to increase HLA-DR expression during T cell expansion can provide valuable insights to optimize CTLs-based therapies.

Here, we explored the dynamics of T cell expansion, emphasizing the importance of HLA-DR expression as a determinant of CTLs functionality. Our study evaluates various stimulation and expansion conditions to determine the optimal methods for generating highly functional CTLs.

Our findings on T cell expansion conditions and their influence on HLA-DR expression and CTLs cytotoxicity have deepened our understanding of how to optimize adoptive T cell therapies for cancer treatment. These insights hold

significant potential for advancing therapeutic strategies that fully leverage CTLs' ability to target and eliminate cancer cells.

3.1. Optimization of T cell expansion protocol

Optimizing T cell expansion protocols is crucial for enhancing the efficacy of adoptive T cell therapy in cancer treatment. Here, we aimed to identify the most effective conditions for the *ex vivo* expansion of CTLs by testing various stimulation techniques and cytokine cocktails. The ultimate goal is to generate a robust population of CTLs capable of targeting and eliminating cancer cells more effectively.

To achieve this, we explored the dynamics of HLA-DR-expressing CTLs expansion within the PBMCs, following various expansion protocols. Our findings revealed that immune cells could be successfully expanded *ex vivo* for up to 14 days using T cell receptor stimulation combined with cytokine cocktails (Figure III-15).

The most effective expansion protocol utilized anti-CD3 (5 µg/mL) and anti-CD28 (1 µg/mL) antibodies, along with interleukin (IL)-2 and IL-12. This combination was selected for subsequent experiments due to its superior ability to promote T cell proliferation and functionality (Figure III-15).

Although the total number of PBMCs increased over the 14-day expansion period (Figure III-15), we observed that HLA-DR-expressing CTLs specifically increased during the initial days but declined after day 5, regardless of the cytokine cocktail employed (Figure III-16). This highlights the critical need to not only expand T cells in quantity but also preserve or enhance key functional markers like HLA-DR to ensure their sustained efficacy in targeting and eliminating cancer cells.

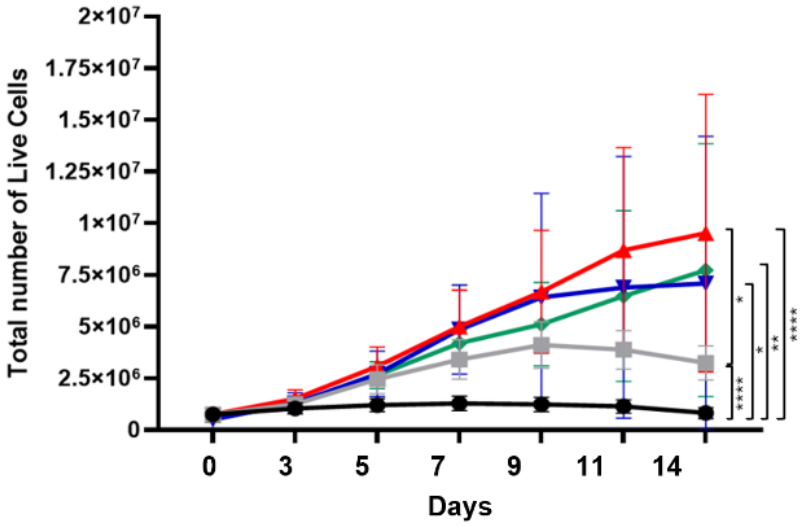


Figure III-15: Total number of live PBMCs during ex vivo expansion for up to 14 days. The cells survived for 14 days but did not proliferate without stimulation (black line, n=6). PBMCs stimulation with anti-CD3 (5 µg/mL) and anti-CD28 (1 µg/mL) slightly increased the number of viable cells for at least 10 days (grey line, n=6). The number of viable cells increased even more when the expansion protocol included the previous stimulation plus different cytokine cocktails, namely interleukin (IL)-2, IL-7, IL-12, and IL-15 (green line, n=4) or IL-2, IL-12, and IL-15 (blue line, n=4). The best expansion protocol was obtained with only IL-2 and IL-12 in addition to the anti-CD3 and anti-CD28 (red line, n=6). Data are represented as mean ± SD, **p < 0.05, **p < 0.01, ****p < 0.0001*.

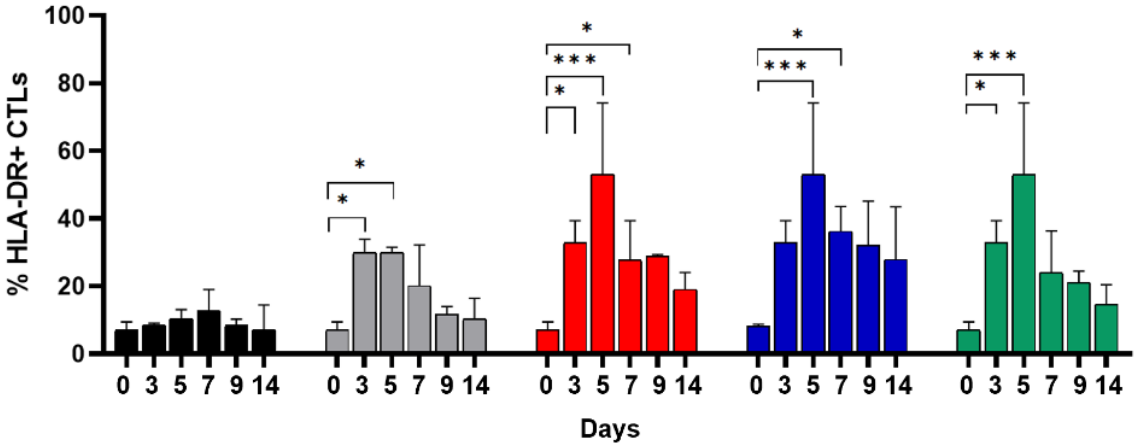


Figure III-16: Percentage of HLA-DR-expressing CTLs under different stimulating conditions. This percentage increased in the initial 5 days of the *ex vivo* expansion and decreased after this period across all the conditions (n=4). Black bars refer to the unstimulated condition. Grey bars refer to the condition where PBMCs were stimulated with anti-CD3 (5 µg/mL) and anti-CD28 (1 µg/mL), green bars refer to the condition of stimulation with anti-CD3 and anti-CD28 in combination with IL-2, IL-7, IL-12, and IL-15. Blue bars refer to the condition of stimulation with anti-CD3 and anti-CD28 plus IL-2, IL-12, and IL-15. Finally, red bars represent PBMCs stimulated with IL-2 and IL-12 in addition to the two stimulatory antibodies. Data are represented as mean ± SD, **p < 0.05, ***p < 0.001*.

3.2. Short-term vs. Long-term stimulation: Effects on HLA-DR expression and CTLs cytotoxicity

We observed that the duration of PBMCs stimulation significantly influences the expression of HLA-DR on CTLs, which likely impacts their functionality and therapeutic potential. Therefore, to further investigate this, we compared the effect of short-term and long-term expansion protocol on the frequency of HLA-DR-expressing CTLs within the PBMCs and their cytotoxic capabilities. Our findings provide insights into the optimal duration for the stimulation protocol to achieve the best therapeutic outcomes, particularly considering the importance of HLA-DR-expressing CTLs for therapeutic success.

Indeed, the results demonstrated that the short-term stimulation protocol (5 days) led to an upregulation of HLA-DR expression on CTLs compared to prolonged expansion methods (14 days) (Figure III-17). This finding highlights the critical impact of expansion duration on the expression of key functional markers.

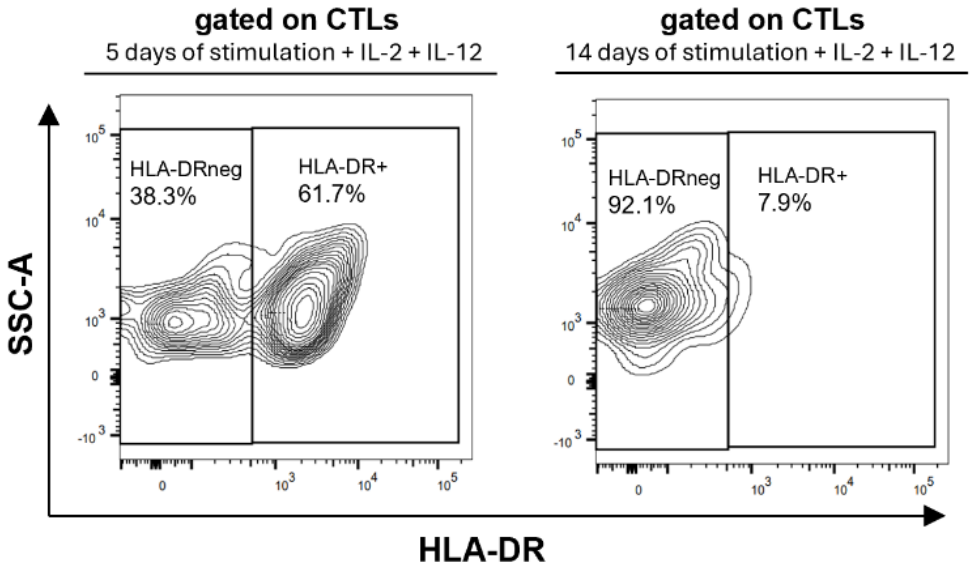


Figure III-17: Short-term stimulation protocols led to an upregulation of HLA-DR expression on CTLs. Representative image of a contour plot illustrating that the short-term stimulation protocol with anti-CD3 and anti-CD28 plus IL-2 and IL-12 (5 days - left side) led to a significant increase in HLA-DR expressing CTLs, contrary to prolonged incubation (14 days - right side) with the same cocktail.

Additionally, *in vitro* experiments employing 3D co-culture models, revealed that cells expanded for longer periods (14 days) exhibited diminished cytotoxic capacity compared to cells subjected to short-term stimulation (5 days) (Figure III-18).

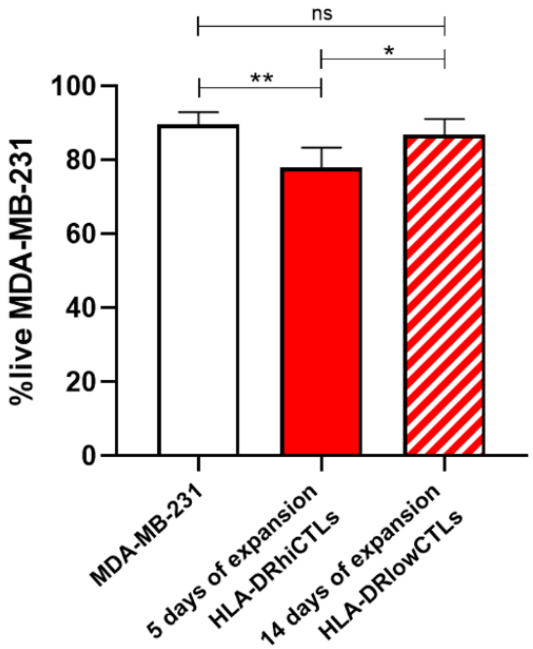


Figure III-18: Therapeutic potential of CTLs could be boosted through short-term expansion. *In vitro* 3D co-cultures composed of BC cell line MDA-MB-231 in a spheroid structure alone (white bar, n=4), with short-term stimulated PBMCs (5 days) (red bar, n=9), or with long-term stimulated PBMCs (14 days) (red striped bar, n=9). Short-term stimulation intensifies the cytotoxic activity of the PBMCs, as the viability of MDA-MB-231 cells was significantly decreased when co-cultured with PBMCs expanded using the short-term stimulation protocol (p=0.0036), but barely affected when co-cultured with PBMCs stimulated for a superior time. Data are represented as mean ± SD, **p < 0.05, **p < 0.01, ns= non-statistical*.

Notably, even when cells expanded for a longer period were added to breast cancer (BC) cells at a higher ratio (8 PBMCs to 1 BC cell), they exhibited reduced cytotoxic capacity compared to cells expanded for a shorter duration, which were added to BC cells at a ratio of 3 PBMCs to 1 BC cell (Figure III-19). Specifically, PBMCs expanded for 14 days, despite their superior number (8000 cells), did not lead to a significant decrease in the viability of MDA-MB-231 cells (Figure III-19). In contrast, the less expanded PBMCs, which were present in reduced numbers (3000 cells) in the co-culture, showed a more substantial cytotoxic effect. This higher cytotoxic capacity exhibited by less expanded PBMCs is most likely due to the fact that short-term stimulation better

promotes HLA-DR expression in CTLs than long-term stimulation, as shown in Figure III-17.

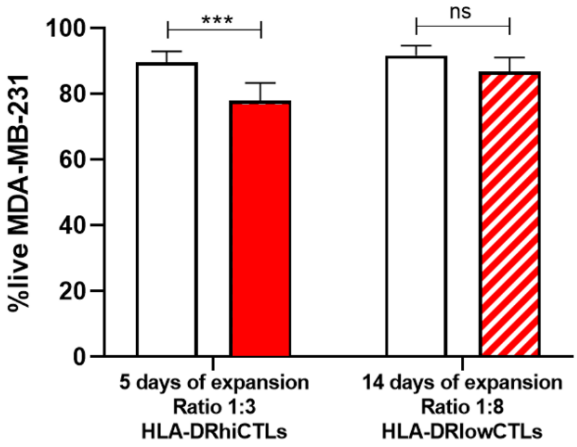


Figure III-19: Effective cytotoxic capabilities of CTLs were obtained with short-term stimulation despite lower ratios. *In vitro* 3D co-cultures composed of BC cell line MDA-MB-231 in a spheroid structure alone (white bars), with short-term stimulated PBMCs (5 days), added to the culture at a ratio of 3 PBMCs:1BC cell (red bars, n=5) and the BC cell line MDA-MB-231 in a spheroid structure alone (white bars), with long-term stimulated PBMCs (14 days), added to the culture at a ratio of 8 PBMCs:1BC cell (red striped bar, n=5). The viability of the BC cell line was significantly reduced when co-cultured with PBMCs stimulated for a shorter period, even at a lower ratio, while the viability of the BC cell line was not influenced by higher numbers of PBMCs stimulated during more days. Data are represented as mean ± SD, ****p < 0.001, ns= non-statistical.*

These results underscore the importance of prioritizing expansion protocols that ensure the effective cytotoxic capabilities of CTLs, which we demonstrated to be strongly dependent on HLA-DR expression achieved through short-term stimulation. This insight is crucial for developing effective adoptive T cell therapies.

4. Development of strategies to augment HLA-DR expression and enhance the cytotoxicity of CTLs

Enhancing the effectiveness of T cells is crucial for achieving more effective cancer treatment outcomes[159]. Cytotoxic T lymphocytes (CTLs) are fundamental to the immune system's ability to target and eradicate cancer cells, however, their efficacy can be limited by insufficient expression of key molecules and diminished cytotoxic capabilities due to various suppressive factors. The ability of CTLs to become and remain activated is essential for their anti-tumor capacity[160].

Due to its critical role in CTLs activation and function, as demonstrated in the previous sections, we consider HLA-DR as a crucial molecule, whose expression on CTLs could significantly amplify their anti-tumor responses.

Thus, here, we delve into potential strategies, including the employment of immunomodulatory agents and metabolic interventions, to enhance HLA-DR expression on CTLs and, consequently, their cytotoxicity against breast cancer (BC) cells. Utilizing the 3D co-culture model previously established by our team[140], we have screened various agents for their ability to improve HLA-DR-mediated CTLs function. Additionally, we investigated the effects of metabolic modulation to assess how alterations in cellular metabolism could further enhance CTLs cytotoxicity. Our objective was to identify strategies that can heighten CTLs-based therapies.

So, we assessed several agents for their potential to enhance HLA-DR expression, focusing on agonists that specifically target co-receptors more expressed in HLA-DR+ CTLs compared to their HLA-DR- CTLs counterparts. Additionally, we explored metabolic modulation strategies, namely the inhibition of lactate production[161], to alleviate metabolic limitations and further boost the cytotoxic potential of CTLs.

By analyzing the effect of these strategies on CTLs activity, we aim to offer valuable insights for optimizing adoptive T cell therapies and improving clinical outcomes for patients with BC.

4.1. Demonstration of the ability of anti-PD-1 treatment to enhance CTLs cytotoxicity and HLA-DR expression

We investigate a range of monoclonal antibodies, used in the clinical setting or in clinical trials, aimed at augmenting HLA-DR expression on CTLs, with the objective of enhancing their cytotoxic efficacy against breast cancer (BC) cells. To achieve this, we employed our established 3D co-culture model[140], which provides a suitable platform for assessing the impact of various immunomodulatory agents on CTLs functionality.

Specifically, our study encompasses agonists of different co-receptors (the anti-CD137 and the anti-CD134/Ox40), an immune checkpoint inhibitor (the anti-PD-1, Nivolumab), and an inhibitor of angiogenesis (the anti-VEGF, Bevacizumab) (Table III-1). Each agent was tested for its ability to modulate HLA-DR expression and enhance the overall functionality of CTLs.

The selection of anti-CD137 and anti-CD134/Ox40 was guided by previous flow cytometry analyses, which demonstrated that HLA-DR+ CTLs express these co-receptors at higher levels compared to their HLA-DR- counterparts (Figure III-20). This observation led us to hypothesize that targeting these co-receptors with specific agonists might stimulate the conversion of HLA-DR-negative CTLs into HLA-DR-positive CTLs, potentially enhancing their functionality. Both of these agents have been tested in clinical trials for their ability to boost immune responses.

In addition to these co-receptor agonists, we also evaluated Nivolumab (Table III-1), a well-established immune checkpoint inhibitor that targets PD-1 to enhance the immune response against cancer by blocking the PD-1/PD-L1 interaction[162]. We also included Bevacizumab (Table III-1), a monoclonal antibody that neutralizes vascular endothelial growth factor (VEGF), thereby inhibiting angiogenesis[163] and potentially providing additional immunostimulatory benefits[164,165]. Both Nivolumab and Bevacizumab have demonstrated efficacy in treating different solid cancer types[166–168] and were chosen for our study to assess their potential in modulating HLA-DR expression.

Table III-1: Summary of the drugs tested and the BC cell lines used in a small-size screening developed in the 3D platform established by our team[140], aiming to identify candidates that enhance HLA-DR expression in cytotoxic T lymphocytes (CTLs), and therefore their cytotoxic capacity against the BC cells. Each candidate entry corresponds to a specific agent assessed in the study. The "BC Cell Line" column specifies the particular BC cell line that was used against the agents ("Candidates" columns) that were tested, evaluating their promise and effectiveness. The "Characteristics of BC cell line" column recaps the representativeness of BC subtype and the expression of PD-L1 in the respective cell lines used in the study. Overall, this table summarizes the key results obtained from our screening.

BC cell line	Characteristics of BC cell line[169]	Candidates: Nivolumab (Anti PD-1)	Bevacizumab (Anti VEGF)	Anti CD137	Anti CD134
MDA-MB-231	• Triple-negative BC • High Expression of PD-L1	↑	↓	↓	↓
MCF-7	• Luminal A (ER+ PR+) • Low Expression of PD-L1	↓	↓	⊘	⊘
Hs 578T	• Triple-negative BC • Middle Expression of PD-L1	×	⊘	⊘	⊘
BT-474	• HER2+ • Low Expression of PD-L1	×	⊘	⊘	⊘
HCC-1806	• Triple-negative BC • High Expression of PD-L1	↑	⊘	⊘	⊘

↑ The candidate exhibits anti-tumor capabilities.
↓ The candidate fails to demonstrate anti-tumor properties.
⊘ The candidate's anti-tumor capabilities remain untested.
× Low spheroid viability or inadequate immune cell penetration into spheroids hampered the assessment of candidate anti-tumor efficacy.

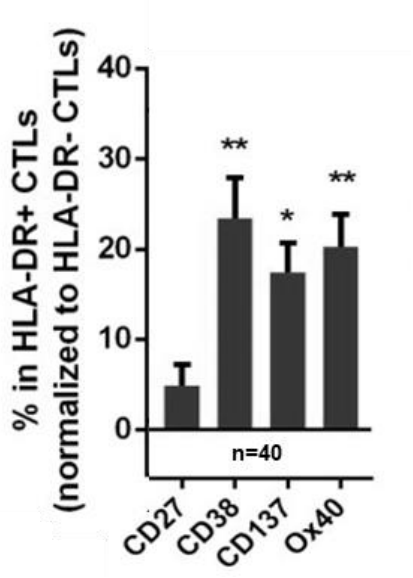


Figure III-20: Immune profile of HLA-DR+ CTLs compared to HLA-DR- CTLs. The % of HLA-DR+CTLs that also express the stimulatory co-receptors CD27, CD38, CD137 and Ox40, related to the % of HLA-DR-CTLs that also express those markers. Results reveal that in general all the markers analyzed are more expressed in HLA-DR+ CTLs compared to HLA-DR- CTLs (n=40). Data are represented as ± SEM, *p < 0.05, **p < 0.01.

The results revealed that Nivolumab significantly enhanced the anti-tumor activity of activated CTLs against MDA-MB-231 and HCC-1806 cell lines (p=0.0163, Figure III-21; Figure III-22 and Table III-1). This effect was consistent when employing either PBMCs from healthy donors or from patients with metastatic BC, who initially had low or undetectable levels of HLA-DR on CTLs (p=0.0487, Figure III-23). Accordingly, *in vitro* Nivolumab treatment increased the percentage of HLA-DR+ CTLs within PBMCs (Figure III-24).

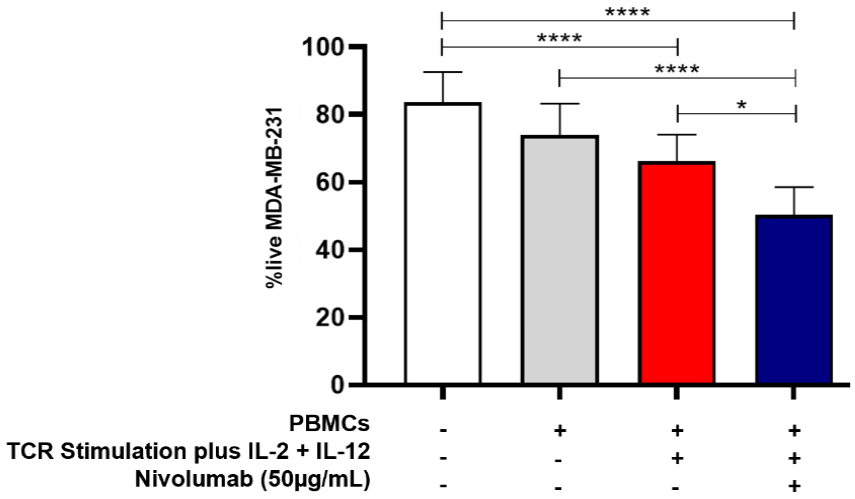


Figure III-21: Anti-PD-1 treatment potentiates the anti-tumor capacity of cytotoxic T lymphocytes (CTLs) against Breast Cancer (BC). The viability of the MDA-MD-231 BC cell line in the 3D co-culture alone (white bar, n=27), incubated in the presence of unstimulated PBMCs (grey bar, n=27), incubated in the presence of stimulated PBMCs (red bar, n=27) and incubated in the presence of stimulated PBMCs plus the addition of the anti-PD-1, Nivolumab (blue bar, n=22). The results demonstrated that the cytotoxic capacity of CTLs against cancer cells was synergistically enhanced with the stimulation protocol previously established and the addition of Nivolumab to the co-culture (p=0.0163). Data are represented as mean ± SD, **p < 0.05, ****p < 0.0001.*

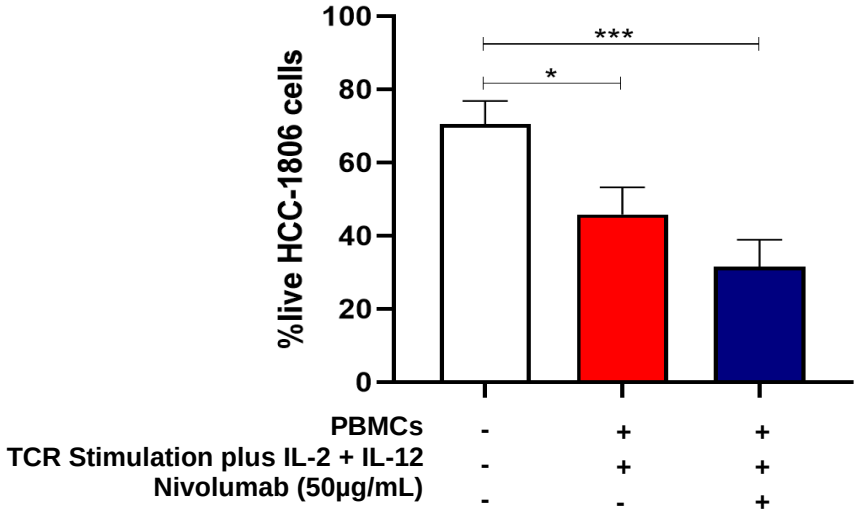


Figure III-22: Anti-PD-1 treatment promotes the anti-tumor capacity of cytotoxic T lymphocytes (CTLs) against the HCC-1806 Breast Cancer (BC) cell line. The viability of the HCC-1806 BC cell line in the 3D co-culture alone (white bar, n=8), incubated in the presence of stimulated PBMCs (red bar, n=8, p=0.0486) and incubated in the presence of stimulated PBMCs plus the addition of the anti-PD-1, Nivolumab (blue bar, n=8, p<0.0001), demonstrating that CTLs cytotoxicity improved synergistically with the stimulation protocol previously established and the addition of Nivolumab to the co-culture. Data are represented as mean ± SD, **p < 0.05, ***p < 0.001.*

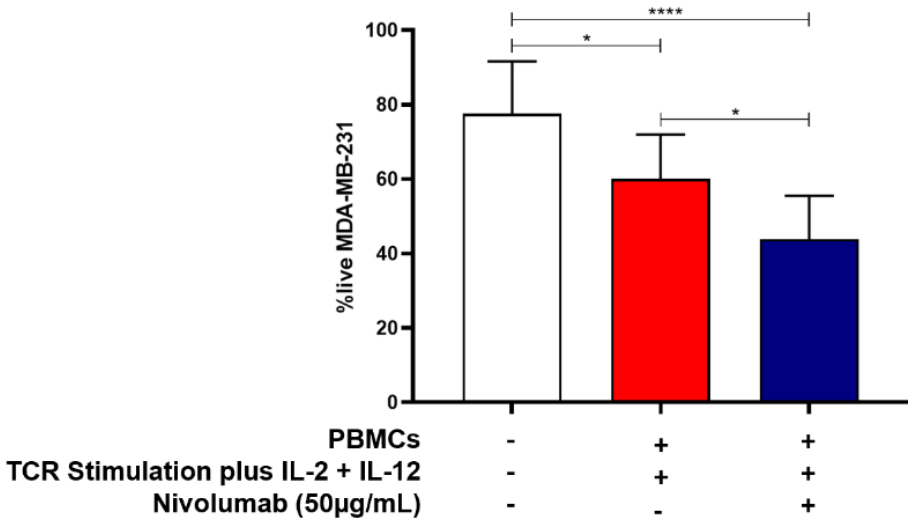


Figure III-23: Anti-PD-1 treatment potentiates the anti-tumor capacity of cytotoxic T lymphocytes (CTLs) against metastatic Breast Cancer (BC). The viability of the MDA-MD-231 BC cell line alone (white bar, n=27), incubated in the presence of stimulated PBMCs which have been isolated from metastatic BC patients (red bar, n=14) and incubated in the presence of stimulated PBMCs plus Nivolumab (blue bar, n=14, p=0.0487). These results were consistent with the results observed in Figure III-21 using PBMCs obtained from healthy donors. Data are represented as mean ± SD, **p < 0.05, ****p < 0.0001*.

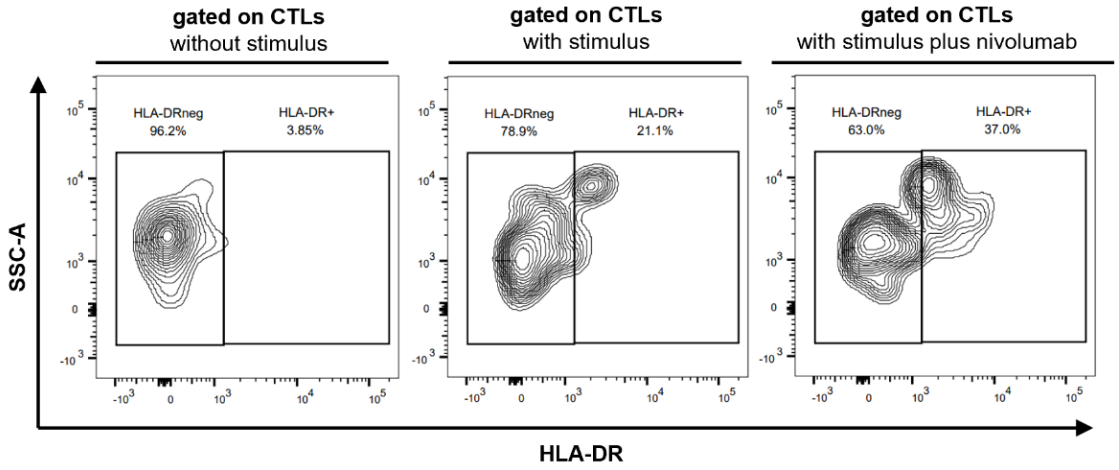


Figure III-24: Representative contour plot showing that anti-PD-1 treatment enhances the frequency of CTLs that express HLA-DR. *In vitro* Nivolumab treatment increased the percentage of HLA-DR+ CTLs within PBMCs.

Moreover, following the recent approval in Portugal (summer of 2023) for anti-PD-1 therapy for BC patients with recurrent, unresectable, or metastatic triple-negative breast cancer (TNBC) expressing PD-L1, we analyzed BC patients undergoing this treatment. Although, so far, we only have the follow-up sample of two patients, we observed an elevated frequency of HLA-DR+ CTLs in their blood four months after initiating anti-PD-1 therapy (Figure III-25), further corroborating our *in vitro* findings that anti-PD-1 treatment increases the percentage of HLA-DR-expressing CTLs.

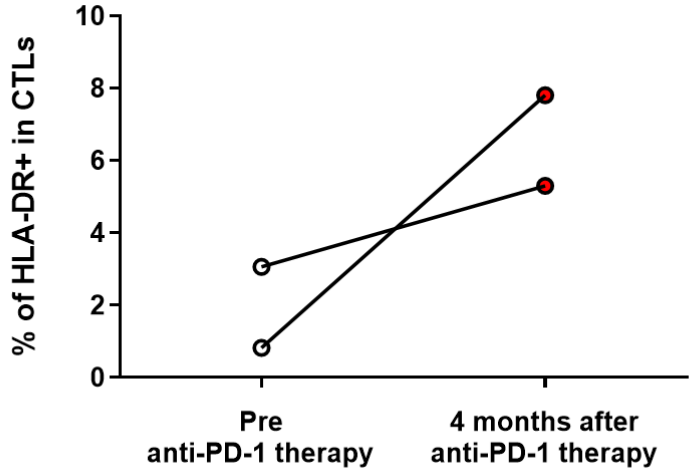


Figure III-25: Breast Cancer (BC) patients submitted to anti-PD-1 therapy exhibited an elevated frequency of Cytotoxic T Lymphocytes (CTLs) expressing HLA-DR in their blood. The BC patients undergoing anti-PD-1 therapy showed a higher percentage of CTLs expressing HLA-DR in their blood 4 months after starting this treatment. Each line represents one patient under anti-PD-1 therapy (n=2).

Contrary to Nivolumab, the other tested agents did not affect the expression of HLA-DR in CTLs nor their ability to kill the cancer cell lines. The results for these other agents are presented in Appendix III.

4.2. Evaluation of the effect of HLA-DR blockade on the efficacy of anti-PD-1 treatment in CTLs

Previously, we demonstrated that treatment with anti-PD-1 significantly increases the percentage of HLA-DR+ cytotoxic T lymphocytes (CTLs) and enhances their anti-tumor activity. Then, following these findings, we explored

the impact of HLA-DR blockade on the efficacy of anti-PD-1 treatment. To investigate this, we incorporated HLA-DR blockade into our 3D co-culture models and assessed how inhibiting HLA-DR affects CTLs cytotoxicity, in the presence of anti-PD-1 treatment.

Our results revealed that while Nivolumab enhanced the cytotoxicity of CTLs probably due to the increase of HLA-DR expression, the HLA-DR blockade diminished this effect (Figure III-26). Specifically, when HLA-DR was blocked during Nivolumab treatment, the CTLs cytotoxic capacity against BC cells decreased, evidenced by a lower loss of viability in this condition. This result strongly suggests that PD-1 treatment *per se* is not sufficient to increase CTLs function and that HLA-DR indeed plays a critical role in mediating the enhanced anti-tumor response (Figure III-26).

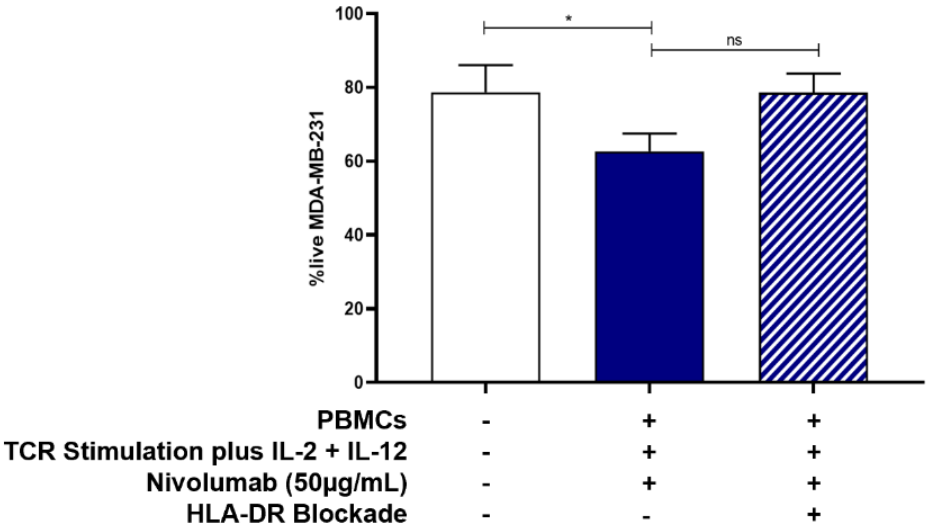


Figure III-26: HLA-DR blockade diminished the efficacy of anti-PD-1 treatment in cytotoxic T lymphocytes (CTLs). The viability of the MDA-MD-231 BC cell line alone (white bar, n=5), incubated in the presence of stimulated PBMCs plus anti-PD-1/Nivolumab (blue bar, n=5, p=0.0159), and incubated in the presence of stimulated PBMCs plus Nivolumab and an anti-HLA-DR blocking antibody (blue striped bar, n=5, non-statistical). This demonstrates that blocking HLA-DR impairs CTLs cytotoxicity against BC cells, even in the presence of Nivolumab. Data are represented as mean ± SD, **p < 0.05, ns= non-statistical.*

These findings further underscore the importance of taking HLA-DR expression into consideration when optimizing CTLs-based therapies. These data, together with results from the previous subsection, suggest that anti-PD-1 treatment has significant potential to boost CTLs cytotoxicity through

HLA-DR upregulation, especially in BC subtypes with high PD-L1 expression (Figure III-21, Figure III-22, Table III-1).

Despite the fact that anti-PD-1 immunotherapy has been primarily applied to triple-negative breast cancer (TNBC), which is known for its high immunogenicity, our analysis of immune features across the various BC subtypes - TNBC, ER+, and HER2+ - revealed similar frequencies of infiltrating CTLs and comparable PD-L1 expression levels (Figure III-27). This suggests that the benefits of CTLs-based therapy, after the cells are treated with anti-PD-1, could extend to other BC subtypes. Notably, since ER+ and HER2+ subtypes showed fewer HLA-DR-expressing CTLs (Figure III-27), these subtypes might derive even greater aid from such immunomodulatory treatment.

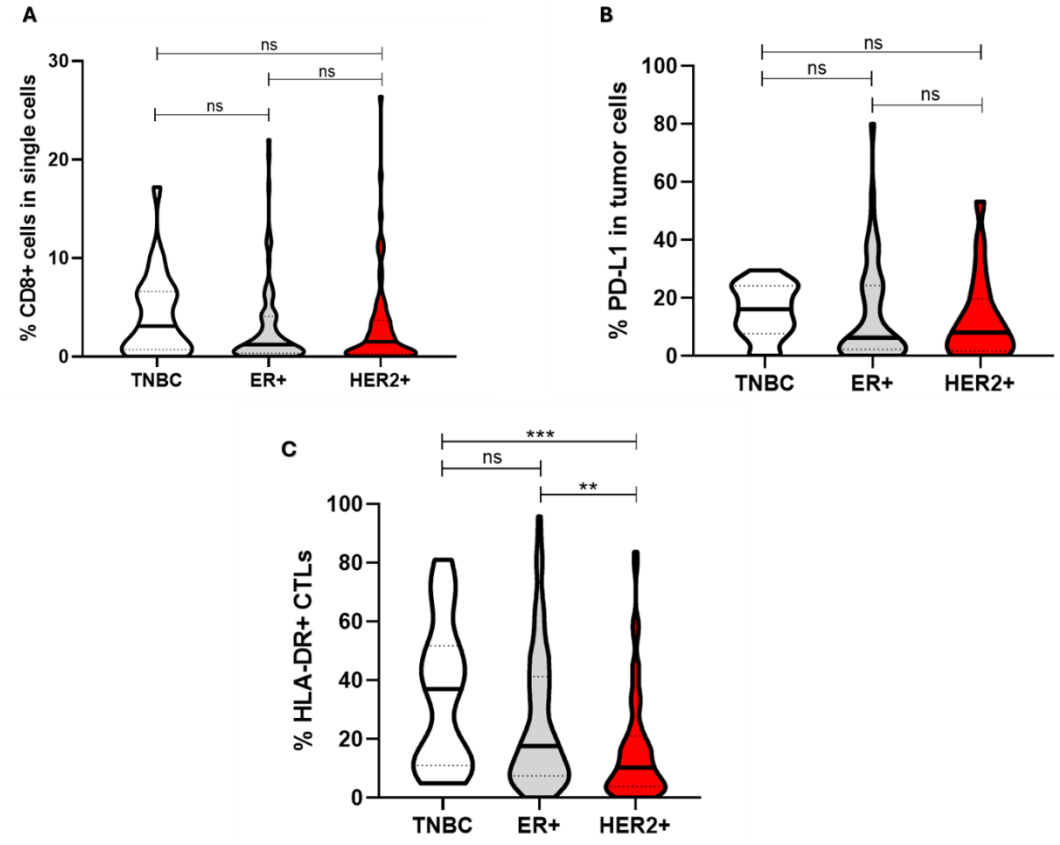


Figure III-27: Phenotyping of patients' biopsies across the three main Breast Cancer (BC) subtypes. Flow cytometry analysis of the immune profile of biopsies from a cohort of 195 BC patients. Only three relevant parameters were shown. **(A)** Percentage of infiltrating CTLs, presenting no significant differences between the analyzed subtypes: TNBC (n=26), HER2+ (n=74), and Luminal A and B (ER+, n=95). **(B)** Percentage of tumor cells expressing PD-L1, showing consistency of this marker expression across the analyzed subtypes: TNBC (n=16), HER2+ (n=20) and ER+ (n=47). **(C)** Percentage of HLA-DR-expressing infiltrating CTLs, demonstrating a significant difference between this parameter in TNBC cases (n=25, p= 0.0003) in comparison with HER2+ (n=69) and ER+ (n=91). Data are represented as mean ± SD, ***p < 0.01, ***p < 0.001, ns= non-statistical.*

4.3. Evaluation of sodium oxamate metabolic modulation on lactate production and CTLs activation

To further optimize CTLs efficacy we also explored metabolic modulation, alternatively to immunomodulation strategies.

Recent research has underscored the critical role of metabolic pathways in determining T cell functionality[170]. The idea of metabolic modulation to enhance CTLs arises from these findings, particularly the understanding that glycolysis can significantly impact T cell efficacy[171,172].

It has been demonstrated that the accumulation of lactate, a byproduct of glycolysis, creates an immunosuppressive environment that impairs T cell activation and efficacy[161,173,174]. Moreover, increasing evidence indicates that restricting glycolysis can help to preserve T cell effector functions[172].

These insights led us to hypothesize that metabolic modulation might lead to improved HLA-DR expression and consequently improve CTLs performance. To test this hypothesis, we investigated whether reducing lactate production could enhance CTLs activation. We used sodium oxamate, a well-known and widely used inhibitor of lactate production, as it hampers the activity of lactate dehydrogenase (LDH), the enzyme responsible for converting pyruvate into lactate. This conversion is a key step in glycolysis, a process that is often upregulated in tumor cells as they shift from oxidative phosphorylation to glycolysis for energy production, a phenomenon known as the Warburg effect[175–177]. In our study, we evaluated the effects of sodium oxamate on lactate levels in the supernatants of peripheral blood mononuclear cells (PBMCs) cultures and on CTLs functionality. We tested various concentrations of sodium oxamate to identify the optimal balance between reducing lactate levels and maintaining cell viability. Indeed, we observed that higher concentrations of sodium oxamate led to a slight decrease in the viability of immune cells (CD45+ cells, Figure III-28). However, our findings also corroborate that this metabolic inhibitor has the potential to reduce lactate production by PBMCs (Figure III-29).

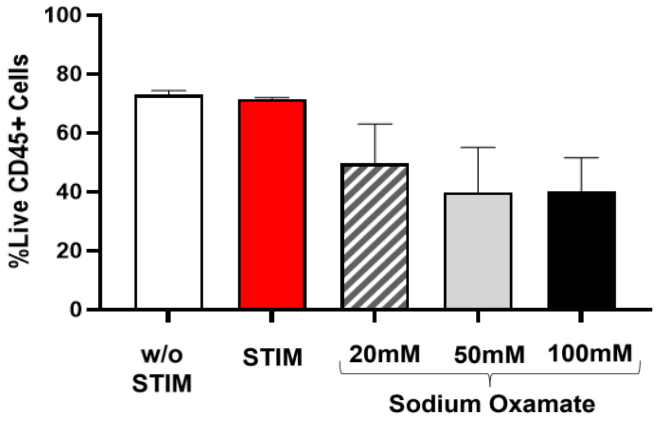


Figure III-28: Impact of sodium oxamate on the viability of immune cells (CD45+ cells). Viability of CD45+ immune cells in peripheral blood mononuclear cell (PBMC) cultures under different conditions: unstimulated PBMCs (white bar, n=3), PBMCs stimulated with T cell receptor activation (red bar, n=3), stimulated PBMCs treated with 20 mM sodium oxamate (grey striped bar, n=3), 50 mM sodium oxamate (grey bar, n=3), and 100 mM sodium oxamate (black bar, n=3). The data show a trend indicating that higher concentrations of sodium oxamate are associated with decreased viability of CD45+ cells. Data are expressed as mean ± SD.

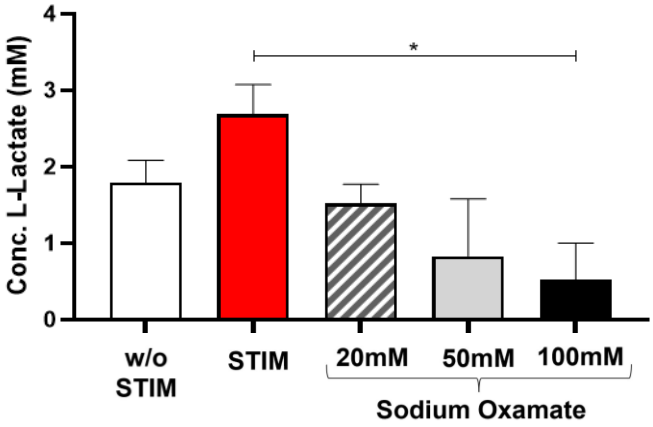


Figure III-29: Impact of sodium oxamate on lactate production in PBMCs cultures. The concentration of L-lactate (mM) was measured in the supernatants of peripheral blood mononuclear cells (PBMCs) cultures under various conditions: unstimulated PBMCs (white bar, n=3), PBMCs stimulated with T cell receptor activation (red bar, n=3), stimulated PBMCs treated with 20 mM sodium oxamate (grey striped bar, n=3), stimulated PBMCs treated with 50 mM sodium oxamate (grey bar, n=3), and stimulated PBMCs treated with 100 mM sodium oxamate (black bar, n=3). The data suggest a trend towards reduced lactate levels with increasing concentrations of sodium oxamate, although this reduction was not statistically significant across all tested concentrations. Results are presented as mean ± SD, *$*p < 0.05$*.

We chose to proceed with a lower concentration (20 mM) for subsequent experiments, as it strikes a good balance between minimizing loss of viability and reducing lactate production. However, treatment with 20 mM sodium oxamate for 72 hours did not lead to a significant increase of the percentage of CTLs expressing the classical activation markers, including HLA-DR (Figure III-30), which we previously identified as critical for CTLs cytotoxicity. Consequently, we concluded that metabolic modulation with sodium oxamate did not lead to the expected enhancement of CTLs functionality.

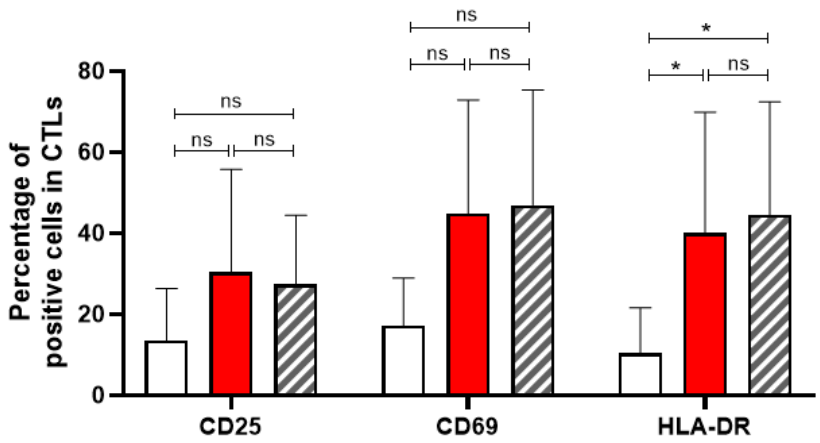


Figure III-30: Effect of sodium oxamate on CTLs activation markers. This figure shows the impact of 20 mM sodium oxamate treatment on CTLs activation markers (CD25, CD69, and HLA-DR) after a 72-hour incubation period. Cells were cultured under the following conditions: untreated (white bar, n=7), stimulated with T cell receptor activation (red bar, n=7), and stimulated with T cell receptor activation plus 20 mM sodium oxamate treatment (grey striped bar, n=7). Activation markers were evaluated to assess the effect of sodium oxamate on CTLs functionality. The results indicate that treatment with sodium oxamate did not result in significant improvements in activation markers of CTLs. Data are presented as mean ± SD, *$*p < 0.05$, ns= non-statistical.*

Several possible factors could explain the limited impact of sodium oxamate on CTLs activation. Firstly, while sodium oxamate effectively reduced lactate levels, it primarily targets glycolysis, which may not fully address the metabolic needs of different CTLs subsets. Different T cell subsets rely on distinct metabolic pathways to support their survival, function, and lineage generation. Naive T cells predominantly use oxidative phosphorylation (OXPHOS) for their energy needs, whereas effector T cells rely on glycolysis, fatty acid synthesis, and glutaminase to support proliferation and cytokine

production[178,179]. Sodium oxamate's inhibition of lactate production from glycolysis alone may be insufficient to reprogram CTLs effectively. Thus, targeting multiple metabolic pathways or providing additional metabolic support might be necessary to enhance CTLs functionality comprehensively. Furthermore, sodium oxamate's impact on cell viability at higher concentrations also suggests that achieving a balance between lactate reduction and cell viability is challenging. Future studies could explore alternative LDH inhibitors, alone or in combination, that have fewer effects on cell viability while providing stronger inhibition of lactate production.

Overall, although sodium oxamate did not improve CTLs functionality as anticipated, these results highlight the need for a more comprehensive strategy that targets multiple metabolic pathways to optimize CTLs-based cancer therapies. Unfortunately, due to time constraints, we were unable to further investigate this approach within the scope of this thesis.

Instead, given the promising effect of anti-PD-1 treatment in increasing HLA-DR+ CTLs within stimulated PBMCs and enhancing their anti-tumor activity, we decided to further investigate the underlying molecular mechanism. The following section will focus on our *in silico* analysis aimed at unraveling the regulatory networks involving HLA-DR and PD-1 in CTLs.

5. Potential links between HLA-DR and PD-1 pathways in CTLs

The specific interactions between HLA-DR and PD-1 molecules, especially in tumor-infiltrating immune cells such as cytotoxic T lymphocytes (CTLs), remain poorly understood. Comprehending the intricate regulatory networks involving the *HLA-DR* and *PD-1* genes is therefore critical for advancing immunotherapeutic strategies in the context of cancer treatment.

Thus, here, we aimed to explore the potential common regulatory networks involving HLA-DR and PD-1 in CTLs. By understanding these interactions at a deeper level, we can gain insights into how these molecules co-regulate and influence each other within the tumor microenvironment. This understanding is crucial for identifying new therapeutic targets and improving the efficacy of existing immunotherapies.

To achieve this goal, we collaborated with Carolina Mathias and Suelen Baal from LabCHO at the Department of Genetics, Universidade Federal do Paraná, Brazil, employing an *in silico* approach using single-cell RNA sequencing (scRNA-seq) data from breast cancer (BC) patients. This method allows us to analyze gene expression profiles at the single-cell level, providing a detailed view of the cellular heterogeneity and the specific genetic and molecular characteristics of CTLs. By understanding how distinct genetic profiles may influence cells' functionality, we can uncover the key regulatory elements involved in the modulation of HLA-DR and PD-1 expression. This is particularly important in the context of immunotherapy, where treatment efficacy can vary widely.

By focusing on individual cells, we can selectively evaluate the pathways that regulate the expression of HLA-DR and PD-1 specifically in CTLs (CD8+ T lymphocytes). Through this exploration, we hope to contribute to the development of more effective immunotherapeutic strategies and ultimately improve clinical outcomes for cancer patients.

5.1. Investigation of the common regulatory networks of HLA-DR and PD-1 in CTLs

To investigate the regulatory networks involved in HLA-DR and PD-1, we employed scRNA-seq data from BC patients sourced from the TIGER database (available at http://tiger.canceromics.org/#/), which serves as a repository for them. Our focus was on the gene expression profiles of CTLs (CD8+ T lymphocytes).

The analysis was conducted using R (version 4.4.0). Our methodology was systematically structured into three stages to identify genes correlated with *HLA-DR* and *PD-1*. In the first stage, we examined genes that were positively and negatively correlated with *HLA-DR* expression. In the second stage, we performed a parallel analysis for genes correlated with *PD-1*. In the final stage, we identified genes that demonstrated a correlation with both *HLA-DR* and *PD-1*. To ensure the robustness and reliability of our results, we applied a stringent correlation threshold of $|r| > 0.5$ and a p-value < 0.05, focusing on genes that were significant in at least three of the largest available datasets.

Interestingly, our analysis did not reveal any significant negative correlations for either *HLA-DR* or *PD-1*. As a result, our investigation focused exclusively on genes that were positively correlated with both markers.

For the HLA-DR analysis, we utilized data from five single-cell RNA sequencing (scRNA-seq) datasets of CD8+ T cells: BRCA_GSE150660, BRCA_GSE110686, BRCA_GSE114727_10X, BRCA_GSE161529, and BRCA_EMTAB8107 (Figure III-31). In parallel, for the PD-1 analysis, we examined data from four datasets: BRCA_GSE110686, BRCA_GSE114727_10X, BRCA_GSE161529, and BRCA_EMTAB8107 (Figure III-32).

Detailed information regarding the correlations and the identified genes is provided in Appendix IV to XII. We identified four genes - *CD74*, *CCL4*, *CCR5*, and *COTL1* - that were consistently present across all datasets related to genes positively correlated with *HLA-DR*. Similarly, for *PD-1*, ten genes - *TIGIT*, *TNFRSF9*, *IFNG*, *CXCR6*, *CTLA4*, *ZBED2*, *CD2*, *CLEC2D*, *CD3D*, and *PTPN7* - appeared in all analyzed datasets. These results underscore the essential roles of these gene sets in regulating immune responses, with HLA-DR-related

genes contributing to antigen processing and immune cell recruitment, while PD-1-associated genes are involved in immune regulation and T cell activation.

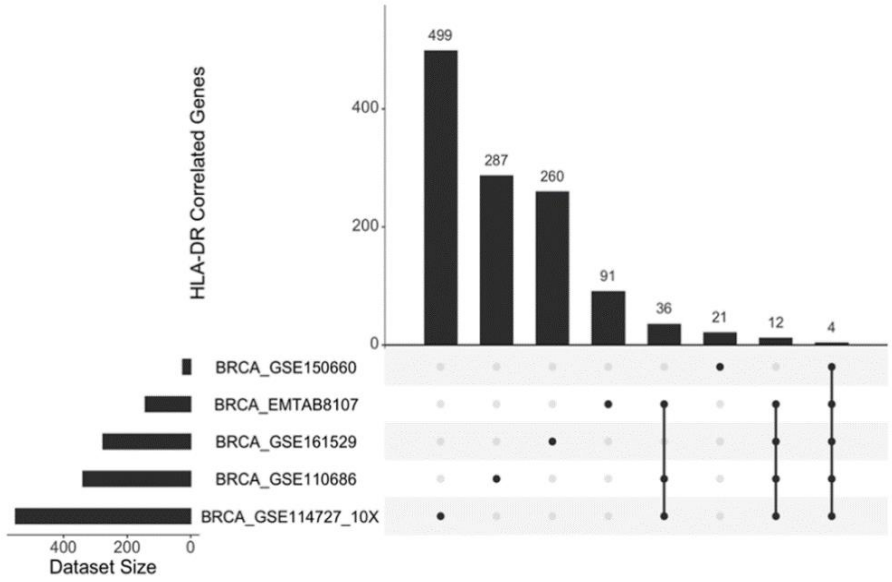


Figure III-31: Upset plot representing the number of genes positively correlated with HLA-DR. For HLA-DR, the dataset BRCA_GSE114727_10x was the one with the highest number of genes correlated with HLA-DR (n=499), and the dataset BRCA_GSE150660 the one with the lowest number (n=21).

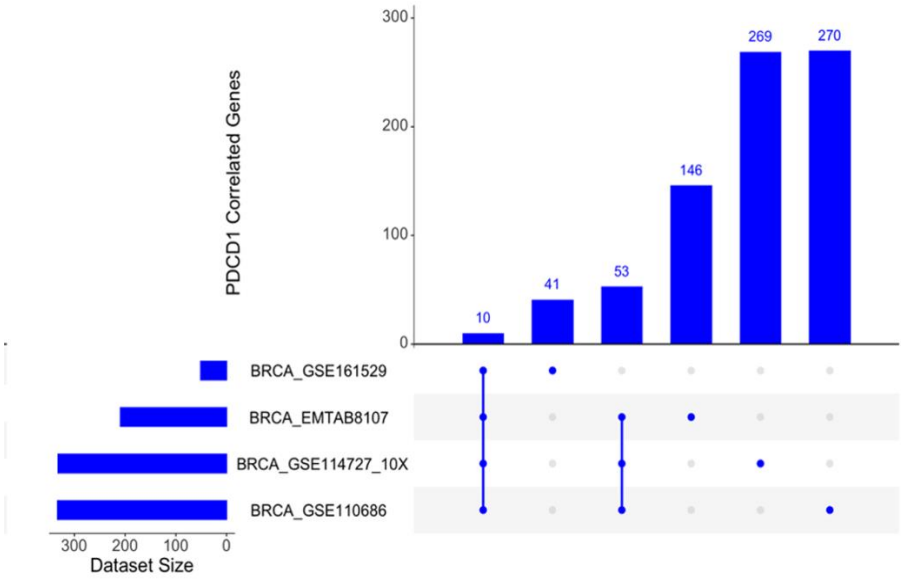


Figure III-32: Upset plot representing the number of genes positively correlated with PD-1. For PD-1 (PDCD1), the datasets BRCA_GSE110686 and BRCA_GSE114727_10X were the ones with the highest number of genes (n=270 and n=269, respectively).

Notably, our analysis identified 34 genes that were positively correlated with both *HLA-DR* and *PD-1* (Figure III-33). This subset of genes is particularly significant as it highlights potential shared regulatory mechanisms that could influence the co-expression of *HLA-DR* and *PD-1*. Understanding these interconnected pathways could reveal novel targets for enhancing immunotherapy strategies, potentially leading to more effective treatments.

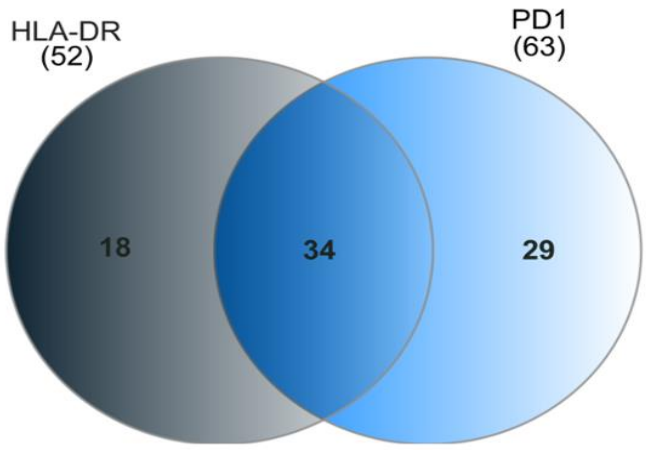


Figure III-33: Venn diagram representing exclusive and shared genes among HLA-DR and PD-1. The diagram highlights 34 genes that are positively correlated and shared among these genes.

5.2. Identification of non-coding RNAs correlated with HLA-DR and PD-1

In addition to identifying genes positively correlated with both *HLA-DR* and *PD-1*, our analysis revealed that several long non-coding RNAs (lncRNAs) are significantly associated with these molecules. Specifically, four of the five datasets containing 52 genes related to *HLA-DR* demonstrated positive correlations with lncRNAs. Similarly, for *PD-1*, among the 63 genes identified across at least three datasets, we also observed positive correlations with lncRNAs. Table III-2 presents all lncRNAs positively correlated with *HLA-DR* or *PD-1*, with *MIR155HG* and *KCNQ1OT1* highlighted due to their consistent presence across multiple datasets. The repeated identification of the lncRNAs

MIR155HG and *KCNQ1OT1* underscores their potential importance in the regulatory network linking HLA-DR and PD-1 expression.

Table III-2: Long non-coding RNA (lncRNAs) positively correlate with HLA-DR and PD-1 in each dataset. The lncRNAs *MIR155HG* and *KCNQ1OT1* are highlighted in bold as they are genes shared between *HLA-DR* and *PD-1*.

Dataset	Gene 1	Gene 2	Correlation value
BRCA_GSE110686	***HLA-DR***	***MIR155HG***	0.79
		KCNQ1OT1	0.81
		LINC01480	0.54
	PD-1	***MIR155HG***	0.79
		KCNQ1OT1	0.79
		AC133644.2	0.79
		LINC01480	0.60
BRCA_GSE114727_10X	***HLA-DR***	***MIR155HG***	0.68
		KCNQ1OT1	0.63
	PD-1	***MIR155HG***	0.85
		KCNQ1OT1	0.78
		AC133644.2	0.74
		AC017002.1	0.67
		LINC01480	0.61
BRCA_GSE161529	***HLA-DR***	***MIR155HG***	0.52
		LINC01857	0.55
	PD-1	*LINC01943*	0.56
BRCA_EMTAB8107	***HLA-DR***	***KCNQ1OT1***	0.67
		MIR155HG	0.59
	PD-1	***MIR155HG***	0.76
		KCNQ1OT1	0.74
		LINC01480	0.70
		AC133644.2	0.66

Effectively, although both HLA-DR and PD-1 have been extensively studied in tumor biology, their roles in tumor-infiltrating immune cells, particularly lymphocytes, and the links between these molecules are not well understood. Our analysis highlighted the lncRNAs *MIR155HG* and *KCNQ1OT1*, which are positively correlated with both *HLA-DR* and *PD-1*, exhibiting high correlation values. These data suggest that these genes are involved in the regulatory axis of *HLA-DR* expression in response to *PD-1* treatment, as previously observed in our *in vitro* experiments.

This finding not only deepens our understanding of the molecular interactions between HLA-DR and PD-1 but also emphasizes the need for continued investigation into the role of non-coding RNAs in shaping immune responses within the tumor microenvironment. Such research could provide insights into how lncRNAs modulate immune cell behavior and response to immunotherapy. It is crucial to explore the functional roles of *MIR155HG* and *KCNQ1OT1* further, as they might offer new avenues for therapeutic intervention. By unraveling these complex regulatory networks, we can enhance our ability to modulate immune responses for better clinical outcomes in cancer treatment.

CHAPTER IV

Discussion

1. Discussion

Breast cancer (BC) remains the most frequently diagnosed cancer among women and continues to be the leading cause of cancer-related mortality in this population, representing a substantial public health challenge. With up to 2 million new cases reported annually and an estimated 1 in 7 women expected to be diagnosed in their lifetime[79,143,144], the incidence and mortality rates of BC are projected to rise significantly in the coming years. This underscores the urgent need for collective efforts to mitigate this growing burden[84].

The multidisciplinary approach required to treat BC includes surgery, chemotherapy, radiotherapy, endocrine therapy, targeted therapy, and immunotherapy, with treatment plans tailored to the disease stage and patient-specific factors. For non-metastatic BC, surgery remains the cornerstone of treatment. Preoperative systemic therapies, particularly chemotherapy, play a crucial role in reducing tumor size, increasing the likelihood of breast-conserving surgery, and potentially preventing the need for axillary lymph node dissection. In contrast, the management of metastatic BC prioritizes systemic therapies, with surgery primarily reserved for palliative purposes in selected cases. Recent advancements in endocrine and targeted therapies, as well as immunotherapy, have expanded treatment options for both metastatic and non-metastatic BC patients. Additionally, ongoing research into novel therapies such as gene therapy, cancer vaccines, and adoptive cell therapies continues to show promising results[180].

However, despite recent advancements, BC treatment continues to face challenges due to its heterogeneity. BC is divided into four subtypes based on hormone receptor (HR) status and HER2 levels: luminal A (HR+/HER2−), luminal B (HR+/HER2+/-), HER2+ (HR−/HER2+) and triple-negative breast cancer (TNBC; HR−/HER2−). Each subtype presents distinct risk factors, therapeutic responses, and disease progression patterns, complicating the development of collectively effective treatments[87,129].

Neoadjuvant chemotherapy (NACT) is widely accepted for high-risk, locally advanced, or inoperable BC tumors[131]. However, less than 50% of patients achieve a pathological complete response (pCR). Nonetheless, the lack of

effective options for patients with chemotherapy-resistant tumors remains a substantial clinical challenge, especially for those ineligible for prompt surgical intervention[181,182].

Indeed, although the presence of tumor-infiltrating lymphocytes (TILs) within the tumor microenvironment (TME) has emerged as a crucial indicator of an anti-tumor immune response, often correlating with better patient prognosis and enhanced response to chemotherapy, the heterogeneity of immune infiltrates across different BC subtypes presents a significant challenge in developing efficient therapeutic strategies[97].

The identification of predictive biomarkers is essential for tailoring treatments that effectively restore or trigger anti-tumor immunity in individual patients. While PD-L1 expression assessed by immunohistochemistry is currently the most commonly used biomarker, especially for immunotherapy, its predictive value appears to be limited, primarily to metastatic TNBC[97].

Effectively, recognizing the challenges posed by immune escape mechanisms, immunotherapies offer a promising avenue for BC treatment, although their effectiveness can vary due to differences in patients' immune competency, tumor heterogeneity, and immune checkpoint expression levels[133]. The recent introduction of immune checkpoint inhibitors (ICIs) has revolutionized BC treatment, defying the prior assumption that breast tumors lack immunogenicity[96]. The success of ICIs in other malignancies and the growing evidence of the immune system's role in cancer progression and treatment response have driven a surge in clinical trials exploring various immune-based therapeutic strategies in BC. These trials emphasize the importance of understanding the TME and its immune constituents[97].

However, the immune evasion mechanisms employed by tumor cells, particularly the suppression of activated T cell responses, present significant challenges. This suppression is mediated by inhibitory interactions between immune checkpoint molecules such as PD-1, TIM-3, LAG-3, TIGIT, and CTLA-4, and their ligands on cancer cells, leading to T cell exhaustion and reduced therapeutic responsiveness. Consequently, the efficacy of ICIs as monotherapy in advanced BC has been modest[180].

Among the most extensively studied targets is the PD-1/PD-L1 axis, with numerous monoclonal antibodies developed to inhibit this pathway. Beyond PD-1, other immune checkpoints and stimulatory molecules are under investigation, with recent initiatives focusing on combination therapies aimed at enhancing response rates and achieving better survival outcomes for BC patients. Clinical trials have highlighted the limited response rates of ICIs when used as single agents in metastatic TNBC. Additionally, ICIs combined with chemotherapy have shown encouraging results in BC. For example, the KEYNOTE-522 trial demonstrated that combining pembrolizumab (anti-PD-1) with chemotherapy improved both pCR rates and event-free survival in TNBC patients. Similarly, the Impassion031 trial reported increased pCR rates with the addition of atezolizumab (anti-PD-L1) to standard chemotherapy regimens in TNBC. However, there is still limited data on the efficacy of ICIs in luminal and HER2-positive subtypes, highlighting the need for further research[180].

CAR-T therapy is also emerging as a potential immune-based treatment for BC, involving the engineering of patient-derived T cells to express chimeric antigen receptors (CARs) that specifically target cancer cells. While CAR-T therapy has shown remarkable success in treating hematologic malignancies, its application in solid tumors, such as BC, remains challenging due to its limited efficacy and feasibility in clinical trials. Currently, no CAR-T therapies are FDA-approved for solid tumors, including BC. Adoptive T-cell therapy (ATC) has also shown promise, particularly through the infusion of autologous T lymphocytes engineered to target neo-antigens, as seen in recent cases. Despite the potential of these therapies, their clinical integration is hindered by high costs, technical complexities, and associated toxicities[134,135].

Building on the concept that HLA-DR-expressing cytotoxic T lymphocytes (CTLs) play a crucial role in the tumor microenvironment by aiding in the elimination of cancer cells and exhibiting potent anti-tumor properties, we proposed that increasing the presence of CTLs with elevated HLA-DR expression could improve therapeutic approaches for BC[137,145]. Therefore, this thesis delves into the significance of HLA-DR-expression in CTLs and its potential to enhance the efficacy of CTL-based immunotherapies. To contribute to increase the effectiveness of anti-tumor treatments and

advance BC therapies, various strategies were explored, including the validation of the extraordinary ability of HLA-DR-expressing CTLs to kill tumor cells, the investigation of their interaction with CD4+ T cells, the optimization of a stimulation/expansion protocol and the identification of methods to increase HLA-DR-expression in CTLs. Additionally, an *in silico* analysis of BC scRNA-seq databases was performed to investigate genes commonly correlated with HLA-DR and PD-1 in CTLs.

To achieve these objectives, human biologic samples, including BC patients' blood and biopsies, were utilized.

1.1. HLA-DR expression on CTLs has predictive value and allows breast cancer cells' elimination

Following the prior research from the laboratory, establishing that high levels of HLA-DR on CTLs correlate with improved therapeutic outcomes following NACT[137,145], here, we validated the established predictive model in a new cohort of BC patients. This model uses HLA-DR expression to forecast responses to NACT, demonstrating its accuracy and reliability. Despite the small cohort, this validation reinforces the clinical utility of HLA-DR as a biomarker for predicting NACT efficacy, thereby supporting its potential integration into the clinical setting.

Moreover, other studies have been corroborating the importance of HLA-DR expression in CTLs to achieve effective responses to NACT in BC patients, particularly associating it with elevated plasma levels of IL-12 and IFN-γ[150].

Given the heterogeneity of BC, which poses a significant challenge in its therapeutic management, more effective and personalized treatment strategies are needed[183]. Thus, we also delve into the functional role of HLA-DR-expressing CTLs.

Our *ex vivo* experiments revealed that peripheral blood mononuclear cells (PBMCs) enriched with HLA-DR-expressing CTLs demonstrate superior cytotoxicity against BC cells compared to PBMCs with lower or no HLA-DR expression in CTLs. Additionally, *in vivo* validation using a zebrafish xenograft model, performed in collaboration with the Fior Lab (Champalimaud

Foundation), confirmed these findings, showing that an increased frequency of HLA-DR-expressing CTLs within the circulating immune cells leads to a significant reduction in tumor size.

Overall, this part of the work highlights the potential of HLA-DR both as a predictive biomarker and a functional enhancer of anti-tumor immunity in BC. Our findings suggest that therapeutic strategies designed to increase HLA-DR expression on CTLs could hold significant clinical value by enhancing the effectiveness of anti-tumor immune responses and improving treatment outcomes for BC patients.

1.2. HLA-DR in CTLs and CD4+ T cells are essential for effective CTLs activation and immune response

Then, we investigated the influence of HLA-DR expression on CTLs' activation and cytotoxic function. Since HLA-DR+ CTLs possess the machinery for antigen processing[184] and presentation to CD4+ T cells, we also investigated the necessity of these CD4+ T cells in boosting CTLs activity.

Specifically, our experiments revealed that blocking HLA-DR or depleting CD4+ T cells significantly reduced CTLs activation and cytotoxicity, as evidenced by decreased expression of key activation markers (CD25, CD69) and cytotoxic molecules (Granzyme B). This reduction emphasizes the necessity of CD4+ T cells, as well as HLA-DR in CTLs to support their activation and function.

According to the idea that HLA-DR presents antigens to the CD4+ T cells and that T-cell-T-cell synapses have been described[185], we hypothesize that HLA-DR-mediated antigen presentation not only primes CTLs for efficient recognition and elimination of tumor cells, but also reinforces their activation through interactions with CD4+ T cells. These CD4+ T cells, in turn, release cytokines such as IFN-γ, ensuring a more robust and sustained CTLs activity, ultimately improving their tumor-killing potential (Figure IV-1).

Indeed, our results underscore the importance of CD4+ T cells in enhancing CTLs-mediated anti-tumor responses. And, when placing our findings within

the broader context of the literature, recent studies also highlight the role of CD4+ T cells in cancer immunotherapy. While cancer immunotherapy has traditionally focused on CD8+ T cells due to their direct cytotoxic activity, emerging research shows that CD4+ T cells also play a direct anti-tumor role. Subsets of CD4+ T cells, such as Th1 cells, promote inflammation and tumor destruction, while regulatory T cells (Tregs) can suppress immune responses and limit the effectiveness of immunotherapy. This dual role of CD4+ T cells presents a challenge in designing immunotherapies: enhancing the Th1 response could boost anti-tumor activity, while increased Tregs activity might inhibit it[186]. Thus, the notion that HLA-DR-mediated interactions between CTLs and CD4+ T cells, lead to increased IFN-γ production, underscores the significance of pro-inflammatory, tumor-destructive Th1 cells in the success of immunotherapies. This supports the idea that targeting the HLA-DR-CTLs-CD4+ T cell axis could bolster anti-tumor immune responses. Consequently, future research should simultaneously take into consideration both CTLs and CD4+ T cells to improve cancer treatments, with a focus on optimizing HLA-DR signaling to ensure robust CTLs activation and more effective anti-tumor responses.

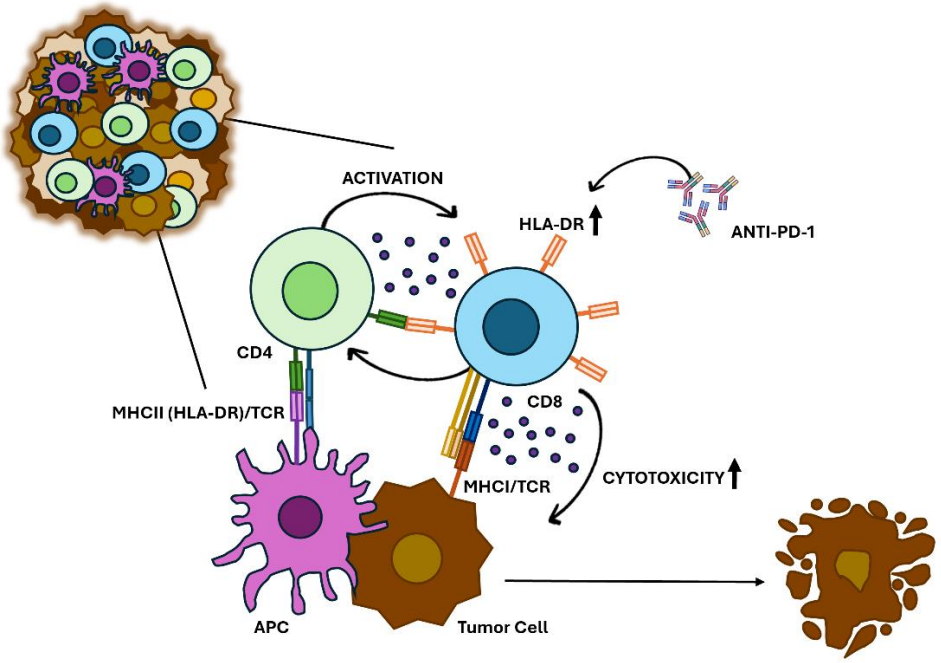


Figure IV-1: Proposed model for HLA-DR-expressing CTLs in anti-cancer immune response. HLA-DR-mediated antigen presentation primes CTLs to recognize and eliminate breast cancer cells. HLA-DR-expressing CTLs may also interact with CD4+ T cells, which release cytokines such as IFN-γ, reinforcing CTL activation and sustaining a robust immune response against tumors. Additionally, anti-PD-1 treatment increases the HLA-DR expression in CTLs and significantly boosts their cytotoxicity.

1.3. Therapeutic potential of CTLs could be boosted through short-term expansion

Subsequently, we investigated a T cell expansion protocol intended to increase the number of cells with cytotoxic capabilities. Our findings demonstrate that short-term stimulation of PBMCs with anti-CD3, anti-CD28, IL-2, and IL-12 significantly increases HLA-DR-expressing CTLs, which correlates with enhanced cytotoxicity of CTLs against BC cells. On the contrary, prolonged stimulation results in a higher total cell count but leads to reduced HLA-DR-expressing CTLs and diminished cytotoxic capacity.

These results are aligned with recent research exploring strategies to boost tumor immunity. For instance, pre-clinical models have shown that targeting IL-2 to CD8+ T cells promotes robust effector T cell responses and strong anti-tumor immunity[187].

Globally, our results underline the need for optimized T cell expansion protocols that balance cell quantity with functionality. By prioritizing both the functionality of CTLs and the quality of the immune response, we can pave the way for more effective and targeted BC therapies.

Still in this context, Nathan Frank, a senior scientist, highlighted the importance of optimizing T cell expansion protocols by noting that simply increasing the number of cells does not guarantee improved therapeutic outcomes. He emphasizes that there is a point at which expanding cells beyond a certain threshold does not enhance their efficacy and that achieving the right balance in the number of cells and their functional quality is crucial[188]. This perspective reinforces our approach of balancing cell quantity with functionality, underscoring that effective cancer immunotherapy requires a strategic focus on both aspects.

The emphasis on preserving immune cell functionality, rather than merely increasing cell numbers, is relevant when considering the existence of T cell exhaustion. Exhausted CD8+ T cells, which lose their ability to effectively combat cancer due to prolonged exposure to the tumor microenvironment, pose a significant challenge in cancer immunotherapy. This exhaustion is characterized by reduced cytotoxic activity and altered expression of

functional markers[189]. Our results suggest that maintaining HLA-DR expression through short-term stimulation helps to mitigate the loss of CTLs functionality, thus supporting more effective anti-tumor responses.

Furthermore, cancer immunotherapy has focused heavily on targeting tumor-associated antigens and neoantigens, with the assumption that the immune system's ability to recognize and attack these antigens is a key factor in therapeutic success. Neoantigens, which arise from mutations in cancer cells, are especially critical because they can generate specific immune responses that avoid tolerance mechanisms seen with normal tissue antigens. However, recent advancements in cancer immunotherapy have shifted the focus from the quantity of neoantigens present in tumors to their quality. While it was historically believed that a higher number of neoantigens would lead to stronger immune responses, it is now understood that the quality of these neoantigens, such as their ability to stimulate and sustain T cell responses, is equally important. Factors such as T cell receptor (TCR) affinity for neoantigens, antigen presentation via MHC molecules, and the tumor microenvironment significantly influence the effectiveness of these immune responses[190]. Indeed, although neoantigen-based therapies continue to play a central role in immunotherapy, our results highlight that this alone may not be sufficient for therapeutic efficacy. Therefore, maintaining the functionality of HLA-DR-expressing CTLs is essential. In this context, overexpressing HLA-DR on CTLs, with a focus on generating high-quality, effective immune responses, may be a promising approach to enhance cancer therapy outcomes.

1.4. Anti-PD-1 treatment contributes to increase HLA-DR in CTLs and amplifies their anti-tumor activity

Effective cancer treatment relies on optimizing the functionality of CTLs, which are essential for targeting and eliminating cancer cells. However, their efficacy is often limited in part by insufficient expression of key activation markers and various suppressive factors. HLA-DR, a recognized activation marker, plays a crucial role in CTLs activation and function. Considering this, here, we explored strategies to enhance HLA-DR expression on CTLs and

improve their cytotoxicity against BC cells. Utilizing our 3D co-culture model, we assessed various agents potentially able to modulate HLA-DR expression and CTLs function, including immune checkpoint inhibitors and co-receptor agonists. Additionally, we also explored metabolic modulation to determine if changing cellular metabolism could further improve CTLs functionality.

Our findings demonstrated that Nivolumab, an anti-PD-1 antibody, significantly enhances both HLA-DR expression and consequently the CTLs' cytotoxicity. These results in BC closely align with recent research by Skadborgin Signe K. *et al.* (2024) in glioblastoma, where Nivolumab was also shown to activate T cells and bolster the anti-tumor immune response[191]. Our observation that Nivolumab increases HLA-DR expression and enhances CTLs cytotoxicity suggests that treating PBMCs with anti-PD-1 could enhance CTL-mediated anti-tumor responses, not only because this antibody blocks inhibitory signals from PD-1/PD-L1 interactions, but also because it upregulates HLA-DR expression, probably promoting a positive feedback loop of cytokine release and sustained activation (Figure IV-1). Although HLA-DR and PD-1 have been extensively studied in tumor biology[150,184,185,187,192], there is limited understanding of the specific role of HLA-DR in tumor-infiltrating CTLs and its interplay with PD-1. For example, a study by Heng Yu *et al.* (2023) on laryngeal squamous cell carcinoma demonstrated that high tumor expression of HLA-DR correlates with an improved response to anti-PD-1 therapy. However, this study focused on HLA-DR expressed by tumor cells, not CTLs[193]. Similarly, studies in metastatic melanoma have observed a link between HLA-DR expression and response to anti-PD-1 therapy, but again, the focus was on tumor cells, featuring a gap in the current understanding of HLA-DR's role specifically in CTL-mediated responses during immunotherapy[194,195].

In addition to Nivolumab, we tested other agents, including anti-CD137, anti-CD134/OX40, and Bevacizumab. CD137 is known to regulate the activation of various immune cells[196], including CD8+ T cells, while the interaction between OX40 and OX40L (OX40/OX40L) promotes the expansion and proliferation of T cells and reduces immunosuppression by regulatory T cells (Tregs)[197]. Bevacizumab, an anti-angiogenic agent, has been shown to influence the clinical response in cancer patients by reducing the proportion of Tregs and

sustaining the circulation of effector T cells[164]. Thus, while all these agents were investigated for their potential ability to enhance HLA-DR expression and CTLs function, only Nivolumab produced a significant increase in HLA-DR+ CTLs and boosted their cytotoxic activity. Our lack of significant results with Bevacizumab aligns with the findings of Guelfi S. *et al.* (2024), who note that, despite the long history of targeting endothelial cells with functional-blocking antibodies, this approach has achieved only modest success[198].

Furthermore, we investigated the impact of HLA-DR blockade on anti-PD-1 therapy's efficacy. Our results indicated that blocking HLA-DR during Nivolumab treatment reduced the enhancement of CTLs cytotoxicity, emphasizing HLA-DR's critical role in mediating the anti-tumor response.

In contrast, our efforts to use metabolic modulation with sodium oxamate to reduce lactate production did not significantly improve CTLs functionality. It has been reported that glycolysis can impact T cell efficacy[171,172], and restricting glycolysis may help to preserve T cell effector functions[172]. Additionally, the accumulation of lactate, a byproduct of glycolysis, contributes to an immunosuppressive environment that hampers T cell activation and efficacy[173,174]. Nonetheless, our findings suggest that metabolic constraints alone may not fully account for CTLs performance, indicating that a broader approach targeting multiple metabolic pathways may be necessary to optimize CTLs function.

Overall, our study highlights the role of anti-PD-1 treatment in enhancing CTLs functionality through HLA-DR upregulation. Our findings suggest that combining short-stimulation of PBMCs with anti-PD-1 treatment could be a promising strategy to offer a more effective approach to increase HLA-DR in CTLs, thereby improving CTLs-based therapies and therapeutic outcomes in BC.

Future research should focus on understanding the regulatory networks involving HLA-DR and PD-1 in CTLs, exploring the synergistic effects of combined therapies.

1.5. Several ncRNAs are correlated with the expression of *HLA-DR* and *PD-1* genes in CTLs

Our findings indicate that anti-PD-1 treatment of PBMCs significantly enhances HLA-DR-expressing CTLs, thereby improving their anti-tumor activity. Although it is known that upon anti-PD-1 treatment, PD-1 expression typically increases in lymphocytes, as part of the normal activation process[199,200], the interplay between this molecule and the rise of HLA-DR in lymphocytes under stimulatory conditions, remains unclear.

Therefore, we delve into the intricate regulatory networks involving HLA-DR and PD-1 in CTLs.

To further investigate these interactions, in collaboration with LabCHO at the Department of Genetics, Universidade Federal do Paraná, Brazil, we utilized single-cell RNA sequencing (scRNA-seq) data from BC patients. Our analysis identified several genes consistently associated with *HLA-DR* and *PD-1* expression in CD8+ T Lymphocytes. Notably, genes such as *CD74*, *CCL4*, *CCR5*, and *COTL1* were positively correlated with *HLA-DR*, while genes related to *PD-1* included *TIGIT* and *IFN-γ*. Importantly, we identified 34 genes that were positively correlated with both *HLA-DR* and *PD-1*, suggesting potential shared regulatory mechanisms that could influence their co-expression.

In addition to protein-coding genes, we discovered significant involvement of long non-coding RNAs (lncRNAs) in regulating HLA-DR and PD-1 expression. LncRNAs are transcripts longer than 500 nucleotides, previously recognized primarily for their regulatory roles, but now also known to encode small functional peptides[201,202]. Among the lncRNAs identified in our study, *MIR155HG* and *KCNQ1OT1* emerged as key players in this regulatory network.

MIR155HG has been identified as a prognostic biomarker across various cancers, including BC. Its expression levels correlate with immune cell infiltration and the expression of immune checkpoint molecules such as PD-L1 and CTLA-4. This correlation suggests that *MIR155HG* plays a critical role in modulating immune responses and contributing to immune evasion by cancer cells. The interaction between *MIR155HG* and immune checkpoint

molecules highlights its potential as a therapeutic target for enhancing immune responses in cancer treatment[203].

Similarly, *KCNQ1OT1*, located on chromosome 11p15, has been implicated in several mechanisms affecting cancer progression. It influences gene regulation by interacting with RNA molecules and proteins and promotes immune escape by regulating PD-L1 ubiquitination through the miR-30a-5p/USP22 pathway[204]. This suggests that *KCNQ1OT1* is involved in modulating the immune microenvironment and contributing to cancer progression[204,205].

These findings highlight the crucial role of lncRNAs in both cancer progression and immune regulation. Recent research has demonstrated that lncRNAs, such as those identified in our study, can significantly impact immune responses and contribute to immunotherapy resistance[206]. LncRNAs influence immune checkpoints, modulate the tumor microenvironment, and interact with key immune signaling pathways, thus affecting the overall efficacy of immunotherapy[206–208]. Therefore, by regulating immune cell activation and differentiation, lncRNAs can either enhance or inhibit immune responses, presenting new opportunities for therapeutic interventions.

Our study highlights the critical role of lncRNAs in the regulatory networks involving HLA-DR and PD-1, providing valuable insights into how these molecules influence immune responses. Specifically, our data suggest that *MIR155HG* and *KCNQ1OT1* may be key players in the regulatory axis controlling HLA-DR expression in response to PD-1 modulation. Therefore, further in-depth studies to unravel the underlying molecular mechanisms and pathways are needed. In particular, we intend to expand bioinformatic approaches to explore these pathways, uncovering new strategies to enhance HLA-DR expression and offering promising directions for the development of targeted therapies that improve cancer treatment outcomes.

2. Perspectives and limitations of the current work

This thesis underscores the therapeutic potential of HLA-DR-expressing CTLs in BC and explores strategies to amplify their presence and functionality. It highlights the critical role that HLA-DR expression plays in the efficacy of CTLs

for tumor eradication. We propose that manipulating HLA-DR expression in CTLs could significantly enhance their ability to kill tumor cells, especially in BC patients with lower HLA-DR expression on their CTLs. This approach has the potential to be effective across multiple BC subtypes. Consequently, the insights derived from this study pave the way for developing personalized therapeutic interventions using the patient's own immune cells. Such advancements could transform BC treatment paradigms and potentially lead to higher rates of cancer-free survival.

However, despite these promising results, several avenues for future research remain. A deeper investigation into the specific regulatory networks involving HLA-DR and PD-1 in CTLs is needed. Better understanding the molecular interactions between HLA-DR and PD-1, particularly within the tumor microenvironment, could provide valuable insights for optimizing anti-PD-1 therapies and enhancing HLA-DR expression. Additionally, the roles of the lncRNAs *MIR155HG* and *KCNQ1OT1* in regulating HLA-DR and PD-1 need further exploration. These lncRNAs may play crucial roles in immune modulation and cancer progression, and their potential as therapeutic targets or biomarkers for personalized BC treatments should be investigated.

Another critical area for future research is the optimization of T cell expansion protocols. Our findings indicate that short-term stimulation is beneficial as it increases HLA-DR expression on CTLs, which is essential for their cytotoxic efficacy. However, refining these protocols to balance the quantity and quality of expanded cells is necessary for maintaining optimal cell functionality.

Moreover, while metabolic modulation with sodium oxamate did not show a significant improvement in CTLs' functionality in this study, exploring broader metabolic pathways and their effects on immune cell efficacy remains a promising research area. Understanding how metabolism influences CTLs performance could lead to new strategies for optimizing cancer immunotherapy. Examining the roles of epigenetic modifications and metabolic changes in CTLs could provide a more comprehensive understanding of the signaling pathways and regulatory mechanisms involved.

Additionally, it is also important to acknowledge the limitations of this study. The use of *in vitro* and xenograft models may not fully recapitulate the complexities of the tumor microenvironment and immune interactions observed *in vivo*. Furthermore, our 3D co-culture experiments employed allogeneic models, which might yield different results compared to autologous systems where both BC and immune cells are derived from the same patient. Also, to gain a comprehensive understanding of the interplay between HLA-DR expression, CTLs activation, and immune checkpoint blockade, validation in clinical settings with larger patient cohorts is essential. Such validation efforts could provide crucial insights into developing novel immunotherapeutic strategies targeting HLA-DR pathways to enhance anti-tumor immune responses. Furthermore, while our study focused primarily on short-term outcomes, further evaluation of the durability of immune responses and the long-term effects of the proposed therapies is necessary.

3. Major contributions of the current study to the research field

While immunotherapy has significantly advanced the field of cancer treatment, there remains a pressing need for more effective and personalized approaches. The complexity of cancer immunity and the tumor microenvironment present considerable challenges in developing universal therapies. While targeting T cells remains a promising approach[135], combining it with agents that modulate the immune response has become relevant, as currently available treatments may not be effective for all patients with advanced cancers. This underscores the importance of rational and precision immunotherapy strategies, which are patient-specific, informed by tumor type, and guided by biomarkers. A critical concern in this context is the already described hyperprogression phenomenon, where certain tumors grow faster following immunotherapy[209], underscoring the need for a more careful approach to treatment selection.

Our findings contribute to this precision-based field by demonstrating that enhancing HLA-DR expression in CTLs, through the combination of anti-PD-1 therapy and short-term stimulation, holds great promise for improving T cell-based therapies, particularly for BC patients with low or absent HLA-DR

expression in their CTLs. By focusing on the quality of CTLs rather than their quantity, we propose a strategy that could significantly enhance the effectiveness of immunotherapies, especially in a cancer as diverse as BC. This approach has the potential to shape future research directions, influence clinical practices, and inform treatment policies, ultimately leading to improved therapeutic outcomes for patients.

CHAPTER V

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CHAPTER VI

Appendix

APPENDIX

Appendix I. Informative document provided to breast cancer patients participating in our study**.**

NOVA MEDICAL SCHOOL

INFORMAÇÃO AO DADOR DE AMOSTRAS BIOLÓGICAS

Título do Projeto de Investigação: Estudo do papel dos linfócitos T citotóxicos com expressão de HLA-DR no prognóstico e tratamento do cancro da mama.

Objetivo do Estudo: A quimioterapia neoadjuvante é o tratamento padrão utilizado para o cancro da mama localmente avançado, embora vários pacientes não respondam a esta terapêutica. Para direcionar prontamente os doentes que à partida serão não-respondedores à quimioterapia neoadjuvante para outros tratamentos alternativos e mais eficientes, poupando-se tempo, recursos e evitando-se a exposição dos doentes à potencial toxicidade associada a esta terapêutica, existe uma necessidade urgente de se encontrar novos biomarcadores preditivos da resposta ao tratamento convencional e de se estabelecer terapias alternativas mais personalizadas. Nos últimos anos a utilização do sistema imunitário em tratamentos de cancro tem recebido especial foco pela comunidade científica devido à sua elevada eficácia e aos baixos efeitos secundários para os pacientes. O objetivo deste estudo é validar os linfócitos T citotóxicos (células do sistema imunitário) que expressam HLA-DR (um reconhecido marcador da ativação das células T) como um biomarcador de prognóstico da resposta dos pacientes à quimioterapia. Temos ainda como foco a otimização de um método de expansão *ex vivo* de linfócitos T citotóxicos que expressem HLA-DR, com o objetivo da sua utilização em protocolos de terapia celular. Assim, acreditamos que este estudo venha a contribuir para a implementação de métodos que revelem *a priori* como um paciente reagirá ao tratamento convencional (quimioterapia) e, portanto, seja útil para selecionar candidatos para uma terapia mais direcionada e menos agressiva.

Procedimentos: A participação neste estudo implica a colheita de amostras de sangue e/ou biópsias do tumor. A colheita de sangue ocorre antes do ínicio do tratamento. A colheita do sangue e da biópsia será efetuada sem alterar os procedimentos médicos habituais, preferencialmente no momento da colheita das análises regulares. As amostras serão preservadas em condições apropriadas e as informações clínicas relacionadas serão introduzidas numa base de dados, passando a sua identificação pessoal a estar codificada e não acessível aos utilizadores das amostras. Caso aceite participar neste estudo, a doação das amostras biológicas de sangue e/ou biópsia tumoral serão utilizadas única e exclusivamente para fins de investigação científica do projeto em cima citado. Os participantes que aceitem doar amostras para este projeto não seram sujeitos a qualquer tipo de risco adicional uma vez que todas as colheitas serão integradas nos protocolos de rotina de saúde.

Identificação das amostras e Confidencialidade: Após a colheita, as amostras serão identificadas por um código de forma a preservar a privacidade. Durante o desenvolvimento do projeto de investigação, a equipa de investigação terá necessidade de recolher informação do processo clínico para a execução do estudo. O anonimato será, contudo mantido, ou seja os dados constantes do seu processo clínico serão fornecidos ao investigador, mas sem qualquer tipo de identificação. Os dados serão tratados confidencialmente e resultados dos estudos realizados serão alvo de publicação de uma forma anónima e agregada, em termos de percentagens ou de dados numéricos, nunca individualmente.

Possíveis Benefícios para os Participantes: A sua participação proporcionará a aquisição de conhecimentos que poderão vir a beneficiá-lo a si ou a terceiros no futuro.

Riscos físicos previsíveis: Na maioria dos casos, os riscos e o desconforto associados serão mínimos ou inexistentes. Nas colheitas associadas a procedimentos com fins diagnósticos ou terapêuticos, os riscos e o desconforto serão os inerentes ao procedimento em si. Em qualquer dos casos, o doente será sempre antecipadamente informado dos riscos e grau de desconforto associados aos procedimentos.

Participação Voluntária e Direitos de Abandono: O dador terá toda a liberdade para se recusar a participar no estudo ou retirar o seu consentimento, suspendendo a participação em qualquer momento através do preenchendo um formulário próprio, mas sem necessidade explicação. Neste caso, as amostras biológicas, derivados e dados associados que estejam armazenados serão totalmente destruídos. A participação é, portanto, voluntária e a sua recusa em participar não envolverá qualquer penalização ou perda de benefícios. A recusa ou abandono não colocarão em risco o direito a receber tratamento ou assistência médica, presentemente ou no futuro.

Appendix II. Template of the informed consent forms utilized in our study.

CONFIDENCIAL

Termo de Consentimento para Doação de Sangue e Tecido

Tendo recebido convite para participar num Projeto de Investigação, a realizar na NOVA Medical School, Faculdade de Ciências Médicas da Universidade Nova de Lisboa, vem o signatário subscrever o presente Termo de Consentimento para Doação de Sangue, que será utilizado para fins de investigação.

Previamente solicitei esclarecimentos e informações, que me foram claramente transmitidos e me permitiram, com conhecimento suficiente, optar por subscrever o presente Termo de Consentimento.

A minha decisão foi ainda suportada pela garantia de que:

1) Todas as respostas às perguntas por mim formuladas tiveram pronta e clara resposta e o Técnico de Saúde que conduzirá o processo de recolha das amostras me informou sobre as matérias da sua área de intervenção;
2) A doação das minhas amostras serão de sangue e serão utilizadas única e exclusivamente para os efeitos supra indicados;
3) Os meus dados pessoais (idade, sexo, etc.) serão anónimos e serão registados separadamente dos componentes do sangue isolados, impossibilitando o rastreio das amostras;
4) Não será possível relacionar a amostra por mim doada com a minha pessoa;
5) Os dados genéticos recolhidos serão mantidos em anonimato, salvo se eu escolher saber esta informação;
6) Não sou atualmente portador de nenhuma doença infectocontagiosa;
7) A minha participação voluntária no estudo não me conferirá direitos a qualquer indemnização ou outros lucros financeiros;
8) Posso terminar a minha participação no estudo assim que quiser, sem repercussão na minha prestação de cuidados de saúde.

Sexo: M F Idade: _____

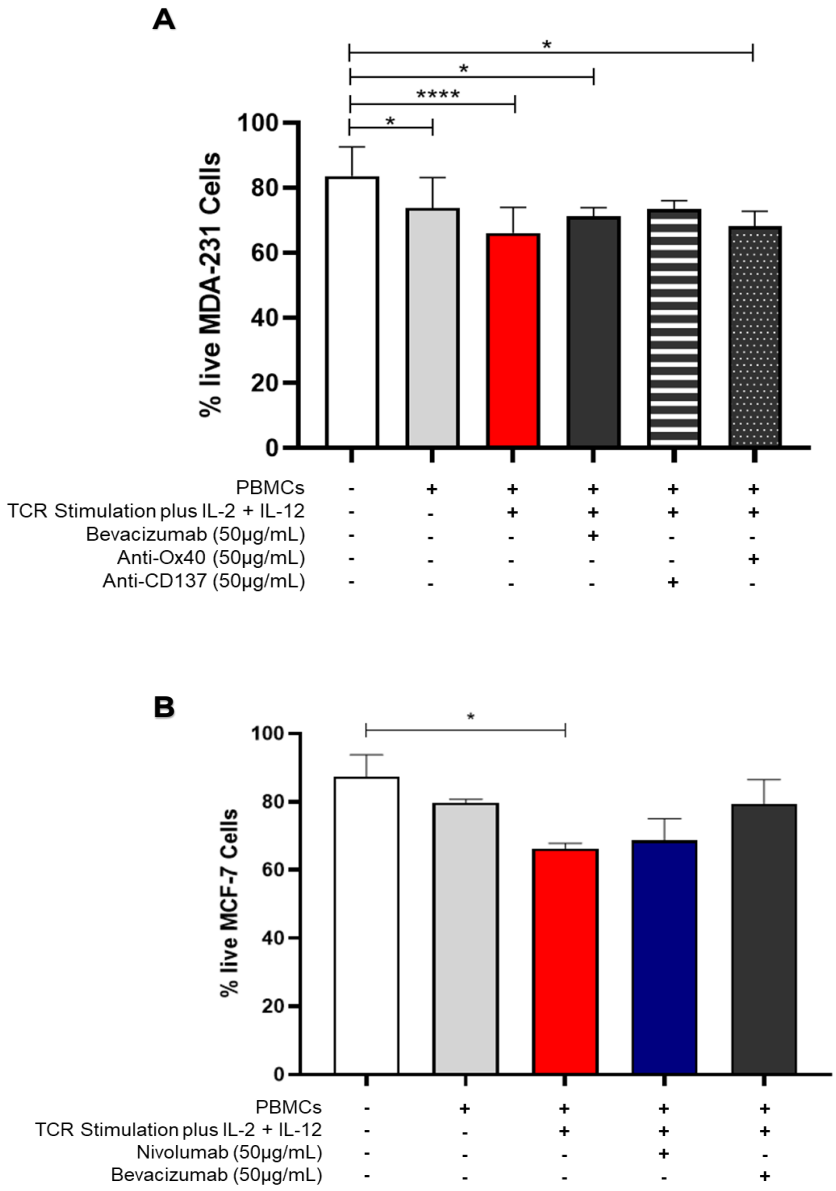
Nome do Médico Responsável: _____

Número da OM: _____

Assinatura ou Rubrica do Participante: _____

Lisboa, _____de _____de_____

Appendix III. Screening of potential agents to enhance the cytotoxic efficacy of cytotoxic T lymphocytes (CTLs) against breast cancer (BC) cells.



Appendix III: Evaluation of agents that could potentiate the anti-tumor activity of cytotoxic T lymphocytes (CTLs) against breast cancer (BC). **(A)** Viability of the MDA-MB-231 BC cell line in 3D co-culture under various conditions: co-culture alone (white bar, n=27), with unstimulated PBMCs (grey bar, n=27), with stimulated PBMCs (red bar, n=27), with stimulated PBMCs plus Bevacizumab (black bar, n=7), with stimulated PBMCs plus anti-OX40 (striped black and white bar, n=5), and with stimulated PBMCs plus anti-CD137 (dotted black and white bar, n=5). The results demonstrate that while the stimulation protocol effectively enhanced CTLs cytotoxicity, the addition of the tested agents did not provide any further improvement. **(B)** Viability of the MCF-7 BC cell line in 3D co-culture under similar conditions: co-culture alone (white bar, n=3), with unstimulated PBMCs (grey bar, n=3), with stimulated PBMCs (red bar, n=3), with stimulated PBMCs plus Nivolumab (blue bar, n=3) and with stimulated PBMCs plus Bevacizumab (black bar, n=5). The results similarly indicate that although the stimulation protocol enhanced CTLs cytotoxicity, the addition of the tested agents did not yield any further enhancement. Data are represented as mean ± SD, *$*p < 0.05$, $****p < 0.0001$.*

Appendix IV. List of genes significantly correlated with HLA-DR from BRCA_GSE150660 database.

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CD74*	CD8T	0.778
HLA-DRA	*GZMH*	CD8T	0.714
HLA-DRA	*CCL4*	CD8T	0.71
HLA-DRA	*CST7*	CD8T	0.685
HLA-DRA	*CCR5*	CD8T	0.646
HLA-DRA	*TMSB4X*	CD8T	0.643
HLA-DRA	*CCL5*	CD8T	0.623
HLA-DRA	*EOMES*	CD8T	0.603
HLA-DRA	*GZMA*	CD8T	0.597
HLA-DRA	*HIGD2A*	CD8T	0.58
HLA-DRA	*CD81*	CD8T	0.574
HLA-DRA	*SERF2*	CD8T	0.567
HLA-DRA	*ITGB2*	CD8T	0.554
HLA-DRA	*GZMK*	CD8T	0.549
HLA-DRA	*OAZ1*	CD8T	0.549
HLA-DRA	*COTL1*	CD8T	0.549
HLA-DRA	*CD8B*	CD8T	0.542
HLA-DRA	*NKG7*	CD8T	0.53
HLA-DRA	*EIF1*	CD8T	0.521
HLA-DRA	*CD8A*	CD8T	0.515
HLA-DRA	*PLEK*	CD8T	0.512

Appendix V. List of genes significantly correlated with HLA-DR from BRCA_GSE110686 database**.**

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CD74*	CD8T	0.884
HLA-DRA	*CXCL13*	CD8T	0.878
HLA-DRA	*LSP1*	CD8T	0.872
HLA-DRA	*CCL3*	CD8T	0.871
HLA-DRA	*GZMB*	CD8T	0.866
HLA-DRA	*HAVCR2*	CD8T	0.864
HLA-DRA	*CCL4*	CD8T	0.861
HLA-DRA	*TIGIT*	CD8T	0.859
HLA-DRA	*FABP5*	CD8T	0.857
HLA-DRA	*DUSP4*	CD8T	0.854
HLA-DRA	*PTMS*	CD8T	0.852
HLA-DRA	*GZMH*	CD8T	0.85
HLA-DRA	*LAG3*	CD8T	0.839
HLA-DRA	*ANXA5*	CD8T	0.835
HLA-DRA	*PRF1*	CD8T	0.832
HLA-DRA	*ID2*	CD8T	0.832
HLA-DRA	*GAPDH*	CD8T	0.829
HLA-DRA	*CXCR6*	CD8T	0.816
HLA-DRA	*AC069363.1*	CD8T	0.813
HLA-DRA	*VCAM1*	CD8T	0.812
HLA-DRA	*FKBP1A*	CD8T	0.806
HLA-DRA	*GZMA*	CD8T	0.806
HLA-DRA	*PDCD1*	CD8T	0.801
HLA-DRA	*PTTG1*	CD8T	0.8
HLA-DRA	*SIRPG*	CD8T	0.799
HLA-DRA	*CD27*	CD8T	0.798
HLA-DRA	*ACP5*	CD8T	0.797
HLA-DRA	*COTL1*	CD8T	0.796

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*GNLY*	CD8T	0.795
HLA-DRA	*DYNLL1*	CD8T	0.795
HLA-DRA	*PARK7*	CD8T	0.794
HLA-DRA	*MIR155HG*	CD8T	0.792
HLA-DRA	*NKG7*	CD8T	0.791
HLA-DRA	*YWHAH*	CD8T	0.789
HLA-DRA	*FASLG*	CD8T	0.788
HLA-DRA	*RABAC1*	CD8T	0.786
HLA-DRA	*IFNG*	CD8T	0.78
HLA-DRA	*APOBEC3G*	CD8T	0.778
HLA-DRA	*CLEC2B*	CD8T	0.771
HLA-DRA	*CCR5*	CD8T	0.767
HLA-DRA	*CST7*	CD8T	0.767
HLA-DRA	*CD8A*	CD8T	0.764
HLA-DRA	*RHOA*	CD8T	0.761
HLA-DRA	*CSF1*	CD8T	0.759
HLA-DRA	*APOBEC3C*	CD8T	0.758
HLA-DRA	*RGS2*	CD8T	0.757
HLA-DRA	*SH2D1A*	CD8T	0.757
HLA-DRA	*SIT1*	CD8T	0.756
HLA-DRA	*SUMO2*	CD8T	0.755
HLA-DRA	*PHLDA1*	CD8T	0.753
HLA-DRA	*TNFRSF9*	CD8T	0.749
HLA-DRA	*TRAC*	CD8T	0.749
HLA-DRA	*SYNGR2*	CD8T	0.748
HLA-DRA	*ABI3*	CD8T	0.748
HLA-DRA	*AC133644.2*	CD8T	0.745
HLA-DRA	*IDH2*	CD8T	0.741
HLA-DRA	*CLIC1*	CD8T	0.739
HLA-DRA	*IFI27L2*	CD8T	0.737

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*ALOX5AP*	CD8T	0.73
HLA-DRA	*PSMB3*	CD8T	0.729
HLA-DRA	*CCR1*	CD8T	0.725
HLA-DRA	*ITM2A*	CD8T	0.719
HLA-DRA	*SCAMP2*	CD8T	0.718
HLA-DRA	*KLRC1*	CD8T	0.711
HLA-DRA	*CD2*	CD8T	0.709
HLA-DRA	*PTPN6*	CD8T	0.709
HLA-DRA	*LITAF*	CD8T	0.709
HLA-DRA	*ITGB1*	CD8T	0.708
HLA-DRA	*CD8B*	CD8T	0.707
HLA-DRA	*BHLHE40*	CD8T	0.706
HLA-DRA	*UCP2*	CD8T	0.705
HLA-DRA	*IVNS1ABP*	CD8T	0.705
HLA-DRA	*CHST12*	CD8T	0.705
HLA-DRA	*DBI*	CD8T	0.704
HLA-DRA	*SRI*	CD8T	0.704
HLA-DRA	*MT1E*	CD8T	0.699
HLA-DRA	*LGALS1*	CD8T	0.698
HLA-DRA	*LAYN*	CD8T	0.694
HLA-DRA	*COPZ1*	CD8T	0.693
HLA-DRA	*SUB1*	CD8T	0.69
HLA-DRA	*CAP1*	CD8T	0.687
HLA-DRA	*SH2D2A*	CD8T	0.686
HLA-DRA	*EZR*	CD8T	0.682
HLA-DRA	*TXNDC17*	CD8T	0.682
HLA-DRA	*CCND2*	CD8T	0.678
HLA-DRA	*PGAM1*	CD8T	0.678
HLA-DRA	*BATF*	CD8T	0.677
HLA-DRA	*IFI27*	CD8T	0.675

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*MYL6*	CD8T	0.675
HLA-DRA	*HERPUD1*	CD8T	0.674
HLA-DRA	*CD63*	CD8T	0.673
HLA-DRA	*NRBP1*	CD8T	0.672
HLA-DRA	*CAPZB*	CD8T	0.67
HLA-DRA	*PKM*	CD8T	0.669
HLA-DRA	*CKS2*	CD8T	0.669
HLA-DRA	*CMC1*	CD8T	0.668
HLA-DRA	*HNRNPLL*	CD8T	0.668
HLA-DRA	*TRBC1*	CD8T	0.666
HLA-DRA	*CTSC*	CD8T	0.666
HLA-DRA	*HNRNPA3*	CD8T	0.664
HLA-DRA	*SOX4*	CD8T	0.664
HLA-DRA	*RHOC*	CD8T	0.663
HLA-DRA	*ID3*	CD8T	0.663
HLA-DRA	*IGFLR1*	CD8T	0.661
HLA-DRA	*CDK2AP2*	CD8T	0.659
HLA-DRA	*CD3D*	CD8T	0.659
HLA-DRA	*TRAPPC1*	CD8T	0.658
HLA-DRA	*ARPC2*	CD8T	0.658
HLA-DRA	*SH3BGRL3*	CD8T	0.657
HLA-DRA	*CARD16*	CD8T	0.656
HLA-DRA	*AIF1*	CD8T	0.656
HLA-DRA	*BUB3*	CD8T	0.655
HLA-DRA	*PRDX6*	CD8T	0.652
HLA-DRA	*COMMD7*	CD8T	0.649
HLA-DRA	*CD82*	CD8T	0.646
HLA-DRA	*HMGB1*	CD8T	0.641
HLA-DRA	*ZBED2*	CD8T	0.64
HLA-DRA	*CD79A*	CD8T	0.64

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*MT1X*	CD8T	0.638
HLA-DRA	*SRGN*	CD8T	0.637
HLA-DRA	*SERF2*	CD8T	0.634
HLA-DRA	*CCL5*	CD8T	0.631
HLA-DRA	*COX5A*	CD8T	0.631
HLA-DRA	*SRP14*	CD8T	0.63
HLA-DRA	*RBX1*	CD8T	0.629
HLA-DRA	*MCM5*	CD8T	0.629
HLA-DRA	*SLA*	CD8T	0.626
HLA-DRA	*CD2BP2*	CD8T	0.626
HLA-DRA	*HSPB1*	CD8T	0.625
HLA-DRA	*ENSA*	CD8T	0.623
HLA-DRA	*SCP2*	CD8T	0.623
HLA-DRA	*ADGRE5*	CD8T	0.623
HLA-DRA	*GALM*	CD8T	0.622
HLA-DRA	*HMGN3*	CD8T	0.621
HLA-DRA	*TPI1*	CD8T	0.618
HLA-DRA	*HSBP1*	CD8T	0.618
HLA-DRA	*HNRNPK*	CD8T	0.616
HLA-DRA	*NDUFC1*	CD8T	0.615
HLA-DRA	*DOK2*	CD8T	0.613
HLA-DRA	*BSG*	CD8T	0.613
HLA-DRA	*KLRC2*	CD8T	0.612
HLA-DRA	*VCP*	CD8T	0.612
HLA-DRA	*TMED9*	CD8T	0.609
HLA-DRA	*PSMB2*	CD8T	0.608
HLA-DRA	*CTLA4*	CD8T	0.608
HLA-DRA	*POMP*	CD8T	0.608
HLA-DRA	*UBB*	CD8T	0.607
HLA-DRA	*CFL1*	CD8T	0.607

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*SOD1*	CD8T	0.606
HLA-DRA	*NDUFB3*	CD8T	0.606
HLA-DRA	*LCK*	CD8T	0.605
HLA-DRA	*CALM3*	CD8T	0.603
HLA-DRA	*KIF20B*	CD8T	0.602
HLA-DRA	*CTSW*	CD8T	0.602
HLA-DRA	*KRT86*	CD8T	0.601
HLA-DRA	*BAX*	CD8T	0.6
HLA-DRA	*CALM2*	CD8T	0.599
HLA-DRA	*ARPP19*	CD8T	0.599
HLA-DRA	*PSMC4*	CD8T	0.598
HLA-DRA	*RGS1*	CD8T	0.598
HLA-DRA	*BIN1*	CD8T	0.597
HLA-DRA	*CLIC3*	CD8T	0.595
HLA-DRA	*NUDT5*	CD8T	0.594
HLA-DRA	*IL2RG*	CD8T	0.594
HLA-DRA	*CARHSP1*	CD8T	0.593
HLA-DRA	*PCMT1*	CD8T	0.593
HLA-DRA	*PYCARD*	CD8T	0.592
HLA-DRA	*TNFRSF1B*	CD8T	0.592
HLA-DRA	*H2AFZ*	CD8T	0.588
HLA-DRA	*OAZ1*	CD8T	0.588
HLA-DRA	*MCL1*	CD8T	0.587
HLA-DRA	*SAMSN1*	CD8T	0.586
HLA-DRA	*NELFCD*	CD8T	0.585
HLA-DRA	*SNRPD1*	CD8T	0.585
HLA-DRA	*PSMA7*	CD8T	0.583
HLA-DRA	*PPDPF*	CD8T	0.583
HLA-DRA	*B3GNT2*	CD8T	0.583
HLA-DRA	*PRDX5*	CD8T	0.583

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CCDC28B*	CD8T	0.582
HLA-DRA	*NUSAP1*	CD8T	0.582
HLA-DRA	*SURF4*	CD8T	0.581
HLA-DRA	*AP2S1*	CD8T	0.58
HLA-DRA	*TMEM165*	CD8T	0.58
HLA-DRA	*AIP*	CD8T	0.578
HLA-DRA	*SKA2*	CD8T	0.577
HLA-DRA	*ATP6V1E1*	CD8T	0.576
HLA-DRA	*PSMA1*	CD8T	0.575
HLA-DRA	*UBE2A*	CD8T	0.573
HLA-DRA	*EOMES*	CD8T	0.573
HLA-DRA	*PDIA3*	CD8T	0.572
HLA-DRA	*TANK*	CD8T	0.571
HLA-DRA	*CD247*	CD8T	0.57
HLA-DRA	*COX8A*	CD8T	0.57
HLA-DRA	*DNAJC8*	CD8T	0.569
HLA-DRA	*ACTB*	CD8T	0.567
HLA-DRA	*CLEC2D*	CD8T	0.565
HLA-DRA	*UBE2L3*	CD8T	0.564
HLA-DRA	*YWHAQ*	CD8T	0.561
HLA-DRA	*FERMT3*	CD8T	0.56
HLA-DRA	*YWHAE*	CD8T	0.558
HLA-DRA	*SLBP*	CD8T	0.558
HLA-DRA	*EID1*	CD8T	0.558
HLA-DRA	*CORO1A*	CD8T	0.557
HLA-DRA	*CD3E*	CD8T	0.556
HLA-DRA	*PRR13*	CD8T	0.555
HLA-DRA	*RNF167*	CD8T	0.554
HLA-DRA	*PFN1*	CD8T	0.554
HLA-DRA	*TMCO1*	CD8T	0.554

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*BRK1*	CD8T	0.554
HLA-DRA	*HSPB11*	CD8T	0.554
HLA-DRA	*NAP1L4*	CD8T	0.553
HLA-DRA	*SEC11A*	CD8T	0.552
HLA-DRA	*LARP7*	CD8T	0.551
HLA-DRA	*THYN1*	CD8T	0.551
HLA-DRA	*PDIA6*	CD8T	0.551
HLA-DRA	*HM13*	CD8T	0.55
HLA-DRA	*ARPC3*	CD8T	0.55
HLA-DRA	*PMF1*	CD8T	0.55
HLA-DRA	*DNPH1*	CD8T	0.548
HLA-DRA	*CMTM3*	CD8T	0.548
HLA-DRA	*SRSF9*	CD8T	0.547
HLA-DRA	*PSMB9*	CD8T	0.547
HLA-DRA	*ITM2C*	CD8T	0.547
HLA-DRA	*RAB11B*	CD8T	0.546
HLA-DRA	*CTSD*	CD8T	0.546
HLA-DRA	*ARPC1B*	CD8T	0.545
HLA-DRA	*PPP4C*	CD8T	0.544
HLA-DRA	*UBL5*	CD8T	0.544
HLA-DRA	*PRKAR1A*	CD8T	0.543
HLA-DRA	*LINC01480*	CD8T	0.543
HLA-DRA	*LBH*	CD8T	0.542
HLA-DRA	*MRPS16*	CD8T	0.541
HLA-DRA	*HMGN1*	CD8T	0.541
HLA-DRA	*LAMTOR5*	CD8T	0.54
HLA-DRA	*BLOC1S1*	CD8T	0.539
HLA-DRA	*KXD1*	CD8T	0.538
HLA-DRA	*GSTP1*	CD8T	0.538
HLA-DRA	*TMEM179B*	CD8T	0.538

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*AKR1B1*	CD8T	0.538
HLA-DRA	*HSD17B10*	CD8T	0.537
HLA-DRA	*SAP18*	CD8T	0.537
HLA-DRA	*SLC1A5*	CD8T	0.536
HLA-DRA	*PEF1*	CD8T	0.536
HLA-DRA	*MTIF*	CD8T	0.534
HLA-DRA	*RHBDD2*	CD8T	0.531
HLA-DRA	*SERPINB9*	CD8T	0.53
HLA-DRA	*ACTG1*	CD8T	0.53
HLA-DRA	*NDUFV2*	CD8T	0.529
HLA-DRA	*ARHGEF1*	CD8T	0.528
HLA-DRA	*HMGN2*	CD8T	0.528
HLA-DRA	*MRPS34*	CD8T	0.527
HLA-DRA	*EIF4E*	CD8T	0.527
HLA-DRA	*CYB5B*	CD8T	0.527
HLA-DRA	*PSMD4*	CD8T	0.526
HLA-DRA	*ASNA1*	CD8T	0.526
HLA-DRA	*CYCS*	CD8T	0.526
HLA-DRA	*PSMB1*	CD8T	0.523
HLA-DRA	*COX6A1*	CD8T	0.522
HLA-DRA	*NDUFA13*	CD8T	0.522
HLA-DRA	*SHKBP1*	CD8T	0.522
HLA-DRA	*PTPN7*	CD8T	0.522
HLA-DRA	*RASAL3*	CD8T	0.521
HLA-DRA	*B2M*	CD8T	0.521
HLA-DRA	*AP2M1*	CD8T	0.521
HLA-DRA	*TXN*	CD8T	0.521
HLA-DRA	*ARL6IP1*	CD8T	0.52
HLA-DRA	*RNF181*	CD8T	0.52
HLA-DRA	*PSMD8*	CD8T	0.519

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*FKBP8*	CD8T	0.519
HLA-DRA	*SMC4*	CD8T	0.519
HLA-DRA	*COMMD3*	CD8T	0.518
HLA-DRA	*CALR*	CD8T	0.517
HLA-DRA	*PSMD7*	CD8T	0.517
HLA-DRA	*COPE*	CD8T	0.516
HLA-DRA	*SNU13*	CD8T	0.516
HLA-DRA	*TMX1*	CD8T	0.516
HLA-DRA	*FDPS*	CD8T	0.514
HLA-DRA	*SEC61G*	CD8T	0.513
HLA-DRA	*PLEKHJ1*	CD8T	0.513
HLA-DRA	*ITGB2*	CD8T	0.513
HLA-DRA	*ITGB7*	CD8T	0.513
HLA-DRA	*TMEM59*	CD8T	0.513
HLA-DRA	*PHPT1*	CD8T	0.513
HLA-DRA	*HNRNPC*	CD8T	0.513
HLA-DRA	*CAPZA1*	CD8T	0.512
HLA-DRA	*TSPO*	CD8T	0.512
HLA-DRA	*CYBA*	CD8T	0.511

Appendix VI. List of genes significantly correlated with HLA-DR from BRCA_GSE114727_10X database.

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*GZMH*	CD8T	0.864
HLA-DRA	*CTSC*	CD8T	0.84
HLA-DRA	*ANXA5*	CD8T	0.828
HLA-DRA	*FABP5*	CD8T	0.82
HLA-DRA	*APOBEC3G*	CD8T	0.819
HLA-DRA	*SUB1*	CD8T	0.817
HLA-DRA	*IDH2*	CD8T	0.81
HLA-DRA	*LSP1*	CD8T	0.808
HLA-DRA	*APOBEC3C*	CD8T	0.806
HLA-DRA	*PTTG1*	CD8T	0.803
HLA-DRA	*CAP1*	CD8T	0.794
HLA-DRA	*COTL1*	CD8T	0.788
HLA-DRA	*PSMB9*	CD8T	0.78
HLA-DRA	*LAG3*	CD8T	0.777
HLA-DRA	*PTMS*	CD8T	0.773
HLA-DRA	*ARPC2*	CD8T	0.765
HLA-DRA	*CAPZB*	CD8T	0.765
HLA-DRA	*PPP1CA*	CD8T	0.763
HLA-DRA	*CXCR6*	CD8T	0.761
HLA-DRA	*CD74*	CD8T	0.755
HLA-DRA	*EVL*	CD8T	0.753
HLA-DRA	*POMP*	CD8T	0.752
HLA-DRA	*ALOX5AP*	CD8T	0.751
HLA-DRA	*RHOA*	CD8T	0.75
HLA-DRA	*CLIC1*	CD8T	0.744
HLA-DRA	*PDCD1*	CD8T	0.744
HLA-DRA	*CARD16*	CD8T	0.743
HLA-DRA	*CTSD*	CD8T	0.739

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*IFNG*	CD8T	0.737
HLA-DRA	*TPM3*	CD8T	0.737
HLA-DRA	*PRF1*	CD8T	0.736
HLA-DRA	*HMGB1*	CD8T	0.734
HLA-DRA	*COX5A*	CD8T	0.732
HLA-DRA	*set/07*	CD8T	0.732
HLA-DRA	*LCP1*	CD8T	0.73
HLA-DRA	*GZMA*	CD8T	0.728
HLA-DRA	*PYCARD*	CD8T	0.726
HLA-DRA	*CD2*	CD8T	0.725
HLA-DRA	*DUSP4*	CD8T	0.725
HLA-DRA	*RBCK1*	CD8T	0.722
HLA-DRA	*ZYX*	CD8T	0.722
HLA-DRA	*LCK*	CD8T	0.721
HLA-DRA	*GAPDH*	CD8T	0.721
HLA-DRA	*PSTPIP1*	CD8T	0.72
HLA-DRA	*ANXA6*	CD8T	0.718
HLA-DRA	*CCL3*	CD8T	0.718
HLA-DRA	*PSME2*	CD8T	0.717
HLA-DRA	*UBE2L3*	CD8T	0.717
HLA-DRA	*ACTB*	CD8T	0.716
HLA-DRA	*RAB1B*	CD8T	0.714
HLA-DRA	*PARK7*	CD8T	0.714
HLA-DRA	*CCR5*	CD8T	0.714
HLA-DRA	*REEP5*	CD8T	0.712
HLA-DRA	*IFI16*	CD8T	0.71
HLA-DRA	*WDR1*	CD8T	0.708
HLA-DRA	*MAP4K1*	CD8T	0.707
HLA-DRA	*ARPC3*	CD8T	0.706
HLA-DRA	*MSC*	CD8T	0.706

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CFL1*	CD8T	0.705
HLA-DRA	*RABAC1*	CD8T	0.704
HLA-DRA	*DBI*	CD8T	0.703
HLA-DRA	*BATF*	CD8T	0.702
HLA-DRA	*DYNLRB1*	CD8T	0.702
HLA-DRA	*PSMB3*	CD8T	0.702
HLA-DRA	*SHISA5*	CD8T	0.702
HLA-DRA	*CDK2AP2*	CD8T	0.702
HLA-DRA	*YWHAB*	CD8T	0.701
HLA-DRA	*MSN*	CD8T	0.7
HLA-DRA	*UBE2L6*	CD8T	0.7
HLA-DRA	*GNG5*	CD8T	0.7
HLA-DRA	*IFI27L2*	CD8T	0.699
HLA-DRA	*BTG3*	CD8T	0.699
HLA-DRA	*ADGRE5*	CD8T	0.698
HLA-DRA	*HSPB11*	CD8T	0.697
HLA-DRA	*PDIA6*	CD8T	0.697
HLA-DRA	*CD3D*	CD8T	0.695
HLA-DRA	*GZMB*	CD8T	0.694
HLA-DRA	*ITGAE*	CD8T	0.694
HLA-DRA	*FERMT3*	CD8T	0.691
HLA-DRA	*LY6E*	CD8T	0.69
HLA-DRA	*COPE*	CD8T	0.69
HLA-DRA	*GNAS*	CD8T	0.689
HLA-DRA	*CALR*	CD8T	0.689
HLA-DRA	*MYO1G*	CD8T	0.688
HLA-DRA	*B2M*	CD8T	0.687
HLA-DRA	*WAS*	CD8T	0.686
HLA-DRA	*ENSA*	CD8T	0.686
HLA-DRA	*PSMB8*	CD8T	0.686

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*PSMB10*	CD8T	0.685
HLA-DRA	*ACTR3*	CD8T	0.685
HLA-DRA	*AKIRIN2*	CD8T	0.685
HLA-DRA	*PSMD8*	CD8T	0.684
HLA-DRA	*EZR*	CD8T	0.683
HLA-DRA	*JAML*	CD8T	0.683
HLA-DRA	*MIR155HG*	CD8T	0.683
HLA-DRA	*SH3BP1*	CD8T	0.683
HLA-DRA	*PPIB*	CD8T	0.679
HLA-DRA	*LCP2*	CD8T	0.679
HLA-DRA	*ITM2A*	CD8T	0.678
HLA-DRA	*CORO1A*	CD8T	0.678
HLA-DRA	*ANXA2*	CD8T	0.678
HLA-DRA	*CSK*	CD8T	0.677
HLA-DRA	*DYNLL1*	CD8T	0.677
HLA-DRA	*MYH9*	CD8T	0.676
HLA-DRA	*BIN1*	CD8T	0.676
HLA-DRA	*SEC11A*	CD8T	0.676
HLA-DRA	*GPI*	CD8T	0.675
HLA-DRA	*RALY*	CD8T	0.675
HLA-DRA	*MYL6*	CD8T	0.674
HLA-DRA	*COPZ1*	CD8T	0.674
HLA-DRA	*TBCB*	CD8T	0.672
HLA-DRA	*CHST12*	CD8T	0.672
HLA-DRA	*CYBA*	CD8T	0.671
HLA-DRA	*RAC1*	CD8T	0.671
HLA-DRA	*TAP1*	CD8T	0.67
HLA-DRA	*PKM*	CD8T	0.669
HLA-DRA	*PSMA7*	CD8T	0.669
HLA-DRA	*PIN1*	CD8T	0.669

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*COMMD7*	CD8T	0.669
HLA-DRA	*TWF2*	CD8T	0.667
HLA-DRA	*ASNA1*	CD8T	0.666
HLA-DRA	*HNRNPA3*	CD8T	0.666
HLA-DRA	*PSMA6*	CD8T	0.666
HLA-DRA	*MRPL28*	CD8T	0.666
HLA-DRA	*TXN*	CD8T	0.665
HLA-DRA	*RBX1*	CD8T	0.665
HLA-DRA	*IL2RG*	CD8T	0.665
HLA-DRA	*PPP4C*	CD8T	0.664
HLA-DRA	*CASP4*	CD8T	0.664
HLA-DRA	*CCL4*	CD8T	0.664
HLA-DRA	*YWHAZ*	CD8T	0.662
HLA-DRA	*UBE2A*	CD8T	0.662
HLA-DRA	*BSG*	CD8T	0.661
HLA-DRA	*APBB1IP*	CD8T	0.66
HLA-DRA	*YWHAQ*	CD8T	0.659
HLA-DRA	*TNFRSF1B*	CD8T	0.658
HLA-DRA	*RAB11B*	CD8T	0.657
HLA-DRA	*UBE2D3*	CD8T	0.657
HLA-DRA	*ARPC5L*	CD8T	0.657
HLA-DRA	*PSMB1*	CD8T	0.656
HLA-DRA	*PLEKHF1*	CD8T	0.656
HLA-DRA	*MDH2*	CD8T	0.656
HLA-DRA	*TLN1*	CD8T	0.656
HLA-DRA	*HCLS1*	CD8T	0.656
HLA-DRA	*CD53*	CD8T	0.653
HLA-DRA	*CD3G*	CD8T	0.653
HLA-DRA	*CDCA7*	CD8T	0.652
HLA-DRA	*PSMB6*	CD8T	0.652

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*TXNDC17*	CD8T	0.651
HLA-DRA	*PPM1G*	CD8T	0.651
HLA-DRA	*PDZD11*	CD8T	0.651
HLA-DRA	*DEK*	CD8T	0.651
HLA-DRA	*ACP5*	CD8T	0.65
HLA-DRA	*SMC4*	CD8T	0.649
HLA-DRA	*PSMB2*	CD8T	0.649
HLA-DRA	*ARF1*	CD8T	0.649
HLA-DRA	*MT2A*	CD8T	0.649
HLA-DRA	*SRGN*	CD8T	0.648
HLA-DRA	*PGAM1*	CD8T	0.645
HLA-DRA	*NKG7*	CD8T	0.645
HLA-DRA	*GYG1*	CD8T	0.644
HLA-DRA	*ABI3*	CD8T	0.644
HLA-DRA	*PPM1M*	CD8T	0.643
HLA-DRA	*ARHGAP9*	CD8T	0.643
HLA-DRA	*GBP5*	CD8T	0.641
HLA-DRA	*ARHGDIA*	CD8T	0.641
HLA-DRA	*PSMA5*	CD8T	0.641
HLA-DRA	*FASLG*	CD8T	0.641
HLA-DRA	*TRAPPC1*	CD8T	0.639
HLA-DRA	*MTIF*	CD8T	0.639
HLA-DRA	*GNB2*	CD8T	0.639
HLA-DRA	*ARF5*	CD8T	0.639
HLA-DRA	*VAMP8*	CD8T	0.638
HLA-DRA	*RNF167*	CD8T	0.638
HLA-DRA	*CAPN2*	CD8T	0.637
HLA-DRA	*AP2M1*	CD8T	0.636
HLA-DRA	*CD63*	CD8T	0.636
HLA-DRA	*CD99*	CD8T	0.635

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*PPP1R18*	CD8T	0.634
HLA-DRA	*CALM3*	CD8T	0.634
HLA-DRA	*PARP1*	CD8T	0.634
HLA-DRA	*AC069363.1*	CD8T	0.633
HLA-DRA	*TMEM120A*	CD8T	0.632
HLA-DRA	*OTUB1*	CD8T	0.632
HLA-DRA	*CLTB*	CD8T	0.632
HLA-DRA	*TMEM50A*	CD8T	0.632
HLA-DRA	*RAC2*	CD8T	0.632
HLA-DRA	*MIEN1*	CD8T	0.632
HLA-DRA	*NDUFS6*	CD8T	0.631
HLA-DRA	*PDIA3*	CD8T	0.631
HLA-DRA	*BLOC1S1*	CD8T	0.63
HLA-DRA	*SSBP4*	CD8T	0.629
HLA-DRA	*SUMO2*	CD8T	0.629
HLA-DRA	*FKBP8*	CD8T	0.629
HLA-DRA	*BUB3*	CD8T	0.628
HLA-DRA	*PPP1R7*	CD8T	0.627
HLA-DRA	*NDUFA6*	CD8T	0.627
HLA-DRA	*RARRES3*	CD8T	0.627
HLA-DRA	*RNF213*	CD8T	0.626
HLA-DRA	*ZCRB1*	CD8T	0.625
HLA-DRA	*CTSW*	CD8T	0.625
HLA-DRA	*TBC1D10C*	CD8T	0.624
HLA-DRA	*SLC7A5*	CD8T	0.624
HLA-DRA	*CLEC2B*	CD8T	0.623
HLA-DRA	*IL32*	CD8T	0.623
HLA-DRA	*EWSR1*	CD8T	0.622
HLA-DRA	*GTF3C6*	CD8T	0.622
HLA-DRA	*VDAC1*	CD8T	0.619

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*AP2S1*	CD8T	0.619
HLA-DRA	*RPN1*	CD8T	0.619
HLA-DRA	*SRI*	CD8T	0.617
HLA-DRA	*ARPP19*	CD8T	0.616
HLA-DRA	*TMEM59*	CD8T	0.616
HLA-DRA	*NDUFB3*	CD8T	0.616
HLA-DRA	*SH3BGRL*	CD8T	0.616
HLA-DRA	*ACTG1*	CD8T	0.615
HLA-DRA	*F2R*	CD8T	0.613
HLA-DRA	*RNF181*	CD8T	0.612
HLA-DRA	*MFSD10*	CD8T	0.611
HLA-DRA	*SPCS2*	CD8T	0.61
HLA-DRA	*PRDX1*	CD8T	0.61
HLA-DRA	*SLC9A3R1*	CD8T	0.609
HLA-DRA	*SURF4*	CD8T	0.609
HLA-DRA	*UBE2N*	CD8T	0.609
HLA-DRA	*ITGB7*	CD8T	0.609
HLA-DRA	*GLIPR1*	CD8T	0.608
HLA-DRA	*ITGB2*	CD8T	0.607
HLA-DRA	*SH3BGRL3*	CD8T	0.607
HLA-DRA	*RGS1*	CD8T	0.606
HLA-DRA	*CD52*	CD8T	0.606
HLA-DRA	*RNF187*	CD8T	0.606
HLA-DRA	*NUDT21*	CD8T	0.605
HLA-DRA	*SP100*	CD8T	0.605
HLA-DRA	*CALCOCO2*	CD8T	0.605
HLA-DRA	*BRK1*	CD8T	0.605
HLA-DRA	*RER1*	CD8T	0.604
HLA-DRA	*CCDC167*	CD8T	0.604
HLA-DRA	*FKBP1A*	CD8T	0.604

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*EID1*	CD8T	0.604
HLA-DRA	*ZBP1*	CD8T	0.604
HLA-DRA	*LAMTOR5*	CD8T	0.604
HLA-DRA	*SCAMP2*	CD8T	0.603
HLA-DRA	*ORMDL2*	CD8T	0.603
HLA-DRA	*DCTN3*	CD8T	0.603
HLA-DRA	*ZNHIT1*	CD8T	0.603
HLA-DRA	*AIP*	CD8T	0.603
HLA-DRA	*HSBP1*	CD8T	0.602
HLA-DRA	*BUD31*	CD8T	0.602
HLA-DRA	*GSDMD*	CD8T	0.602
HLA-DRA	*DRAP1*	CD8T	0.602
HLA-DRA	*UBC*	CD8T	0.601
HLA-DRA	*SASH3*	CD8T	0.601
HLA-DRA	*TPI1*	CD8T	0.601
HLA-DRA	*HNRNPK*	CD8T	0.601
HLA-DRA	*UBB*	CD8T	0.601
HLA-DRA	*PSMC4*	CD8T	0.601
HLA-DRA	*BST2*	CD8T	0.601
HLA-DRA	*MYL12B*	CD8T	0.6
HLA-DRA	*MDH1*	CD8T	0.6
HLA-DRA	*CHMP2A*	CD8T	0.6
HLA-DRA	*CPNE7*	CD8T	0.598
HLA-DRA	*RNF7*	CD8T	0.597
HLA-DRA	*PPP2R1A*	CD8T	0.597
HLA-DRA	*SLA*	CD8T	0.596
HLA-DRA	*TBCD*	CD8T	0.595
HLA-DRA	*ARL6IP5*	CD8T	0.595
HLA-DRA	*MAGOH*	CD8T	0.595
HLA-DRA	*SEC11C*	CD8T	0.594

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*VASP*	CD8T	0.594
HLA-DRA	*MEA1*	CD8T	0.594
HLA-DRA	*ADRM1*	CD8T	0.594
HLA-DRA	*DCTN2*	CD8T	0.593
HLA-DRA	*ANP32A*	CD8T	0.593
HLA-DRA	*SLC1A5*	CD8T	0.593
HLA-DRA	*GNAI2*	CD8T	0.593
HLA-DRA	*IAH1*	CD8T	0.593
HLA-DRA	*LPXN*	CD8T	0.592
HLA-DRA	*SSNA1*	CD8T	0.592
HLA-DRA	*GUK1*	CD8T	0.592
HLA-DRA	*COX6A1*	CD8T	0.592
HLA-DRA	*SERPINB1*	CD8T	0.592
HLA-DRA	*GSTP1*	CD8T	0.592
HLA-DRA	*PSMA4*	CD8T	0.592
HLA-DRA	*UBE2I*	CD8T	0.591
HLA-DRA	*AURKAIP1*	CD8T	0.591
HLA-DRA	*KIF5B*	CD8T	0.591
HLA-DRA	*NELFCD*	CD8T	0.591
HLA-DRA	*JOSD2*	CD8T	0.59
HLA-DRA	*CHMP1A*	CD8T	0.59
HLA-DRA	*TSPO*	CD8T	0.59
HLA-DRA	*ANAPC11*	CD8T	0.59
HLA-DRA	*SHKBP1*	CD8T	0.59
HLA-DRA	*PSMC3*	CD8T	0.59
HLA-DRA	*UCP2*	CD8T	0.589
HLA-DRA	*C11orf58*	CD8T	0.589
HLA-DRA	*TMEM258*	CD8T	0.589
HLA-DRA	*AP1S1*	CD8T	0.589
HLA-DRA	*TNFRSF9*	CD8T	0.588

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*SRSF9*	CD8T	0.588
HLA-DRA	*DR1*	CD8T	0.588
HLA-DRA	*TYMP*	CD8T	0.587
HLA-DRA	*COMMD8*	CD8T	0.587
HLA-DRA	*RASAL3*	CD8T	0.586
HLA-DRA	*PSMD4*	CD8T	0.586
HLA-DRA	*NDUFS7*	CD8T	0.586
HLA-DRA	*PSME1*	CD8T	0.586
HLA-DRA	*GBP2*	CD8T	0.585
HLA-DRA	*OPTN*	CD8T	0.585
HLA-DRA	*SCAND1*	CD8T	0.585
HLA-DRA	*POLD4*	CD8T	0.584
HLA-DRA	*CCL5*	CD8T	0.584
HLA-DRA	*ATP6V0E1*	CD8T	0.584
HLA-DRA	*COX7A2*	CD8T	0.584
HLA-DRA	*COX7B*	CD8T	0.583
HLA-DRA	*MPG*	CD8T	0.583
HLA-DRA	*CXCL13*	CD8T	0.583
HLA-DRA	*MRPS36*	CD8T	0.583
HLA-DRA	*RBM42*	CD8T	0.583
HLA-DRA	*SNRPB*	CD8T	0.583
HLA-DRA	*GLUD1*	CD8T	0.582
HLA-DRA	*CD2BP2*	CD8T	0.582
HLA-DRA	*SNRPB2*	CD8T	0.582
HLA-DRA	*PRR13*	CD8T	0.582
HLA-DRA	*BAX*	CD8T	0.581
HLA-DRA	*VAMP5*	CD8T	0.581
HLA-DRA	*CCL4L2*	CD8T	0.58
HLA-DRA	*TSTA3*	CD8T	0.58
HLA-DRA	*SIT1*	CD8T	0.58

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*TMEM179B*	CD8T	0.579
HLA-DRA	*FIBP*	CD8T	0.579
HLA-DRA	*TIGIT*	CD8T	0.579
HLA-DRA	*NDUFA1*	CD8T	0.578
HLA-DRA	*SNRPG*	CD8T	0.576
HLA-DRA	*PUF60*	CD8T	0.575
HLA-DRA	*WDR83OS*	CD8T	0.575
HLA-DRA	*NDUFB10*	CD8T	0.575
HLA-DRA	*PGK1*	CD8T	0.574
HLA-DRA	*SUMO1*	CD8T	0.574
HLA-DRA	*TALDO1*	CD8T	0.574
HLA-DRA	*LAMTOR2*	CD8T	0.574
HLA-DRA	*SNX6*	CD8T	0.574
HLA-DRA	*GOLGA7*	CD8T	0.573
HLA-DRA	*COX8A*	CD8T	0.573
HLA-DRA	*POLR2G*	CD8T	0.573
HLA-DRA	*LGALS1*	CD8T	0.572
HLA-DRA	*SELPLG*	CD8T	0.572
HLA-DRA	*TIMM8B*	CD8T	0.572
HLA-DRA	*VPS28*	CD8T	0.572
HLA-DRA	*CASP1*	CD8T	0.571
HLA-DRA	*NAA38*	CD8T	0.571
HLA-DRA	*SEC61B*	CD8T	0.571
HLA-DRA	*ITGB1BP1*	CD8T	0.57
HLA-DRA	*CMTM3*	CD8T	0.57
HLA-DRA	*PFN1*	CD8T	0.57
HLA-DRA	*TCIRG1*	CD8T	0.569
HLA-DRA	*MIIP*	CD8T	0.569
HLA-DRA	*UBL5*	CD8T	0.569
HLA-DRA	*PET100*	CD8T	0.568

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*IL2RB*	CD8T	0.567
HLA-DRA	*YWHAH*	CD8T	0.567
HLA-DRA	*SF3B6*	CD8T	0.567
HLA-DRA	*TMEM14A*	CD8T	0.566
HLA-DRA	*XRCC5*	CD8T	0.566
HLA-DRA	*CTLA4*	CD8T	0.566
HLA-DRA	*C4orf48*	CD8T	0.566
HLA-DRA	*CALM2*	CD8T	0.566
HLA-DRA	*ANXA11*	CD8T	0.564
HLA-DRA	*PDCD6*	CD8T	0.564
HLA-DRA	*GABARAPL1*	CD8T	0.564
HLA-DRA	*TADA3*	CD8T	0.563
HLA-DRA	*TPM4*	CD8T	0.563
HLA-DRA	*LSM2*	CD8T	0.562
HLA-DRA	*ILK*	CD8T	0.562
HLA-DRA	*TRAC*	CD8T	0.562
HLA-DRA	*HCST*	CD8T	0.562
HLA-DRA	*ARRDC1*	CD8T	0.562
HLA-DRA	*TMX4*	CD8T	0.562
HLA-DRA	*HMGN2*	CD8T	0.561
HLA-DRA	*CDC42*	CD8T	0.56
HLA-DRA	*CKS2*	CD8T	0.56
HLA-DRA	*SEC61G*	CD8T	0.56
HLA-DRA	*HSD17B10*	CD8T	0.56
HLA-DRA	*MOB1A*	CD8T	0.559
HLA-DRA	*CST7*	CD8T	0.559
HLA-DRA	*OASL*	CD8T	0.558
HLA-DRA	*LSM8*	CD8T	0.558
HLA-DRA	*SAMD9L*	CD8T	0.557
HLA-DRA	*MPC2*	CD8T	0.556

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CD8A*	CD8T	0.556
HLA-DRA	*ROMO1*	CD8T	0.556
HLA-DRA	*MAD2L2*	CD8T	0.556
HLA-DRA	*PAIP2*	CD8T	0.555
HLA-DRA	*RAB7A*	CD8T	0.555
HLA-DRA	*IFI35*	CD8T	0.555
HLA-DRA	*COX6B1*	CD8T	0.555
HLA-DRA	*MAP1LC3B*	CD8T	0.555
HLA-DRA	*ISG15*	CD8T	0.554
HLA-DRA	*SAP18*	CD8T	0.554
HLA-DRA	*ITGB1*	CD8T	0.554
HLA-DRA	*CFLAR*	CD8T	0.553
HLA-DRA	*ARHGAP30*	CD8T	0.553
HLA-DRA	*SF3B2*	CD8T	0.553
HLA-DRA	*DUSP23*	CD8T	0.552
HLA-DRA	*NABP2*	CD8T	0.552
HLA-DRA	*SDF2L1*	CD8T	0.552
HLA-DRA	*NDUFAB1*	CD8T	0.551
HLA-DRA	*SH2D2A*	CD8T	0.551
HLA-DRA	*RAP1B*	CD8T	0.551
HLA-DRA	*CAPZA1*	CD8T	0.55
HLA-DRA	*CD3E*	CD8T	0.55
HLA-DRA	*APOBEC3H*	CD8T	0.55
HLA-DRA	*PTPN7*	CD8T	0.549
HLA-DRA	*EIF6*	CD8T	0.549
HLA-DRA	*LIME1*	CD8T	0.549
HLA-DRA	*NDUFS3*	CD8T	0.549
HLA-DRA	*TRIM22*	CD8T	0.548
HLA-DRA	*AC133644.2*	CD8T	0.548
HLA-DRA	*WIPF1*	CD8T	0.547

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*PSMC1*	CD8T	0.546
HLA-DRA	*COX17*	CD8T	0.546
HLA-DRA	*NUDT5*	CD8T	0.545
HLA-DRA	*SLTM*	CD8T	0.545
HLA-DRA	*SLC25A5*	CD8T	0.545
HLA-DRA	*CHCHD2*	CD8T	0.545
HLA-DRA	*NDUFB7*	CD8T	0.544
HLA-DRA	*ENO1*	CD8T	0.544
HLA-DRA	*S100A6*	CD8T	0.544
HLA-DRA	*GSTK1*	CD8T	0.543
HLA-DRA	*PHPT1*	CD8T	0.543
HLA-DRA	*PRDX5*	CD8T	0.543
HLA-DRA	*CCNDBP1*	CD8T	0.543
HLA-DRA	*ARPC5*	CD8T	0.543
HLA-DRA	*LDHA*	CD8T	0.542
HLA-DRA	*KDELR2*	CD8T	0.542
HLA-DRA	*KRTCAP2*	CD8T	0.542
HLA-DRA	*NDUFS8*	CD8T	0.542
HLA-DRA	*ACTR2*	CD8T	0.541
HLA-DRA	*NDUFA2*	CD8T	0.541
HLA-DRA	*BTN3A2*	CD8T	0.541
HLA-DRA	*C9orf16*	CD8T	0.541
HLA-DRA	*BCL2L1*	CD8T	0.541
HLA-DRA	*NDUFB2*	CD8T	0.541
HLA-DRA	*TCEA1*	CD8T	0.541
HLA-DRA	*GSTO1*	CD8T	0.541
HLA-DRA	*PLEKHJ1*	CD8T	0.54
HLA-DRA	*TMX1*	CD8T	0.539
HLA-DRA	*NDUFC1*	CD8T	0.539
HLA-DRA	*ITPA*	CD8T	0.539

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*RHOG*	CD8T	0.539
HLA-DRA	*PSMA3*	CD8T	0.538
HLA-DRA	*RAD21*	CD8T	0.538
HLA-DRA	*DBNL*	CD8T	0.538
HLA-DRA	*ATP6V1F*	CD8T	0.538
HLA-DRA	*UQCR11*	CD8T	0.538
HLA-DRA	*COX5B*	CD8T	0.538
HLA-DRA	*HNRNPF*	CD8T	0.538
HLA-DRA	*ATP6V0B*	CD8T	0.537
HLA-DRA	*DAD1*	CD8T	0.536
HLA-DRA	*ATP6V0C*	CD8T	0.536
HLA-DRA	*UBE2D2*	CD8T	0.536
HLA-DRA	*CHCHD5*	CD8T	0.535
HLA-DRA	*FMNL1*	CD8T	0.535
HLA-DRA	*ECHS1*	CD8T	0.535
HLA-DRA	*GABARAP*	CD8T	0.534
HLA-DRA	*SYNGR2*	CD8T	0.533
HLA-DRA	*LGALS3*	CD8T	0.533
HLA-DRA	*CARHSP1*	CD8T	0.533
HLA-DRA	*THRAP3*	CD8T	0.533
HLA-DRA	*P4HB*	CD8T	0.533
HLA-DRA	*PLP2*	CD8T	0.533
HLA-DRA	*YIPF3*	CD8T	0.533
HLA-DRA	*CCND2*	CD8T	0.532
HLA-DRA	*SIRPG*	CD8T	0.532
HLA-DRA	*HERPUD1*	CD8T	0.532
HLA-DRA	*DEF6*	CD8T	0.532
HLA-DRA	*MRPL41*	CD8T	0.532
HLA-DRA	*FLNA*	CD8T	0.532
HLA-DRA	*VIM*	CD8T	0.532

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*PRDX6*	CD8T	0.532
HLA-DRA	*HNRNPLL*	CD8T	0.532
HLA-DRA	*EMC7*	CD8T	0.532
HLA-DRA	*CISD3*	CD8T	0.531
HLA-DRA	*H2AFZ*	CD8T	0.53
HLA-DRA	*CYB561D2*	CD8T	0.53
HLA-DRA	*OAZ1*	CD8T	0.529
HLA-DRA	*IDH3G*	CD8T	0.529
HLA-DRA	*H3F3B*	CD8T	0.529
HLA-DRA	*TMEM160*	CD8T	0.528
HLA-DRA	*LAMTOR1*	CD8T	0.528
HLA-DRA	*ELOVL1*	CD8T	0.528
HLA-DRA	*ISG20*	CD8T	0.528
HLA-DRA	*NUDT1*	CD8T	0.528
HLA-DRA	*SNRPD3*	CD8T	0.527
HLA-DRA	*TEX264*	CD8T	0.527
HLA-DRA	*APOL6*	CD8T	0.526
HLA-DRA	*CBX3*	CD8T	0.526
HLA-DRA	*METTL9*	CD8T	0.526
HLA-DRA	*FGFR1OP2*	CD8T	0.526
HLA-DRA	*RHOF*	CD8T	0.526

Appendix VII. List of genes significantly correlated with HLA-DR from BRCA_GSE161529 database.

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CD74*	CD8T	0.961
HLA-DRA	*LAPTM5*	CD8T	0.876
HLA-DRA	*CD53*	CD8T	0.851
HLA-DRA	*EVI2B*	CD8T	0.832
HLA-DRA	*GPSM3*	CD8T	0.823
HLA-DRA	*HCLS1*	CD8T	0.813
HLA-DRA	*CTSS*	CD8T	0.808
HLA-DRA	*RGS1*	CD8T	0.801
HLA-DRA	*NCF1*	CD8T	0.796
HLA-DRA	*CYBB*	CD8T	0.787
HLA-DRA	*CD83*	CD8T	0.786
HLA-DRA	*CD37*	CD8T	0.786
HLA-DRA	*ITGB2*	CD8T	0.783
HLA-DRA	*CTSH*	CD8T	0.78
HLA-DRA	*PLEK*	CD8T	0.778
HLA-DRA	*RNASE6*	CD8T	0.777
HLA-DRA	*TYROBP*	CD8T	0.776
HLA-DRA	*AIF1*	CD8T	0.773
HLA-DRA	*GMFG*	CD8T	0.772
HLA-DRA	*GPR183*	CD8T	0.772
HLA-DRA	*C1orf162*	CD8T	0.77
HLA-DRA	*FERMT3*	CD8T	0.77
HLA-DRA	*LCP1*	CD8T	0.768
HLA-DRA	*SPI1*	CD8T	0.768
HLA-DRA	*LGALS9*	CD8T	0.765
HLA-DRA	*HCST*	CD8T	0.761
HLA-DRA	*LST1*	CD8T	0.761
HLA-DRA	*PTPN6*	CD8T	0.76

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*TAGAP*	CD8T	0.758
HLA-DRA	*CARD16*	CD8T	0.756
HLA-DRA	*ARHGDIB*	CD8T	0.755
HLA-DRA	*PTPRC*	CD8T	0.755
HLA-DRA	*FCER1G*	CD8T	0.75
HLA-DRA	*LYZ*	CD8T	0.749
HLA-DRA	*MS4A6A*	CD8T	0.747
HLA-DRA	*IL10RA*	CD8T	0.744
HLA-DRA	*CD84*	CD8T	0.744
HLA-DRA	*MNDA*	CD8T	0.744
HLA-DRA	*SRGN*	CD8T	0.742
HLA-DRA	*CD48*	CD8T	0.74
HLA-DRA	*LAT2*	CD8T	0.74
HLA-DRA	*FYB1*	CD8T	0.738
HLA-DRA	*LPXN*	CD8T	0.736
HLA-DRA	*LAIR1*	CD8T	0.733
HLA-DRA	*FCGR2B*	CD8T	0.732
HLA-DRA	*C1QA*	CD8T	0.732
HLA-DRA	*GPR65*	CD8T	0.732
HLA-DRA	*THEMIS2*	CD8T	0.731
HLA-DRA	*LILRB1*	CD8T	0.729
HLA-DRA	*RNASET2*	CD8T	0.726
HLA-DRA	*PSMB9*	CD8T	0.726
HLA-DRA	*C1QB*	CD8T	0.722
HLA-DRA	*CD4*	CD8T	0.722
HLA-DRA	*LILRB4*	CD8T	0.72
HLA-DRA	*IGSF6*	CD8T	0.72
HLA-DRA	*ARRB2*	CD8T	0.719
HLA-DRA	*LCP2*	CD8T	0.715
HLA-DRA	*CASP1*	CD8T	0.713

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*C1QC*	CD8T	0.712
HLA-DRA	*CCL3*	CD8T	0.71
HLA-DRA	*CYTH4*	CD8T	0.71
HLA-DRA	*EVI2A*	CD8T	0.707
HLA-DRA	*ABI3*	CD8T	0.705
HLA-DRA	*TNFSF13B*	CD8T	0.705
HLA-DRA	*LYL1*	CD8T	0.705
HLA-DRA	*CTSZ*	CD8T	0.703
HLA-DRA	*SLC15A3*	CD8T	0.703
HLA-DRA	*CD68*	CD8T	0.701
HLA-DRA	*CD14*	CD8T	0.7
HLA-DRA	*CCL4*	CD8T	0.699
HLA-DRA	*FCGR3A*	CD8T	0.698
HLA-DRA	*FCGR2A*	CD8T	0.698
HLA-DRA	*ALOX5AP*	CD8T	0.697
HLA-DRA	*GNAI2*	CD8T	0.693
HLA-DRA	*CCR1*	CD8T	0.693
HLA-DRA	*CORO1A*	CD8T	0.692
HLA-DRA	*RGS19*	CD8T	0.692
HLA-DRA	*CXCR4*	CD8T	0.691
HLA-DRA	*FCGR1A*	CD8T	0.69
HLA-DRA	*RASSF4*	CD8T	0.688
HLA-DRA	*CSF1R*	CD8T	0.686
HLA-DRA	*SNX10*	CD8T	0.686
HLA-DRA	*TPP1*	CD8T	0.684
HLA-DRA	*CD300A*	CD8T	0.681
HLA-DRA	*MPEG1*	CD8T	0.681
HLA-DRA	*FPR3*	CD8T	0.68
HLA-DRA	*ACP5*	CD8T	0.68
HLA-DRA	*DOK2*	CD8T	0.68

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*C3AR1*	CD8T	0.678
HLA-DRA	*AP1S2*	CD8T	0.678
HLA-DRA	*SAMHD1*	CD8T	0.678
HLA-DRA	*CCL4L2*	CD8T	0.678
HLA-DRA	*ITGAX*	CD8T	0.676
HLA-DRA	*LY96*	CD8T	0.676
HLA-DRA	*IL18*	CD8T	0.675
HLA-DRA	*GIMAP4*	CD8T	0.675
HLA-DRA	*NFKBID*	CD8T	0.674
HLA-DRA	*OLR1*	CD8T	0.672
HLA-DRA	*BCL2A1*	CD8T	0.67
HLA-DRA	*MPP1*	CD8T	0.669
HLA-DRA	*TNFAIP8L2*	CD8T	0.667
HLA-DRA	*CSF2RA*	CD8T	0.667
HLA-DRA	*SELPLG*	CD8T	0.662
HLA-DRA	*LRRC25*	CD8T	0.662
HLA-DRA	*CLEC4A*	CD8T	0.66
HLA-DRA	*CYBA*	CD8T	0.659
HLA-DRA	*FAM49B*	CD8T	0.659
HLA-DRA	*GBP4*	CD8T	0.657
HLA-DRA	*SAMD9L*	CD8T	0.657
HLA-DRA	*IL2RG*	CD8T	0.655
HLA-DRA	*RHOG*	CD8T	0.655
HLA-DRA	*KCTD12*	CD8T	0.655
HLA-DRA	*FGR*	CD8T	0.653
HLA-DRA	*LILRB2*	CD8T	0.653
HLA-DRA	*SIGLEC10*	CD8T	0.652
HLA-DRA	*NCF2*	CD8T	0.652
HLA-DRA	*APOC1*	CD8T	0.651
HLA-DRA	*IFI30*	CD8T	0.65

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*UNC93B1*	CD8T	0.65
HLA-DRA	*CSK*	CD8T	0.649
HLA-DRA	*IRF8*	CD8T	0.648
HLA-DRA	*TREM2*	CD8T	0.648
HLA-DRA	*FGL2*	CD8T	0.646
HLA-DRA	*PARP14*	CD8T	0.646
HLA-DRA	*SLAMF8*	CD8T	0.644
HLA-DRA	*STAT1*	CD8T	0.644
HLA-DRA	*CNPY3*	CD8T	0.641
HLA-DRA	*SLA*	CD8T	0.64
HLA-DRA	*MS4A4A*	CD8T	0.639
HLA-DRA	*CXorf21*	CD8T	0.638
HLA-DRA	*CD52*	CD8T	0.637
HLA-DRA	*IL4I1*	CD8T	0.637
HLA-DRA	*SERPINB9*	CD8T	0.633
HLA-DRA	*VSIG4*	CD8T	0.632
HLA-DRA	*SP110*	CD8T	0.632
HLA-DRA	*EFHD2*	CD8T	0.632
HLA-DRA	*LIMD2*	CD8T	0.631
HLA-DRA	*RNASE1*	CD8T	0.629
HLA-DRA	*TGFB1*	CD8T	0.629
HLA-DRA	*CYTIP*	CD8T	0.626
HLA-DRA	*CD163*	CD8T	0.625
HLA-DRA	*ADAM8*	CD8T	0.625
HLA-DRA	*GRN*	CD8T	0.624
HLA-DRA	*SH2B3*	CD8T	0.623
HLA-DRA	*FCGR1B*	CD8T	0.622
HLA-DRA	*TCIRG1*	CD8T	0.62
HLA-DRA	*CTSD*	CD8T	0.617
HLA-DRA	*SPP1*	CD8T	0.617

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CALHM6*	CD8T	0.616
HLA-DRA	*APOBR*	CD8T	0.616
HLA-DRA	*TNFRSF14*	CD8T	0.615
HLA-DRA	*OSCAR*	CD8T	0.614
HLA-DRA	*IFI44L*	CD8T	0.614
HLA-DRA	*IGKC*	CD8T	0.614
HLA-DRA	*LAP3*	CD8T	0.613
HLA-DRA	*MYO1G*	CD8T	0.613
HLA-DRA	*CCRL2*	CD8T	0.612
HLA-DRA	*C5AR1*	CD8T	0.612
HLA-DRA	*STK4*	CD8T	0.611
HLA-DRA	*ARHGAP9*	CD8T	0.61
HLA-DRA	*XAF1*	CD8T	0.608
HLA-DRA	*SGK1*	CD8T	0.605
HLA-DRA	*LSP1*	CD8T	0.603
HLA-DRA	*CSF2RB*	CD8T	0.601
HLA-DRA	*HHEX*	CD8T	0.6
HLA-DRA	*SIGLEC1*	CD8T	0.597
HLA-DRA	*MOB1A*	CD8T	0.597
HLA-DRA	*ACTR2*	CD8T	0.597
HLA-DRA	*GPR34*	CD8T	0.596
HLA-DRA	*CLEC7A*	CD8T	0.596
HLA-DRA	*SAMSN1*	CD8T	0.594
HLA-DRA	*TLR7*	CD8T	0.592
HLA-DRA	*SLC16A3*	CD8T	0.59
HLA-DRA	*IL1B*	CD8T	0.59
HLA-DRA	*PPP1R18*	CD8T	0.589
HLA-DRA	*CXCL10*	CD8T	0.587
HLA-DRA	*VSIR*	CD8T	0.587
HLA-DRA	*LILRB3*	CD8T	0.586

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*SLAMF7*	CD8T	0.585
HLA-DRA	*CCL3L1*	CD8T	0.583
HLA-DRA	*OTUD1*	CD8T	0.583
HLA-DRA	*ADGRE5*	CD8T	0.582
HLA-DRA	*PLA2G7*	CD8T	0.582
HLA-DRA	*GBP5*	CD8T	0.581
HLA-DRA	*CD40*	CD8T	0.581
HLA-DRA	*TNFRSF1B*	CD8T	0.58
HLA-DRA	*GPR84*	CD8T	0.579
HLA-DRA	*PTGER4*	CD8T	0.578
HLA-DRA	*IL10*	CD8T	0.576
HLA-DRA	*SMIM25*	CD8T	0.576
HLA-DRA	*CLEC4E*	CD8T	0.575
HLA-DRA	*PYCARD*	CD8T	0.574
HLA-DRA	*B2M*	CD8T	0.574
HLA-DRA	*IFI35*	CD8T	0.572
HLA-DRA	*APOBEC3C*	CD8T	0.572
HLA-DRA	*set/06*	CD8T	0.571
HLA-DRA	*RAC2*	CD8T	0.57
HLA-DRA	*CTSC*	CD8T	0.57
HLA-DRA	*NR4A2*	CD8T	0.567
HLA-DRA	*REL*	CD8T	0.567
HLA-DRA	*RNF213*	CD8T	0.566
HLA-DRA	*OSM*	CD8T	0.565
HLA-DRA	*IL18BP*	CD8T	0.564
HLA-DRA	*STAB1*	CD8T	0.563
HLA-DRA	*P2RY13*	CD8T	0.563
HLA-DRA	*CCR5*	CD8T	0.563
HLA-DRA	*GIMAP1*	CD8T	0.562
HLA-DRA	*IFIT3*	CD8T	0.56

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*ADORA3*	CD8T	0.56
HLA-DRA	*JAML*	CD8T	0.558
HLA-DRA	*RGS2*	CD8T	0.558
HLA-DRA	*STK17B*	CD8T	0.558
HLA-DRA	*CAPG*	CD8T	0.557
HLA-DRA	*SLC37A2*	CD8T	0.557
HLA-DRA	*RENBP*	CD8T	0.555
HLA-DRA	*MRC1*	CD8T	0.554
HLA-DRA	*OAS1*	CD8T	0.553
HLA-DRA	*VMO1*	CD8T	0.552
HLA-DRA	*TMEM273*	CD8T	0.551
HLA-DRA	*LINC01857*	CD8T	0.55
HLA-DRA	*SUCNR1*	CD8T	0.549
HLA-DRA	*GAPT*	CD8T	0.549
HLA-DRA	*NPC2*	CD8T	0.548
HLA-DRA	*ADAMDEC1*	CD8T	0.546
HLA-DRA	*SLC11A1*	CD8T	0.545
HLA-DRA	*LGMN*	CD8T	0.541
HLA-DRA	*RILPL2*	CD8T	0.541
HLA-DRA	*POU2F2*	CD8T	0.539
HLA-DRA	*GBP1*	CD8T	0.538
HLA-DRA	*MX1*	CD8T	0.535
HLA-DRA	*LMO2*	CD8T	0.535
HLA-DRA	*SPN*	CD8T	0.535
HLA-DRA	*GAL3ST4*	CD8T	0.534
HLA-DRA	*AKR1B1*	CD8T	0.533
HLA-DRA	*IGLC2*	CD8T	0.533
HLA-DRA	*TXNIP*	CD8T	0.533
HLA-DRA	*CD69*	CD8T	0.533
HLA-DRA	*NLRP3*	CD8T	0.532

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CXCL9*	CD8T	0.53
HLA-DRA	*CTSB*	CD8T	0.53
HLA-DRA	*CLEC2B*	CD8T	0.53
HLA-DRA	*GLRX*	CD8T	0.528
HLA-DRA	*RNASE2*	CD8T	0.528
HLA-DRA	*TGFBI*	CD8T	0.528
HLA-DRA	*ARPC1B*	CD8T	0.527
HLA-DRA	*HAMP*	CD8T	0.526
HLA-DRA	*PARP9*	CD8T	0.525
HLA-DRA	*C2*	CD8T	0.525
HLA-DRA	*IFIT2*	CD8T	0.524
HLA-DRA	*IGLC3*	CD8T	0.524
HLA-DRA	*SDS*	CD8T	0.523
HLA-DRA	*CSF3R*	CD8T	0.521
HLA-DRA	*UCP2*	CD8T	0.52
HLA-DRA	*APOE*	CD8T	0.52
HLA-DRA	*MIR155HG*	CD8T	0.52
HLA-DRA	*PPT1*	CD8T	0.52
HLA-DRA	*COTL1*	CD8T	0.519
HLA-DRA	*MMP9*	CD8T	0.519
HLA-DRA	*ANKRD22*	CD8T	0.518
HLA-DRA	*NR1H3*	CD8T	0.517

Appendix VIII. List of genes significantly correlated with HLA-DR from BRCA_EMTAB8107 database.

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*CD74*	CD8T	0.794
HLA-DRA	*LAG3*	CD8T	0.763
HLA-DRA	*FASLG*	CD8T	0.742
HLA-DRA	*IFNG*	CD8T	0.727
HLA-DRA	*PTMS*	CD8T	0.717
HLA-DRA	*ALOX5AP*	CD8T	0.701
HLA-DRA	*APOBEC3C*	CD8T	0.695
HLA-DRA	*CCL3*	CD8T	0.69
HLA-DRA	*GZMH*	CD8T	0.684
HLA-DRA	*FABP5*	CD8T	0.679
HLA-DRA	*OASL*	CD8T	0.678
HLA-DRA	*AC069363.1*	CD8T	0.677
HLA-DRA	*GZMB*	CD8T	0.673
HLA-DRA	*CXCR6*	CD8T	0.665
HLA-DRA	*CTSC*	CD8T	0.661
HLA-DRA	*IDH2*	CD8T	0.647
HLA-DRA	*MT2A*	CD8T	0.644
HLA-DRA	*GBP5*	CD8T	0.643
HLA-DRA	*ANXA5*	CD8T	0.643
HLA-DRA	*COTL1*	CD8T	0.636
HLA-DRA	*PDCD1*	CD8T	0.631
HLA-DRA	*PRF1*	CD8T	0.629
HLA-DRA	*CCL4*	CD8T	0.624
HLA-DRA	*PSMB9*	CD8T	0.622
HLA-DRA	*APOBEC3G*	CD8T	0.622
HLA-DRA	*ARPC2*	CD8T	0.618
HLA-DRA	*MT1E*	CD8T	0.613
HLA-DRA	*ID2*	CD8T	0.608

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*TAP1*	CD8T	0.607
HLA-DRA	*MT1F*	CD8T	0.605
HLA-DRA	*JAML*	CD8T	0.604
HLA-DRA	*GAPDH*	CD8T	0.603
HLA-DRA	*TIGIT*	CD8T	0.603
HLA-DRA	*MIR155HG*	CD8T	0.598
HLA-DRA	*VCAM1*	CD8T	0.595
HLA-DRA	*CD2*	CD8T	0.591
HLA-DRA	*LGALS1*	CD8T	0.59
HLA-DRA	*PTPN7*	CD8T	0.586
HLA-DRA	*GZMA*	CD8T	0.583
HLA-DRA	*KRT86*	CD8T	0.58
HLA-DRA	*PLPP1*	CD8T	0.578
HLA-DRA	*IFI16*	CD8T	0.578
HLA-DRA	*LY6E*	CD8T	0.575
HLA-DRA	*IFI35*	CD8T	0.572
HLA-DRA	*CCND2*	CD8T	0.571
HLA-DRA	*PLSCR1*	CD8T	0.567
HLA-DRA	*CCR5*	CD8T	0.566
HLA-DRA	*ACP5*	CD8T	0.565
HLA-DRA	*SUB1*	CD8T	0.562
HLA-DRA	*CDKN2A*	CD8T	0.558
HLA-DRA	*IFI27L2*	CD8T	0.556
HLA-DRA	*DUSP4*	CD8T	0.555
HLA-DRA	*ABI3*	CD8T	0.555
HLA-DRA	*SAMD9L*	CD8T	0.555
HLA-DRA	*CTSD*	CD8T	0.551
HLA-DRA	*TNFRSF9*	CD8T	0.549
HLA-DRA	*RBCK1*	CD8T	0.547
HLA-DRA	*CXCL13*	CD8T	0.544

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*ISG15*	CD8T	0.543
HLA-DRA	*PLEKHF1*	CD8T	0.542
HLA-DRA	*ADGRE5*	CD8T	0.542
HLA-DRA	*ACTN4*	CD8T	0.541
HLA-DRA	*IFITM3*	CD8T	0.54
HLA-DRA	*CCL4L2*	CD8T	0.537
HLA-DRA	*PTTG1*	CD8T	0.537
HLA-DRA	*CAPZB*	CD8T	0.536
HLA-DRA	*UBE2L6*	CD8T	0.536
HLA-DRA	*POMP*	CD8T	0.535
HLA-DRA	*set/07*	CD8T	0.53
HLA-DRA	*RHOA*	CD8T	0.528
HLA-DRA	*LSP1*	CD8T	0.528
HLA-DRA	*TYMP*	CD8T	0.526
HLA-DRA	*ITM2A*	CD8T	0.526
HLA-DRA	*MTHFD2*	CD8T	0.525
HLA-DRA	*CST7*	CD8T	0.523
HLA-DRA	*GBP1*	CD8T	0.521
HLA-DRA	*ANXA2*	CD8T	0.521
HLA-DRA	*BST2*	CD8T	0.521
HLA-DRA	*PARK7*	CD8T	0.52
HLA-DRA	*LCP2*	CD8T	0.52
HLA-DRA	*CAPZA1*	CD8T	0.519
HLA-DRA	*SRGN*	CD8T	0.519
HLA-DRA	*CSF1*	CD8T	0.519
HLA-DRA	*CAPN2*	CD8T	0.517
HLA-DRA	*SHISA5*	CD8T	0.515
HLA-DRA	*DRAP1*	CD8T	0.515
HLA-DRA	*CLEC2D*	CD8T	0.514
HLA-DRA	*PDIA6*	CD8T	0.513

Gene	Gene 2	Cell Type	Correlation value
HLA-DRA	*LGALS3*	CD8T	0.512
HLA-DRA	*ACTB*	CD8T	0.511
HLA-DRA	*DYNLL1*	CD8T	0.51

Appendix IX. List of genes significantly correlated with PD1 from BRCA_GSE110686 database.

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*TIGIT*	CD8T	0.889
PDCD1	*PHLDA1*	CD8T	0.881
PDCD1	*CCL3*	CD8T	0.879
PDCD1	*TNFRSF9*	CD8T	0.865
PDCD1	*FABP5*	CD8T	0.854
PDCD1	*GAPDH*	CD8T	0.845
PDCD1	*CXCL13*	CD8T	0.843
PDCD1	*VCAM1*	CD8T	0.838
PDCD1	*HAVCR2*	CD8T	0.834
PDCD1	*BHLHE40*	CD8T	0.83
PDCD1	*GZMB*	CD8T	0.822
PDCD1	*DUSP4*	CD8T	0.821
PDCD1	*IFNG*	CD8T	0.82
PDCD1	*BATF*	CD8T	0.815
PDCD1	*RGS2*	CD8T	0.813
PDCD1	*SYNGR2*	CD8T	0.812
PDCD1	*YWHAH*	CD8T	0.809
PDCD1	*PKM*	CD8T	0.806
PDCD1	*PTMS*	CD8T	0.805
PDCD1	*LAG3*	CD8T	0.805
PDCD1	*MIR155HG*	CD8T	0.799
PDCD1	*ANXA5*	CD8T	0.798
PDCD1	*SH2D2A*	CD8T	0.797
PDCD1	*AC069363.1*	CD8T	0.797
PDCD1	*ACP5*	CD8T	0.795
PDCD1	*SIRPG*	CD8T	0.793
PDCD1	*FASLG*	CD8T	0.793
PDCD1	*ID2*	CD8T	0.792

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*FKBP1A*	CD8T	0.791
PDCD1	*CXCR6*	CD8T	0.787
PDCD1	*AC133644.2*	CD8T	0.786
PDCD1	*CCND2*	CD8T	0.776
PDCD1	*RHOA*	CD8T	0.775
PDCD1	*LSP1*	CD8T	0.773
PDCD1	*CD27*	CD8T	0.771
PDCD1	*ITM2A*	CD8T	0.767
PDCD1	*SIT1*	CD8T	0.766
PDCD1	*CD82*	CD8T	0.764
PDCD1	*PARK7*	CD8T	0.761
PDCD1	*ID3*	CD8T	0.76
PDCD1	*TNFRSF1B*	CD8T	0.75
PDCD1	*CCL4*	CD8T	0.748
PDCD1	*HSPB1*	CD8T	0.747
PDCD1	*DYNLL1*	CD8T	0.746
PDCD1	*CD74*	CD8T	0.738
PDCD1	*SCAMP2*	CD8T	0.735
PDCD1	*PGAM1*	CD8T	0.733
PDCD1	*CKS2*	CD8T	0.73
PDCD1	*HSBP1*	CD8T	0.727
PDCD1	*CTLA4*	CD8T	0.726
PDCD1	*DNPH1*	CD8T	0.723
PDCD1	*PRDX6*	CD8T	0.721
PDCD1	*COPZ1*	CD8T	0.721
PDCD1	*LITAF*	CD8T	0.72
PDCD1	*NR4A2*	CD8T	0.717
PDCD1	*PRF1*	CD8T	0.71
PDCD1	*KLRC1*	CD8T	0.709
PDCD1	*GNLY*	CD8T	0.708

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*SRI*	CD8T	0.706
PDCD1	*ALOX5AP*	CD8T	0.706
PDCD1	*SOX4*	CD8T	0.705
PDCD1	*SNRPD1*	CD8T	0.704
PDCD1	*ZBED2*	CD8T	0.703
PDCD1	*NDUFA13*	CD8T	0.698
PDCD1	*SLA*	CD8T	0.696
PDCD1	*SUMO2*	CD8T	0.696
PDCD1	*CD63*	CD8T	0.694
PDCD1	*CSF1*	CD8T	0.693
PDCD1	*DBI*	CD8T	0.689
PDCD1	*TRAPPC1*	CD8T	0.688
PDCD1	*CD2*	CD8T	0.685
PDCD1	*LAYN*	CD8T	0.684
PDCD1	*GALM*	CD8T	0.684
PDCD1	*SAMSN1*	CD8T	0.681
PDCD1	*RABAC1*	CD8T	0.68
PDCD1	*GZMH*	CD8T	0.68
PDCD1	*PTTG1*	CD8T	0.679
PDCD1	*SOD1*	CD8T	0.677
PDCD1	*VCP*	CD8T	0.676
PDCD1	*COX8A*	CD8T	0.673
PDCD1	*TRAC*	CD8T	0.673
PDCD1	*CDK2AP2*	CD8T	0.671
PDCD1	*IFI27L2*	CD8T	0.671
PDCD1	*CALM3*	CD8T	0.669
PDCD1	*EZR*	CD8T	0.667
PDCD1	*PRDM1*	CD8T	0.667
PDCD1	*CHST12*	CD8T	0.667
PDCD1	*CLEC2D*	CD8T	0.66

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*PTPN6*	CD8T	0.659
PDCD1	*IGFLR1*	CD8T	0.658
PDCD1	*CCR5*	CD8T	0.655
PDCD1	*APOBEC3C*	CD8T	0.655
PDCD1	*SUB1*	CD8T	0.653
PDCD1	*SRGN*	CD8T	0.651
PDCD1	*PSMB3*	CD8T	0.65
PDCD1	*CD8A*	CD8T	0.649
PDCD1	*CD79A*	CD8T	0.648
PDCD1	*CARHSP1*	CD8T	0.647
PDCD1	*CLEC2B*	CD8T	0.646
PDCD1	*SDC4*	CD8T	0.645
PDCD1	*MYL6*	CD8T	0.643
PDCD1	*CAP1*	CD8T	0.642
PDCD1	*ADGRE5*	CD8T	0.642
PDCD1	*PDIA6*	CD8T	0.641
PDCD1	*TPI1*	CD8T	0.638
PDCD1	*IL2RG*	CD8T	0.637
PDCD1	*TSPO*	CD8T	0.635
PDCD1	*TXNDC17*	CD8T	0.632
PDCD1	*HNRNPK*	CD8T	0.631
PDCD1	*CYCS*	CD8T	0.63
PDCD1	*MCM5*	CD8T	0.628
PDCD1	*CD2BP2*	CD8T	0.628
PDCD1	*PRDX5*	CD8T	0.628
PDCD1	*SERPINB9*	CD8T	0.627
PDCD1	*ARPP19*	CD8T	0.627
PDCD1	*KLRC2*	CD8T	0.627
PDCD1	*NRBP1*	CD8T	0.626
PDCD1	*SRP14*	CD8T	0.626

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*UCP2*	CD8T	0.623
PDCD1	*IFI27*	CD8T	0.622
PDCD1	*CLIC1*	CD8T	0.622
PDCD1	*EID1*	CD8T	0.621
PDCD1	*MCL1*	CD8T	0.62
PDCD1	*MT1E*	CD8T	0.619
PDCD1	*HERPUD1*	CD8T	0.618
PDCD1	*CCR1*	CD8T	0.618
PDCD1	*PRDX1*	CD8T	0.617
PDCD1	*TRBC1*	CD8T	0.617
PDCD1	*HNRNPLL*	CD8T	0.616
PDCD1	*UBB*	CD8T	0.616
PDCD1	*SLC1A5*	CD8T	0.615
PDCD1	*TMED9*	CD8T	0.615
PDCD1	*ARPC2*	CD8T	0.615
PDCD1	*NKG7*	CD8T	0.613
PDCD1	*RGS1*	CD8T	0.613
PDCD1	*PHPT1*	CD8T	0.613
PDCD1	*SNU13*	CD8T	0.612
PDCD1	*APOBEC3G*	CD8T	0.61
PDCD1	*NDUFB3*	CD8T	0.609
PDCD1	*NUSAP1*	CD8T	0.607
PDCD1	*COTL1*	CD8T	0.607
PDCD1	*LGALS1*	CD8T	0.606
PDCD1	*LINC01480*	CD8T	0.606
PDCD1	*NDUFC1*	CD8T	0.605
PDCD1	*KRT86*	CD8T	0.604
PDCD1	*SURF4*	CD8T	0.601
PDCD1	*FDPS*	CD8T	0.6
PDCD1	*PMF1*	CD8T	0.6

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*CTSC*	CD8T	0.599
PDCD1	*LAMTOR5*	CD8T	0.599
PDCD1	*NUDT5*	CD8T	0.598
PDCD1	*KIF20B*	CD8T	0.598
PDCD1	*HMGN3*	CD8T	0.597
PDCD1	*CD3D*	CD8T	0.596
PDCD1	*GZMA*	CD8T	0.595
PDCD1	*BUB3*	CD8T	0.593
PDCD1	*HNRNPA3*	CD8T	0.591
PDCD1	*DCTN3*	CD8T	0.59
PDCD1	*MOB1A*	CD8T	0.589
PDCD1	*SLC25A3*	CD8T	0.588
PDCD1	*SLC25A11*	CD8T	0.588
PDCD1	*HMGN1*	CD8T	0.588
PDCD1	*ITGB1*	CD8T	0.586
PDCD1	*DEF6*	CD8T	0.585
PDCD1	*RHOC*	CD8T	0.584
PDCD1	*CCDC28B*	CD8T	0.584
PDCD1	*COX5A*	CD8T	0.583
PDCD1	*U2AF1L4*	CD8T	0.583
PDCD1	*MRPS34*	CD8T	0.581
PDCD1	*MT1X*	CD8T	0.58
PDCD1	*IVNS1ABP*	CD8T	0.579
PDCD1	*GTF3C6*	CD8T	0.578
PDCD1	*PSMC4*	CD8T	0.575
PDCD1	*ANP32A*	CD8T	0.575
PDCD1	*SH2D1A*	CD8T	0.575
PDCD1	*SDF2L1*	CD8T	0.574
PDCD1	*SKA2*	CD8T	0.574
PDCD1	*CCNDBP1*	CD8T	0.573

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*AIF1*	CD8T	0.57
PDCD1	*DNAJC8*	CD8T	0.569
PDCD1	*COMMD7*	CD8T	0.569
PDCD1	*PSMC3*	CD8T	0.568
PDCD1	*ABI3*	CD8T	0.568
PDCD1	*CFL1*	CD8T	0.567
PDCD1	*CYB5B*	CD8T	0.567
PDCD1	*PFDN2*	CD8T	0.567
PDCD1	*CARD16*	CD8T	0.567
PDCD1	*CAPZB*	CD8T	0.566
PDCD1	*SAP18*	CD8T	0.566
PDCD1	*AP2S1*	CD8T	0.565
PDCD1	*CST7*	CD8T	0.565
PDCD1	*SERF2*	CD8T	0.564
PDCD1	*GTF2A2*	CD8T	0.564
PDCD1	*HSD17B10*	CD8T	0.564
PDCD1	*ARL6IP1*	CD8T	0.562
PDCD1	*COX6C*	CD8T	0.562
PDCD1	*B3GNT2*	CD8T	0.561
PDCD1	*MSN*	CD8T	0.559
PDCD1	*ARHGEF1*	CD8T	0.559
PDCD1	*TMCO1*	CD8T	0.558
PDCD1	*MRPS11*	CD8T	0.558
PDCD1	*EIF4E2*	CD8T	0.558
PDCD1	*COX7A2*	CD8T	0.557
PDCD1	*TRAPPC4*	CD8T	0.557
PDCD1	*TXN*	CD8T	0.556
PDCD1	*EIF4H*	CD8T	0.554
PDCD1	*ENSA*	CD8T	0.554
PDCD1	*TMEM179B*	CD8T	0.553

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*MRPL51*	CD8T	0.552
PDCD1	*ITGB7*	CD8T	0.552
PDCD1	*PSMA7*	CD8T	0.552
PDCD1	*NDUFS6*	CD8T	0.552
PDCD1	*UBE2A*	CD8T	0.551
PDCD1	*KPNA2*	CD8T	0.55
PDCD1	*YIF1A*	CD8T	0.55
PDCD1	*PTPN7*	CD8T	0.548
PDCD1	*CD8B*	CD8T	0.547
PDCD1	*RBX1*	CD8T	0.547
PDCD1	*MLF2*	CD8T	0.547
PDCD1	*KXD1*	CD8T	0.546
PDCD1	*GSTO1*	CD8T	0.545
PDCD1	*HSPA5*	CD8T	0.544
PDCD1	*ROMO1*	CD8T	0.544
PDCD1	*UQCR10*	CD8T	0.544
PDCD1	*PYCARD*	CD8T	0.543
PDCD1	*OAZ1*	CD8T	0.542
PDCD1	*CMTM6*	CD8T	0.541
PDCD1	*RNF181*	CD8T	0.541
PDCD1	*DDA1*	CD8T	0.539
PDCD1	*LSM8*	CD8T	0.537
PDCD1	*RHBDD2*	CD8T	0.537
PDCD1	*LCK*	CD8T	0.536
PDCD1	*PPP4C*	CD8T	0.535
PDCD1	*PSME2*	CD8T	0.535
PDCD1	*H2AFZ*	CD8T	0.534
PDCD1	*MAP2K3*	CD8T	0.534
PDCD1	*TALDO1*	CD8T	0.534
PDCD1	*GGCT*	CD8T	0.534

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*LBH*	CD8T	0.534
PDCD1	*ARPC1B*	CD8T	0.534
PDCD1	*CAPZA1*	CD8T	0.534
PDCD1	*EDF1*	CD8T	0.533
PDCD1	*SMC4*	CD8T	0.532
PDCD1	*SLC16A3*	CD8T	0.532
PDCD1	*DNAJB11*	CD8T	0.53
PDCD1	*YWHAQ*	CD8T	0.53
PDCD1	*IDH2*	CD8T	0.53
PDCD1	*BAX*	CD8T	0.53
PDCD1	*HSP90B1*	CD8T	0.53
PDCD1	*COX6A1*	CD8T	0.529
PDCD1	*PSMA2*	CD8T	0.527
PDCD1	*KCNN4*	CD8T	0.527
PDCD1	*SEC11A*	CD8T	0.526
PDCD1	*PSMA1*	CD8T	0.526
PDCD1	*TANK*	CD8T	0.526
PDCD1	*RPS26*	CD8T	0.525
PDCD1	*AP2M1*	CD8T	0.525
PDCD1	*GTF2H5*	CD8T	0.525
PDCD1	*PEF1*	CD8T	0.525
PDCD1	*set/07*	CD8T	0.524
PDCD1	*TMX1*	CD8T	0.523
PDCD1	*UQCRFS1*	CD8T	0.522
PDCD1	*MPC2*	CD8T	0.52
PDCD1	*SFT2D1*	CD8T	0.519
PDCD1	*GLOD4*	CD8T	0.519
PDCD1	*PDIA3*	CD8T	0.518
PDCD1	*SEC61G*	CD8T	0.518
PDCD1	*CTSW*	CD8T	0.517

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*HNRNPC*	CD8T	0.516
PDCD1	*LSM1*	CD8T	0.516

Appendix X. List of genes significantly correlated with PD1 from BRCA_GSE114727_10X database.

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*MIR155HG*	CD8T	0.851
PDCD1	*CXCL13*	CD8T	0.835
PDCD1	*CTLA4*	CD8T	0.827
PDCD1	*FABP5*	CD8T	0.813
PDCD1	*ITGAE*	CD8T	0.798
PDCD1	*CCL3*	CD8T	0.796
PDCD1	*ANXA5*	CD8T	0.795
PDCD1	*AC069363.1*	CD8T	0.788
PDCD1	*ACP5*	CD8T	0.764
PDCD1	*SIRPG*	CD8T	0.763
PDCD1	*JAML*	CD8T	0.762
PDCD1	*PTTG1*	CD8T	0.76
PDCD1	*RGS1*	CD8T	0.747
PDCD1	*AC133644.2*	CD8T	0.747
PDCD1	*AKAP5*	CD8T	0.742
PDCD1	*CXCR6*	CD8T	0.728
PDCD1	*LAYN*	CD8T	0.719
PDCD1	*IFNG*	CD8T	0.71
PDCD1	*TNFRSF1B*	CD8T	0.709
PDCD1	*PARK7*	CD8T	0.709
PDCD1	*CTSD*	CD8T	0.707
PDCD1	*TIGIT*	CD8T	0.706
PDCD1	*COMMD7*	CD8T	0.703
PDCD1	*LSP1*	CD8T	0.703
PDCD1	*IFI27L2*	CD8T	0.698
PDCD1	*PTMS*	CD8T	0.695
PDCD1	*GZMB*	CD8T	0.692
PDCD1	*CAPZB*	CD8T	0.689

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*TNFRSF9*	CD8T	0.689
PDCD1	*CARD16*	CD8T	0.689
PDCD1	*WDR1*	CD8T	0.684
PDCD1	*ALOX5AP*	CD8T	0.681
PDCD1	*SUB1*	CD8T	0.681
PDCD1	*ITM2A*	CD8T	0.68
PDCD1	*RALY*	CD8T	0.679
PDCD1	*AC017002.1*	CD8T	0.678
PDCD1	*PPM1M*	CD8T	0.677
PDCD1	*BATF*	CD8T	0.677
PDCD1	*ITGB1*	CD8T	0.677
PDCD1	*CD200*	CD8T	0.672
PDCD1	*LAG3*	CD8T	0.669
PDCD1	*CLEC2D*	CD8T	0.666
PDCD1	*BTG3*	CD8T	0.665
PDCD1	*TXNDC17*	CD8T	0.661
PDCD1	*SEC11A*	CD8T	0.66
PDCD1	*PKM*	CD8T	0.66
PDCD1	*SMC4*	CD8T	0.66
PDCD1	*CD63*	CD8T	0.659
PDCD1	*FKBP1A*	CD8T	0.658
PDCD1	*EVL*	CD8T	0.658
PDCD1	*IFI16*	CD8T	0.658
PDCD1	*APOBEC3C*	CD8T	0.657
PDCD1	*HMGB1*	CD8T	0.654
PDCD1	*ENSA*	CD8T	0.653
PDCD1	*KRT86*	CD8T	0.653
PDCD1	*BLOC1S1*	CD8T	0.65
PDCD1	*MSN*	CD8T	0.649
PDCD1	*CTSC*	CD8T	0.647

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*PPM1G*	CD8T	0.647
PDCD1	*PSMD8*	CD8T	0.646
PDCD1	*LCP2*	CD8T	0.645
PDCD1	*NR3C1*	CD8T	0.644
PDCD1	*GAPDH*	CD8T	0.643
PDCD1	*RHOA*	CD8T	0.643
PDCD1	*DUSP4*	CD8T	0.639
PDCD1	*PDIA6*	CD8T	0.638
PDCD1	*ARPC2*	CD8T	0.633
PDCD1	*CARHSP1*	CD8T	0.633
PDCD1	*APBB1IP*	CD8T	0.632
PDCD1	*TMX4*	CD8T	0.631
PDCD1	*SLA*	CD8T	0.631
PDCD1	*TMEM120A*	CD8T	0.628
PDCD1	*CD2BP2*	CD8T	0.627
PDCD1	*POMP*	CD8T	0.626
PDCD1	*COX5A*	CD8T	0.626
PDCD1	*PYCARD*	CD8T	0.625
PDCD1	*DYNLL1*	CD8T	0.624
PDCD1	*set/07*	CD8T	0.623
PDCD1	*FIBP*	CD8T	0.622
PDCD1	*GZMH*	CD8T	0.622
PDCD1	*GBP2*	CD8T	0.62
PDCD1	*LCK*	CD8T	0.618
PDCD1	*MZB1*	CD8T	0.617
PDCD1	*GNAS*	CD8T	0.617
PDCD1	*ARHGAP9*	CD8T	0.615
PDCD1	*CDCA7*	CD8T	0.614
PDCD1	*CSK*	CD8T	0.614
PDCD1	*RAC1*	CD8T	0.613

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*MAP4K1*	CD8T	0.612
PDCD1	*LINC01480*	CD8T	0.611
PDCD1	*CAP1*	CD8T	0.609
PDCD1	*PHYKPL*	CD8T	0.608
PDCD1	*TLN1*	CD8T	0.608
PDCD1	*CD2*	CD8T	0.608
PDCD1	*MYL6B*	CD8T	0.608
PDCD1	*CD3D*	CD8T	0.607
PDCD1	*PPP1CA*	CD8T	0.607
PDCD1	*GNAI2*	CD8T	0.606
PDCD1	*HSBP1*	CD8T	0.606
PDCD1	*CCND2*	CD8T	0.605
PDCD1	*EID1*	CD8T	0.604
PDCD1	*TADA3*	CD8T	0.603
PDCD1	*DEK*	CD8T	0.603
PDCD1	*TWF2*	CD8T	0.602
PDCD1	*PSMB8*	CD8T	0.6
PDCD1	*CDK2AP2*	CD8T	0.599
PDCD1	*COPZ1*	CD8T	0.598
PDCD1	*COTL1*	CD8T	0.597
PDCD1	*PDZD11*	CD8T	0.597
PDCD1	*BUB3*	CD8T	0.597
PDCD1	*TSPO*	CD8T	0.596
PDCD1	*PARP1*	CD8T	0.595
PDCD1	*PRF1*	CD8T	0.593
PDCD1	*CHST12*	CD8T	0.593
PDCD1	*PLEKHF1*	CD8T	0.593
PDCD1	*ACTR3*	CD8T	0.592
PDCD1	*SLC7A5*	CD8T	0.592
PDCD1	*GNG5*	CD8T	0.591

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*AP2M1*	CD8T	0.59
PDCD1	*SH3BP1*	CD8T	0.589
PDCD1	*DYNLRB1*	CD8T	0.589
PDCD1	*ARF5*	CD8T	0.588
PDCD1	*UBE2L3*	CD8T	0.586
PDCD1	*CSF1*	CD8T	0.585
PDCD1	*CD3G*	CD8T	0.584
PDCD1	*UCP2*	CD8T	0.584
PDCD1	*PAQR4*	CD8T	0.581
PDCD1	*UBE2A*	CD8T	0.58
PDCD1	*RAB1B*	CD8T	0.579
PDCD1	*HNRNPA3*	CD8T	0.578
PDCD1	*GEM*	CD8T	0.578
PDCD1	*AKIRIN2*	CD8T	0.578
PDCD1	*PRDX3*	CD8T	0.578
PDCD1	*CD82*	CD8T	0.577
PDCD1	*ZCRB1*	CD8T	0.576
PDCD1	*LAMTOR5*	CD8T	0.575
PDCD1	*RNF187*	CD8T	0.574
PDCD1	*PGAM1*	CD8T	0.574
PDCD1	*WAS*	CD8T	0.574
PDCD1	*IDH2*	CD8T	0.574
PDCD1	*MRPS36*	CD8T	0.573
PDCD1	*CMTM6*	CD8T	0.573
PDCD1	*ASNA1*	CD8T	0.572
PDCD1	*RABAC1*	CD8T	0.569
PDCD1	*TCIRG1*	CD8T	0.569
PDCD1	*PPP4C*	CD8T	0.569
PDCD1	*APOBEC3G*	CD8T	0.569
PDCD1	*EWSR1*	CD8T	0.568

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*SYNGR2*	CD8T	0.568
PDCD1	*SERPINB1*	CD8T	0.568
PDCD1	*OTUB1*	CD8T	0.567
PDCD1	*TPM3*	CD8T	0.567
PDCD1	*PSMB2*	CD8T	0.567
PDCD1	*COPE*	CD8T	0.567
PDCD1	*ACTB*	CD8T	0.565
PDCD1	*ETFB*	CD8T	0.564
PDCD1	*NUDT21*	CD8T	0.564
PDCD1	*MSC*	CD8T	0.563
PDCD1	*MRPL28*	CD8T	0.563
PDCD1	*MPG*	CD8T	0.562
PDCD1	*NDUFB3*	CD8T	0.562
PDCD1	*GBP5*	CD8T	0.562
PDCD1	*TAP1*	CD8T	0.562
PDCD1	*IL2RG*	CD8T	0.562
PDCD1	*RAB11B*	CD8T	0.561
PDCD1	*PSMB3*	CD8T	0.561
PDCD1	*PDCD6*	CD8T	0.56
PDCD1	*SUMO2*	CD8T	0.559
PDCD1	*PSMB9*	CD8T	0.558
PDCD1	*SIT1*	CD8T	0.557
PDCD1	*HCLS1*	CD8T	0.556
PDCD1	*SDF4*	CD8T	0.555
PDCD1	*YWHAB*	CD8T	0.554
PDCD1	*AP2S1*	CD8T	0.554
PDCD1	*BST2*	CD8T	0.554
PDCD1	*AP1S1*	CD8T	0.554
PDCD1	*NUDT5*	CD8T	0.553
PDCD1	*VDAC1*	CD8T	0.552

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*IAH1*	CD8T	0.551
PDCD1	*DBI*	CD8T	0.551
PDCD1	*MTHFD2*	CD8T	0.55
PDCD1	*HNRNPLL*	CD8T	0.55
PDCD1	*DUSP23*	CD8T	0.549
PDCD1	*MAD2L2*	CD8T	0.548
PDCD1	*LSM2*	CD8T	0.548
PDCD1	*CD79B*	CD8T	0.548
PDCD1	*SRSF9*	CD8T	0.547
PDCD1	*ZYX*	CD8T	0.547
PDCD1	*PRDM1*	CD8T	0.547
PDCD1	*LY6E*	CD8T	0.547
PDCD1	*RBX1*	CD8T	0.546
PDCD1	*NAP1L4*	CD8T	0.546
PDCD1	*MDH2*	CD8T	0.545
PDCD1	*BIN1*	CD8T	0.544
PDCD1	*CHMP2A*	CD8T	0.544
PDCD1	*PTPN7*	CD8T	0.543
PDCD1	*UBE2D2*	CD8T	0.543
PDCD1	*SRGN*	CD8T	0.543
PDCD1	*FASLG*	CD8T	0.542
PDCD1	*GGCT*	CD8T	0.542
PDCD1	*TPI1*	CD8T	0.541
PDCD1	*CTSB*	CD8T	0.541
PDCD1	*VAMP5*	CD8T	0.541
PDCD1	*ID2*	CD8T	0.541
PDCD1	*MIEN1*	CD8T	0.54
PDCD1	*NDUFA6*	CD8T	0.54
PDCD1	*ZBED2*	CD8T	0.54
PDCD1	*CFL1*	CD8T	0.54

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*IL2RB*	CD8T	0.54
PDCD1	*GSTO1*	CD8T	0.54
PDCD1	*LGALS3*	CD8T	0.538
PDCD1	*TMEM179B*	CD8T	0.537
PDCD1	*POLR2G*	CD8T	0.537
PDCD1	*HSD17B10*	CD8T	0.536
PDCD1	*NELFCD*	CD8T	0.536
PDCD1	*MKI67*	CD8T	0.536
PDCD1	*NDUFS8*	CD8T	0.535
PDCD1	*MIIP*	CD8T	0.534
PDCD1	*TYMP*	CD8T	0.534
PDCD1	*SLC1A5*	CD8T	0.534
PDCD1	*NABP2*	CD8T	0.533
PDCD1	*CKS2*	CD8T	0.533
PDCD1	*FLII*	CD8T	0.533
PDCD1	*SUMO1*	CD8T	0.533
PDCD1	*UBE2L6*	CD8T	0.533
PDCD1	*EZR*	CD8T	0.533
PDCD1	*CLIC1*	CD8T	0.532
PDCD1	*PIN1*	CD8T	0.532
PDCD1	*TIMM8B*	CD8T	0.532
PDCD1	*BSG*	CD8T	0.531
PDCD1	*ADGRE5*	CD8T	0.531
PDCD1	*PRKAR1A*	CD8T	0.531
PDCD1	*GTF3C6*	CD8T	0.531
PDCD1	*ARPC3*	CD8T	0.531
PDCD1	*B2M*	CD8T	0.53
PDCD1	*ZNHIT1*	CD8T	0.53
PDCD1	*CALM3*	CD8T	0.529
PDCD1	*NDUFC1*	CD8T	0.529

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*MCM5*	CD8T	0.528
PDCD1	*PSMA4*	CD8T	0.528
PDCD1	*YWHAQ*	CD8T	0.528
PDCD1	*CCDC167*	CD8T	0.528
PDCD1	*UBE2N*	CD8T	0.527
PDCD1	*RBCK1*	CD8T	0.527
PDCD1	*NDUFS6*	CD8T	0.527
PDCD1	*NCF4*	CD8T	0.526
PDCD1	*MYL6*	CD8T	0.526
PDCD1	*CALR*	CD8T	0.526
PDCD1	*TPM4*	CD8T	0.526
PDCD1	*GPI*	CD8T	0.526
PDCD1	*VASP*	CD8T	0.525
PDCD1	*PSMB6*	CD8T	0.525
PDCD1	*AIP*	CD8T	0.524
PDCD1	*PPP1R7*	CD8T	0.524
PDCD1	*TBCB*	CD8T	0.523
PDCD1	*CD151*	CD8T	0.523
PDCD1	*SCAND1*	CD8T	0.522
PDCD1	*COMMD8*	CD8T	0.522
PDCD1	*LAMTOR1*	CD8T	0.522
PDCD1	*TMX1*	CD8T	0.522
PDCD1	*CCR5*	CD8T	0.522
PDCD1	*PRDX6*	CD8T	0.521
PDCD1	*FERMT3*	CD8T	0.52
PDCD1	*LPXN*	CD8T	0.52
PDCD1	*REEP5*	CD8T	0.519
PDCD1	*PSMD4*	CD8T	0.519
PDCD1	*CLTB*	CD8T	0.519
PDCD1	*SLTM*	CD8T	0.518

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*SNRPB2*	CD8T	0.518

Appendix XI. List of genes significantly correlated with PD1 from BRCA_EMTAB8107 database.

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*CXCL13*	CD8T	0.869
PDCD1	*TIGIT*	CD8T	0.821
PDCD1	*CXCR6*	CD8T	0.806
PDCD1	*LAG3*	CD8T	0.798
PDCD1	*PTMS*	CD8T	0.796
PDCD1	*IFNG*	CD8T	0.784
PDCD1	*DUSP4*	CD8T	0.775
PDCD1	*MIR155HG*	CD8T	0.765
PDCD1	*PHLDA1*	CD8T	0.76
PDCD1	*AC069363.1*	CD8T	0.745
PDCD1	*CSF1*	CD8T	0.737
PDCD1	*RGS1*	CD8T	0.737
PDCD1	*PLPP1*	CD8T	0.723
PDCD1	*SIRPG*	CD8T	0.721
PDCD1	*CAMK1*	CD8T	0.719
PDCD1	*ACP5*	CD8T	0.717
PDCD1	*LINC01480*	CD8T	0.704
PDCD1	*MTHFD2*	CD8T	0.704
PDCD1	*ZBED2*	CD8T	0.699
PDCD1	*PTPN7*	CD8T	0.696
PDCD1	*PTTG1*	CD8T	0.696
PDCD1	*CD200*	CD8T	0.693
PDCD1	*TYMP*	CD8T	0.691
PDCD1	*GAPDH*	CD8T	0.688
PDCD1	*SUB1*	CD8T	0.68
PDCD1	*CCND2*	CD8T	0.676
PDCD1	*FABP5*	CD8T	0.676
PDCD1	*CD2*	CD8T	0.663

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*ANXA5*	CD8T	0.663
PDCD1	*TNFRSF1B*	CD8T	0.659
PDCD1	*AC133644.2*	CD8T	0.659
PDCD1	*IGFLR1*	CD8T	0.654
PDCD1	*TNFRSF9*	CD8T	0.651
PDCD1	*CLEC2D*	CD8T	0.649
PDCD1	*PGAM1*	CD8T	0.645
PDCD1	*PDIA6*	CD8T	0.642
PDCD1	*ALOX5AP*	CD8T	0.641
PDCD1	*FKBP1A*	CD8T	0.641
PDCD1	*CD74*	CD8T	0.638
PDCD1	*KRT86*	CD8T	0.637
PDCD1	*LY6E*	CD8T	0.636
PDCD1	*ITM2A*	CD8T	0.635
PDCD1	*CTLA4*	CD8T	0.633
PDCD1	*TAP1*	CD8T	0.632
PDCD1	*CLTA*	CD8T	0.629
PDCD1	*PKM*	CD8T	0.627
PDCD1	*GEM*	CD8T	0.627
PDCD1	*BST2*	CD8T	0.622
PDCD1	*HMOX1*	CD8T	0.617
PDCD1	*CCL3*	CD8T	0.617
PDCD1	*APOBEC3C*	CD8T	0.614
PDCD1	*LAP3*	CD8T	0.614
PDCD1	*GNG5*	CD8T	0.612
PDCD1	*IFI16*	CD8T	0.612
PDCD1	*DYNLL1*	CD8T	0.611
PDCD1	*MYL6B*	CD8T	0.608
PDCD1	*CD82*	CD8T	0.607
PDCD1	*OAS1*	CD8T	0.606

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*IFI27L2*	CD8T	0.606
PDCD1	*IFI6*	CD8T	0.602
PDCD1	*SH2D2A*	CD8T	0.602
PDCD1	*BTG3*	CD8T	0.601
PDCD1	*TPI1*	CD8T	0.6
PDCD1	*CAPZB*	CD8T	0.599
PDCD1	*BATF*	CD8T	0.596
PDCD1	*SRGN*	CD8T	0.595
PDCD1	*CCDC50*	CD8T	0.594
PDCD1	*ISG15*	CD8T	0.591
PDCD1	*MT1E*	CD8T	0.591
PDCD1	*ARPC2*	CD8T	0.589
PDCD1	*PARK7*	CD8T	0.589
PDCD1	*PSMB9*	CD8T	0.585
PDCD1	*ITGB1*	CD8T	0.584
PDCD1	*MT2A*	CD8T	0.584
PDCD1	*ID2*	CD8T	0.584
PDCD1	*COX5A*	CD8T	0.583
PDCD1	*CTSC*	CD8T	0.583
PDCD1	*GBP5*	CD8T	0.581
PDCD1	*PSMB8*	CD8T	0.58
PDCD1	*ARPC1B*	CD8T	0.579
PDCD1	*CORO1B*	CD8T	0.578
PDCD1	*RHOA*	CD8T	0.575
PDCD1	*PPM1M*	CD8T	0.569
PDCD1	*CKS2*	CD8T	0.569
PDCD1	*MX1*	CD8T	0.569
PDCD1	*RANBP1*	CD8T	0.564
PDCD1	*RHOB*	CD8T	0.562
PDCD1	*IFI44*	CD8T	0.56

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*FAM166B*	CD8T	0.56
PDCD1	*POMP*	CD8T	0.559
PDCD1	*CD2BP2*	CD8T	0.558
PDCD1	*VAMP5*	CD8T	0.558
PDCD1	*PHPT1*	CD8T	0.557
PDCD1	*PLSCR1*	CD8T	0.557
PDCD1	*TXNDC17*	CD8T	0.555
PDCD1	*MAP1LC3A*	CD8T	0.554
PDCD1	*IFI35*	CD8T	0.553
PDCD1	*IRF7*	CD8T	0.553
PDCD1	*UBE2L6*	CD8T	0.552
PDCD1	*CALM3*	CD8T	0.549
PDCD1	*NBL1*	CD8T	0.548
PDCD1	*OASL*	CD8T	0.546
PDCD1	*LCP2*	CD8T	0.544
PDCD1	*JAML*	CD8T	0.544
PDCD1	*YWHAH*	CD8T	0.541
PDCD1	*ZFP36L1*	CD8T	0.539
PDCD1	*SH3BGRL3*	CD8T	0.539
PDCD1	*RBCK1*	CD8T	0.538
PDCD1	*SUMO2*	CD8T	0.538
PDCD1	*HSBP1*	CD8T	0.538
PDCD1	*FASLG*	CD8T	0.538
PDCD1	*TMEM14A*	CD8T	0.537
PDCD1	*SHISA5*	CD8T	0.537
PDCD1	*PDLIM7*	CD8T	0.536
PDCD1	*NDUFV2*	CD8T	0.535
PDCD1	*COTL1*	CD8T	0.534
PDCD1	*NCF4*	CD8T	0.534
PDCD1	*SAMD9L*	CD8T	0.534

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*ICOS*	CD8T	0.534
PDCD1	*CDKN2A*	CD8T	0.533
PDCD1	*CARD16*	CD8T	0.533
PDCD1	*SDC4*	CD8T	0.53
PDCD1	*CSF2*	CD8T	0.53
PDCD1	*VDAC1*	CD8T	0.528
PDCD1	*SURF4*	CD8T	0.527
PDCD1	*CD3D*	CD8T	0.527
PDCD1	*SERF2*	CD8T	0.526
PDCD1	*COMMD7*	CD8T	0.525
PDCD1	*TPM4*	CD8T	0.525
PDCD1	*C4orf48*	CD8T	0.523
PDCD1	*TNFRSF18*	CD8T	0.522
PDCD1	*CCR1*	CD8T	0.522
PDCD1	*LBH*	CD8T	0.522
PDCD1	*RBX1*	CD8T	0.521
PDCD1	*DEK*	CD8T	0.521
PDCD1	*NPDC1*	CD8T	0.52
PDCD1	*DRAP1*	CD8T	0.52
PDCD1	*KLHDC7B*	CD8T	0.519
PDCD1	*B2M*	CD8T	0.518
PDCD1	*MZB1*	CD8T	0.518
PDCD1	*WDR1*	CD8T	0.518
PDCD1	*C3orf14*	CD8T	0.517
PDCD1	*SCAMP2*	CD8T	0.517
PDCD1	*PPP1CC*	CD8T	0.516
PDCD1	*SNRPG*	CD8T	0.516
PDCD1	*PLEKHF1*	CD8T	0.514

Appendix XII. List of genes significantly correlated with PD1 from BRCA_GSE161529 database.

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*TIGIT*	CD8T	0.758
PDCD1	*CD3D*	CD8T	0.756
PDCD1	*CD2*	CD8T	0.749
PDCD1	*TRBC1*	CD8T	0.741
PDCD1	*TRAC*	CD8T	0.721
PDCD1	*LCK*	CD8T	0.712
PDCD1	*CTLA4*	CD8T	0.698
PDCD1	*IFNG*	CD8T	0.696
PDCD1	*TRBC2*	CD8T	0.693
PDCD1	*CD3G*	CD8T	0.683
PDCD1	*ZBED2*	CD8T	0.682
PDCD1	*CD247*	CD8T	0.679
PDCD1	*GPR171*	CD8T	0.665
PDCD1	*LAIR2*	CD8T	0.65
PDCD1	*CXCR6*	CD8T	0.648
PDCD1	*CD7*	CD8T	0.644
PDCD1	*SPOCK2*	CD8T	0.636
PDCD1	*CD3E*	CD8T	0.636
PDCD1	*SH2D2A*	CD8T	0.635
PDCD1	*IL2RB*	CD8T	0.623
PDCD1	*ICOS*	CD8T	0.621
PDCD1	*PTPN7*	CD8T	0.62
PDCD1	*CLEC2D*	CD8T	0.609
PDCD1	*APOBEC3G*	CD8T	0.607
PDCD1	*SIT1*	CD8T	0.607
PDCD1	*KLRB1*	CD8T	0.6
PDCD1	*TBC1D10C*	CD8T	0.591
PDCD1	*LINC01943*	CD8T	0.568

Gene	Gene 2	Cell Type	Correlation value
PDCD1	*GZMM*	CD8T	0.566
PDCD1	*S1PR4*	CD8T	0.564
PDCD1	*CD8A*	CD8T	0.564
PDCD1	*TNFRSF9*	CD8T	0.557
PDCD1	*CD27*	CD8T	0.555
PDCD1	*CD8B*	CD8T	0.553
PDCD1	*CXCR3*	CD8T	0.552
PDCD1	*GZMA*	CD8T	0.549
PDCD1	*IL2RG*	CD8T	0.549
PDCD1	*RAC2*	CD8T	0.543
PDCD1	*CTSW*	CD8T	0.537
PDCD1	*CORO1A*	CD8T	0.536
PDCD1	*LAT*	CD8T	0.523