TRANSLATING BREAST CANCER IMMUNE FEATURES INTO BIOMARKERS AND THERAPIES

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Tese para obtenção do grau de Doutor em Ciências da Saúde

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Abstract

Breast cancer remains one of the main causes of cancer-related deaths in women worldwide. In the past years, advances in breast cancer treatment have been made, namely with the introduction of preoperative neoadjuvant chemotherapy (NACT) in selected cases of inflammatory/inoperable or advanced tumors (size larger than 2 cm and/or disease extension to the axillary lymph node). This treatment is effective in reducing the size of the primary tumor, allowing breast conservation. However, less than half of the patients achieve a pathological complete response and residual disease after NACT is a strong predictor of relapse. Hence, it is essential to find a suitable marker of response to this treatment, to promptly direct NACT non-responder patients to alternative therapies.

Tumor infiltrating lymphocytes (TILs), specifically CD8+ cytotoxic T cells (CTLs) have been appointed as biomarkers of response. However, the clinical usefulness of this biomarker is still controversial and their evaluation is not yet implemented. This controversy could be explained by the fact that tumor cells can escape the immune system by releasing cytokines or expressing immune checkpoint inhibitors, dampening CTLs activity. HLA-DR, a T cell activation marker, could be a more reliable biomarker of response to NACT, than the presence of CTLs *per se*, since it may reflect the overall immune status of the tumor microenvironment and their functionality.

To get more insights into the immune component of the tumor microenvironment, fresh biopsies, surgical specimens and blood were collected from breast cancer patients. In order to assess the differences between breast cancer aggressiveness, the patients were divided in two groups: the ones that have metastasis in the axillary lymph node and the ones that don't have. Although the immunophenotype was similar in both groups of patients, a significant difference was observed for the expression level of HLA-DR in CTLs and regulatory T cells (Tregs). Indeed, patients without axillary lymph node metastasis had higher level of HLA-DR in CTLs and lower in Tregs when compared to patients with axillary lymph node metastasis.

Given this result, we wondered if this immune trait could be used to predict response to NACT. In biopsies of two independent cohorts of breast cancer patients selected for NACT, we observed that high HLA-DR expression level in CTLs was strongly correlated with good response to NACT, with a high sensitivity (94.12% and 80% in cohort 1 and 2, respectively) and specificity (100% and 85.71% in cohort 1 and 2, respectively). Therefore we propose that HLA-DR expression level in CTLs above a threshold value, calculated by a ROC curve, would identify patients that would be responders to NACT. Additionally, by multivariable analysis, we noted that HLA-DR expression in CTLs was an independent predictor of response to NACT.

Interestingly, HLA-DR expression in CTLs also have the likelihood of being a prognostic marker of breast cancer patients' outcome, since a progression-free survival analysis revealed that patients with low levels of HLA-DR in CTLs tend to relapse sooner.

Moreover, we observed that this immune feature was systemically reflected. Indeed, with the assessment of HLA-DR expression level in circulating CTLs we could differentiate breast cancer aggressiveness and even response to NACT, although in a less striking manner when compared to the analysis of HLA-DR expression level in intratumor CTLs.

To characterize HLA-DR+ CTLs, we performed a gene expression as well as a broad surface markers' expression analysis. When comparing to HLA-DR negative CTLs, HLA-DR+ CTLs had higher expression of cytotoxicity-related molecules *Granzyme B*, *IFN-*γ, *Perforin*, *TNF-*α, *Eomes* and lower expression of *Tbet*. Additionally, they had a high proliferative capacity (high Ki67 levels) and an intermediate level of exhaustion markers, namely PD-1, Tim3 and CD127. These results suggest that HLA-DR+ CTLs had a phenotype closer to effector memory T cells (T_{EM}), which have the capacity to home to tissues and to recirculate, rapidly release effector molecules and differentiate in effector CTLs.

Then, we developed a 3D culture platform to shed some light on the functionality of these HLA-DR+ CTLs and their contribution for NACT success. Namely, we used two different breast cancer cell lines (MCF-7 and MDA-MB-231) and allowed them to spontaneously form spheroids. Peripheral blood mononuclear cells (PBMCs) isolated from NACT-responders and non-responders were added to the culture, infiltrating the 3D tumor-like structure. Interestingly, the PBMCs from NACT-responders were able by themselves to reduce the viability of the breast cancer cell line; whereas the PBMCs from NACT non-responders showed no effect. This emphasizes that the immune cells from NACT-responders are activated and have cytotoxic capacities; on the opposite, the immune cells from NACT non-responders are immunosuppressed and cannot exert their cytotoxic function. Furthermore, we added doxorubicin (a NACT agent) to the 3D co-culture and observed that NACT-responders' PBMCs had a synergistic effect with this drug in decreasing the tumor cells' viability. On the contrary, the addition of doxorubicin and NACT non-responders' PBMCs did not alter the viability of the tumor cells. With these *in vitro* assays we validated the clinical observations.

To confirm that the anti-tumor activity of NACT-responders' PBMCs were indeed due to HLA-DR+ CTLs, which are more abundant in the blood of these patients than in the blood of NACT non-responders, we took advantage of the 3D system implemented. In fact, sorted HLA-DR+ CTLs, but not HLA-DR negative CTLs, reduce the viability of MCF-7, attesting the cytotoxic capacity and anti-tumor properties of HLA-DR+ CTLs.

To investigate if it would be possible to revert the immunosuppressed phenotype of NACT non-responders' CTLs we stimulated their PBMCs with PMA/ionomycin or by T cell receptor (TCR) engagement. Notably, both stimuli raised the HLA-DR levels in CTLs in NACT non-responders' PBMCs allowing these cells to reduce the viability of the breast cancer cells, similarly to NACT-responders' PBMCs. The observed effect was even more striking with the addition of doxorubicin. This result opens the possibility to develop new treatments for breast cancer patients, whose tumors are not susceptible to NACT regimens alone.

In this regard, we tested if immune checkpoint blockade (that are already approved for the treatment of other cancer types) could allow NACT non-responders' CTLs activation. However, neither PD-1 nor PD-L1 blockade increased the cytotoxic effect of NACT non-responders' CTLs, probably due to the low levels of PD-1 in the circulating CTLs of these patients. Nonetheless, our 3D cultures would be an ideal platform to screen other immune-modulating molecules.

Overall, this thesis revealed a new biomarker to predict breast cancer patients' response to NACT – HLA-DR-expressing CTLs, that should be mainly assessed in the biopsy, but with a systemic reflection. Moreover, we developed a 3D co-culture platform of breast cancer cell lines and patient-derived immune cells that allowed us to validate the clinical observations and clarify the anti-tumor function of HLA-DR+ CTLs; and that could be further used to evaluate new therapeutic strategies for NACT non-responders. Actually, considering that CTLs expressing high levels of HLA-DR are required in the tumor microenvironment for NACT to be effective, we believe that stimulating the NACT non-responders' CTLs should be a promising alternative therapy, that we intend to explore in the future.

Resumo

O cancro da mama é uma das principais causas de morte por cancro em mulheres. Recentemente, têm havido diversos avanços no tratamento do cancro da mama, sobretudo com a introdução da quimioterapia neoadjuvante (NACT, na sigla em inglês). A NACT prescreve-se antes da cirurgia em tumores inflamados/inoperáveis ou em casos de cancro da mama avançado, isto é, tumores de dimensão superior a 2 cm e/ou com metástases nos nódulos linfáticos da axila. Esta terapia permite reduzir o tamanho do tumor, possibilitando uma cirurgia que conserve o tecido mamário, ao invés da mastectomia. No entanto, apenas metade das doentes conseguem atingir uma resposta patológica completa. Deste modo, é urgente encontrar marcadores preditivos de resposta à NACT, para prontamente direcionar para terapias alternativas as doentes não-respondedoras.

Os linfócitos capazes de infiltrar o tecido tumoral (TILs, na sigla em inglês), em particular as células T citotóxicas (CTLs, na sigla em inglês), têm sido descritas como biomarcadores de resposta ao tratamento. No entanto, este biomarcador ainda não é usado na rotina clinica, o que pode ser explicado pelo facto de as células tumorais terem mecanismos para escapar ao ambiente imunitário, nomeadamente através da produção e libertação de citocinas anti-inflamatórias e expressão de *checkpoints* imunitários que diminuem a função das CTLs. Um marcador de ativação das células T, como o HLA-DR, é potencialmente um biomarcador da resposta à NACT mais robusto, dado que reflete este ambiente tumoral e a função das CTLs.

Para melhor compreender o microambiente imunitário no tumor usámos biópsias e peças cirúrgicas a fresco, bem como amostras de sangue de doentes com cancro da mama. Para entender as diferenças entre a agressividade do cancro da mama, dividimos as doentes de acordo com o estado dos nódulos linfáticos da axila: com ou sem metástases. A principal diferença encontrada entre os dois grupos foi o estado de ativação das CTLs e das Tregs, isto é, o nível de expressão de HLA-DR nestas células. Em especial, as doentes sem metástases nos nódulos linfáticos da axila apresentavam CTLs com elevado nível de expressão de HLA-DR e Tregs com baixo nível de expressão de HLA-DR, quando comparadas com doentes com metástases nos nódulos linfáticos da axila.

Perguntámo-nos então se estas características imunes também poderiam ser usadas como marcadores preditivos de resposta à NACT. Para tal, estabelecemos duas coortes independentes de doentes de cancro da mama selecionadas para NACT. Observámos que o nível de expressão de HLA-DR, em especial nas CTLs, correlacionava-se com resposta à NACT com alta especificidade (100% e 85.71% na coorte 1 e 2, respetivamente) e elevada sensibilidade (94.12% na coorte 1 e 80% na 2). Assim, propomos que doentes com nível de HLA-DR nas CTLs acima do limite calculado pela curva ROC serão respondedores à NACT. Adicionalmente, com uma análise multivariada, concluímos que o nível de expressão de HLA-DR em CTLs é um biomarcador independente.

O nível de expressão de HLA-DR em CTLs também pode ser um possível marcador de prognóstico, já que a análise da sobrevivência sem progressão da doença demonstrou que doentes com nível baixo de expressão de HLA-DR em CTLs têm recaída da doença mais cedo.

Descobrimos igualmente que este biomarcador reflete-se de um modo sistémico. A análise do nível de expressão de HLA-DR em CTLs em circulação também nos permitiu distinguir entre doentes com e sem resposta à NACT. No entanto, esta distinção com as células do sangue não revelou ser tão eficaz quanto a análise do biomarcador em CTLs tumorais.

Para melhor caracterizar as HLA-DR+ CTLs, procedemos a uma análise de expressão genética e a uma detalhada caracterização de diferentes marcadores de superfície. Quando comparadas às CTLs sem expressão de HLA-DR, descobrimos que as HLA-DR+ CTLs têm maior expressão em moléculas de citotoxicidade, como a *Granzima B*, *Perforina*, *IFN-*γ, *TNF-*α, *Eomes* e menor expressão de *Tbet*. Adicionalmente, as células HLA-DR+ CTLs têm maior capacidade proliferativa, demonstrada pelo nível de Ki67, e apresentam um nível intermédio de marcadores de exaustão, nomeadamente PD-1, Tim3 e CD127. Concluímos que as HLA-DR+ CTLs têm um fenótipo similar ao das células T de memória efectoras (T_{EM}) que têm a capacidade de permanecerem nos tecidos e de recircularem, libertam rapidamente moléculas efectoras e podem diferenciar-se em CTLs efectoras.

Posteriormente, desenvolvemos uma plataforma de culturas 3D para compreender melhor a função das HLA-DR+ CTLs e a sua contribuição para o sucesso da NACT. Nomeadamente, usamos duas linhas celulares de cancro da mama (MCF-7 e MDA-MB-231) e deixamo-las formarem espontaneamente esferoides. As células mononucleares do sangue (PBMCs, na sigla em inglês), isoladas de doentes com e sem resposta à NACT, foram adicionadas à cultura e foram capazes de infiltrar as estruturas 3D. Na co-cultura com as PBMCs de respondedoras à NACT, a viabilidade das células tumorais diminuiu, ao contrário das PBMCs de não-respondedores à NACT. Este resultado demonstra que as células imunes de doentes com resposta à NACT estão ativadas e são citotóxicas; já as células imunes de doentes sem resposta à NACT estão imunossuprimidas e não conseguem realizar a sua função citotóxica. Adicionámos ainda um fármaco normalmente usado na NACT – doxorrubicina, e observámos um efeito sinergístico com as PBMCs das doentes respondedoras à NACT, o qual diminuía ainda mais a viabilidade das células tumorais. Por outro lado, a adição de doxorrubicina a culturas com PBMCs das doentes não-respondedoras à NACT não produziu qualquer efeito na viabilidade das células tumorais.

Para confirmar que a atividade anti-tumoral das respondedoras à NACT era devido à presença de HLA-DR+ CTLs, usamos os modelos 3D criados. Na verdade, HLA-DR+ CTLs sorteadas, ao contrário das CTLs sem HLA-DR, reduziram a viabilidade das MCF-7, atestando a sua capacidade citotóxica e anti-tumoral.

Para verificar se era possível reverter o fenótipo de imunossupressão das PBMCs das doentes não-respondedoras à NACT, estas foram estimuladas de modo a aumentar o nível de expressão de HLA-DR nas CTLs. Deste modo, se previamente estimuladas, as PBMCs das doentes não-

respondedoras à NACT passam a ter um efeito citotóxico e a viabilidade das células tumorais diminui. Este efeito foi potenciado com a adição da doxorrubicina. Este resultado abre a possibilidade de desenvolver novos tratamentos para doentes de cancro da mama em que os tumores não sejam suscetíveis à NACT. Deste modo, testámos se o uso de anticorpos contra os *checkpoints* imunológicos permitia a ativação das CTLs das não-respondedoras. No entanto, nem o bloqueio de PD-1 nem de PD-L1 conseguiu aumentar o efeito citotóxico das CTLs das não-respondedoras, provavelmente devido à reduzida percentagem de CTLs positivas para PD-1 nestas doentes.

No global, nesta tese, revelamos um novo biomarcador capaz de prever a resposta à NACT em doentes com cancro da mama. Este marcador, nível de expressão de HLA-DR nas CTLs, analisa-se em biópsias pré-tratamento mas tem representação sistémica. Desenvolvemos ainda uma cultura 3D com linhas celulares de cancro da mama e células imunes isoladas de doentes que não só validou os resultados clínicos como também pode ser usada como uma plataforma para testar tratamentos alternativos para as doentes não-respondedoras à NACT. De facto, a estimulação das PBMCs, e o consequente aumento do nível de expressão de HLA-DR nas CTLs, é algo que pretendemos explorar no futuro como uma possível terapêutica para o cancro da mama.

Thesis publications

The work presented in this thesis, namely sections 1, 3, 4, 6, 7, 8 of Chapter IV and sections 1-3 of Chapter V was published:

Saraiva DP, Jacinto A, Borralho P, Braga S and Cabral MG "HLA-DR in cytotoxic T lymphocytes predicts breast cancer patients' response to neoadjuvant chemotherapy" (2018) Frontiers in Immunology 9:2605.

The work presented in the first section of Chapter VI was submitted to Frontiers in Oncology as a methods paper:

Saraiva DP, Matias AT, Braga S, Jacinto A and Cabral MG "Establishment of a 3D co-culture with MDA-MB-231 breast cancer cell line and patient-derived immune cells for application in the development of immunotherapies".

The work presented in section 5 of Chapter IV, section 4 of Chapter V and Chapter VI is currently in preparation for submission in the near future:

Saraiva DP, Antunes A, Azeredo S, Jacinto A, Braga S and Cabral MG "Clinical and *in vitro* validation of HLA-DR level in CD8+ T cells as an independent predictive biomarker in breast cancer".

Additionally, during this thesis, we published a review:

Saraiva DP, Cabral MG, Jacinto A and Braga S "How many diseases is Triple Negative Breast Cancer: the protagonism of the immune microenvironment" (2017) ESMO Open 2:e000208.

Two abstracts were published in Annals of Oncology:

Saraiva DP, Jacinto A, Braga S and Cabral MG "Abstract 1937P: A 3D co-culture platform of breast cancer and patient derived immune cells to analyse the response to chemotherapy and immunotherapies" (2019) Annals of Oncology, Vol 30, Issue suppl_5, mdz268.064.

Saraiva DP, Jacinto A, Braga S and Cabral MG "Abstract 120P: A new biomarker of breast cancer stage and patient response to neoadjuvant chemotherapy: HLA-DR expression in cytotoxic and regulatory T cells" (2018) Annals of Oncology, Vol 29, Issue suppl_8, 1.

During the course of this thesis, I also participated in other studies that are under preparation for submission in the near future:

Saraiva DP* and Correia BF*, Salvador R, de Sousa N, Jacinto A, Braga S, Cabral MG "Potential clinical utility of low density neutrophils for prognosis and treatment in human breast cancer"

Domingues N, Gaifém J, Saraiva DP, Bento L, Vaz W, Silvestre R, Vieira O "New insights on the inflammatory profile and recruitment dynamics of immune cells during atheroma formation".

List of abbreviations

2D	Two dimension
3D	Three dimension
APCs	Antigen presenting cells
AUC	Area under the curve
Blimp-1	B lymphocyte-induced maturation protein-1
BMI	Body mass index
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
cDNA	Complementary Deoxyribonucleic Acid
CFSE	Carboxyfluorescein succinimidyl ester
CTLA4	Cytotoxic T lymphocyte associated protein 4
CTLs	Cytotoxic T Lymphocytes
DAMPs	Danger-associated molecular patterns
DAPI	4’,6-diamidino-2-phenylindole
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
Doxo	Doxorubicin
EDTA	Ethylenediamine tetraacetic acid
Eomes	Eomesodermin
ER	Estrogen receptor
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	Federal Drug Administration
FFPE	Formalin-fixed paraffin embedded tissue
FMO	Fluorescence minus one
FOXP3	Forkhead box P3
h	Hour(s)
HCD	Hospital CUF Descobertas
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HER2	Human epidermal growth factor receptor 2
HFF	Hospital Prof Doutor Fernando Fonseca
Hobit	Homolog of Blimp-1 in T cells
HRP	Horseradish peroxidase
HVFX	Hospital Vila Franca de Xira
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
IL-	Interleukin
KLF2	Kruppel Like Factor 2
KLF7	Kruppel Like Factor 7

L	Liter
m^2	Square meter
M1	M1 macrophages
M2	M2 macrophages
mAbs	Monoclonal fluorescent antibodies
MDSCs	Myeloid derived suppressor cells
MFI	Median fluorescent intensity
mg	Milligrams
μg	Micrograms
min	Minutes
mL	Milliliters
μL	Microliters
mM	Millimolar
μM	Micromolar
NACT	Neoadjuvant chemotherapy
ng	Nanograms
NK	Natural killer cells
NLR	Neutrophil-to-lymphocyte ratio
ºC	Degrees Celsius
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
pCR	Pathological complete response
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PMA	Phorbol 12-myristate 13-acetate
PR	Progesterone receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
rpm	Rotations per minute
Sec	Seconds
St	Stimulation with PMA+ionomycin
t-SNE	T-distributed Stochastic Neighbor Embedding
TAMs	Tumor associated macrophages
Tbet	T-box transcription factor
TCF-1	T cell factor 1
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{eff}	Effector T cells
T_{EM}	Effector memory T cells

T_{Exh}	Exhausted T cells
TGF-β	Transforming growth factor beta
Th	Helper T cells
TILs	Tumor infiltrating lymphocytes
Tim3	T cell immunoglobulin and mucin domain 3
TMB	3,3',5,5'-tetramethylbenzidine
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor alpha
Tregs	Regulatory T cells
T_{RM}	Tissue resident memory T cells
T_{SCM}	Stem cell memory T cells
T_{TE}	Terminally differentiated T cells
T_{TM}	Transitional memory T cells
VEGF	Vascular endothelial growth factor

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Chapter I

Introduction

Chapter I - Introduction

1. Cancer Immune Features

For many years, cancer was seen as a group of abnormally proliferating cells that had lost the capacity of apoptosis. This proliferation could arise from genetically differences, new point mutations, or from the accumulation of DNA repair errors. Nowadays, it is known that cancer is a complex and evolving tissue composed of several cell types, namely tumor cells, fibroblasts and immune cells. Altogether, these different cell types compose the tumor microenvironment. Cancer progression depends not only on the attributes of tumor cells but on the complex interactions that occur on the tumor microenvironment.

To help clarify the biology of cancer, Hanahan *et al* have proposed six hallmarks that define tumor origin, growth and dissemination (1). To this first classification, the authors added another four hallmarks (2), that are explained in the next subsection.

1.1. Hallmarks of Cancer

The hallmarks of cancer are an enumeration of the characteristics of a tumor, or by other words, what defines a tumor (Figure I-1). The first six hallmarks were sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (1). Here it is depicted the basis of a tumor – the abnormal proliferation with release of growth factors and evasion to growth suppressive molecules; the inability to undergo apoptosis; the need to release angiogenic factors to develop new blood vessels in order to transport more nutrients and oxygen to a demanding tissue; and the capacity to invade the tissue boundaries and spread to distant locations.

Following this first publication, the authors made a second revision noticing that the six hallmarks were not sufficient to characterize a tumor. In this second revision, the authors included a paradigm shift in the field – the importance of the immune system.

These new four hallmarks were the genome instability and mutation, the deregulation of cellular energetics, the avoidance of immune destruction and the capacity to employ a tumor-promoting inflammation (Figure I-1). The succession of genomic alterations or mutations and the genomic instability are essential for the development of distinct clones. These clones adapt differently to the environment and subsequently, the fittest ones are selected, leading to tumor growth and progression. The need to reprogram cellular energetics arises from the high rates of proliferation seen in tumor cells. Tumor cells opt to switch their metabolism to glycolysis to obtain faster energy, even in the presence of oxygen, in a process called aerobic glycolysis or the Warburg effect (3).

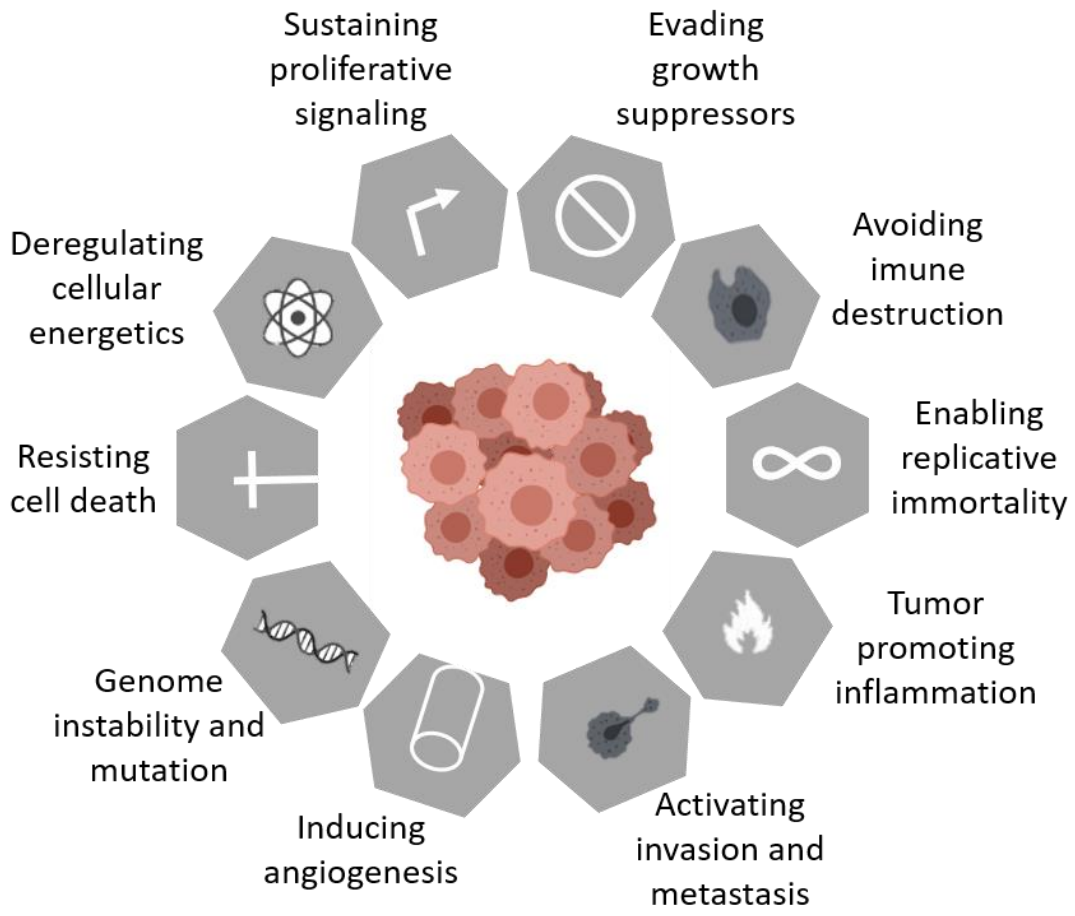


Figure I-1 **Hallmarks of Cancer**. Adapted from Hanahan *et al* Cell 2011 (2).

These first two new hallmarks complete the previously published ones as what defines the functional characteristics that ultimately allow the tumor to survive, proliferate and invade the tissue boundaries. On the presence of an abnormal cell, the immune system should be able to eliminate it and develop a memory to those antigens. Nevertheless, it is important to note that tumor cells evolve and give rise to complex tissues even in the presence of a surveilling immune system. In fact, tumors are heavily infiltrated by different immune populations (4), and have developed several mechanisms to escape the anti-tumor immune response, which will be described in this introduction. Thus, one of the hallmarks is to avoid immune destruction. Additionally, tumors can take advantage of the inflammatory process and use them in their favor, completing the last hallmark – tumor-promoting inflammation. During inflammation, the release, to the tumor microenvironment, of a high amount of growth and survival factors and also pro-tumor cytokines occur. Matrix degrading enzymes can also be produced during inflammation, which can facilitate adjacent tissue invasion by tumor cells. Moreover, TGF-β is normally released in an inflammatory process and it is a known inductor of epithelial to mesenchymal transition, which empowers the tumor cells with a

mesenchymal-like phenotype, acquiring the capacity to invade the tissue and form metastasis (4,5).

1.2. Tumor Immune Surveillance

As referred, the immune system have a pivotal role in eliminating tumor cells or, on the other hand, in assisting tumor growth and progression. Indeed, it is known that virtually all solid cancer types can be infiltrated by immune cells in distinct ranges (6).

The theoretical notion that immune cells can control tumor cells and contribute for tumor elimination or settlement, appeared at the beginning of the 20th century, developed by Paul Ehrlich. In the 50s the formulation of immune surveillance by recognition of tumor-specific antigens was elaborated (7,8). This theoretical hypothesis was validated by experimental procedures with immunosuppressed mice, where an increase in spontaneous or chemically induced tumors was observed (9,10). Additionally, mice with an immunodeficient system had higher susceptibility to develop viral-related tumors. Nevertheless, other groups disproved these results, mainly due to inadequate technical abilities at the time, and the idea of tumor immune surveillance was left behind.

Only in the mid-70s and throughout the 80s and 90s the tumor immune surveillance was again investigated, and experiments with nude mice (with an inhibited immune system) were executed. There, the authors confirmed that these mice, without immune system, develop more chemically induced tumors. Also in these decades, other authors demonstrated that antigen presenting cells had the capacity to process and present tumor antigens to other immune cells (11). Additionally, the capacity to develop effector and memory immune cells were also revealed in these decades (11).

Besides the experimental data, there are clinical evidences that tumor immune surveillance occurs. Namely, there is an increased incidence of cancer (normally from a viral origin (12,13)) in patients with immunodeficiency, such as HIV patients (14). Patients who were submitted to organ transplants and were prescribed with immunosuppressive drugs also have an increased risk of developing cancer (15). Also, the older population has increased incidence of cancer, which could be explained by the fact that with age the capacity of the immune system decreases, resulting in less tumor immune surveillance (16). Additionally, patients with melanoma, breast, ovary, bladder, colorectal and prostate cancer had longer survival rates when the percentage of lymphocytes infiltrating the tumor was increased (17–22).

However, as previously stated, some cancer cells manage to bypass this immune surveillance step. Throughout the years, in order to better understand this relationship between cancer and the immune system, the field of Immuno-Oncology has been growing and nowadays, immune cells and their released cytokines are considered one of the pillars of either tumor

suppression or progression. In the next section, this tumor-immune crosstalk is going to be discussed.

2. Tumor-Immune Interplay at the Cellular Level

The leukocytes that populate the tumor microenvironment can be divided into lymphocytes and myeloid cells. Within lymphocytes there are natural killer (NK) cells, B and T lymphocytes; and within myeloid cells there are neutrophils, macrophages, dendritic cells and myeloid derived suppressor cells. Each subtype of immune cell population will have an important role in tumor elimination or progression. The role in tumor progression/regression of the most noteworthy immune cell populations for this work will be detailed below.

2.1. The role of Tumor Infiltrating Lymphocytes in the Tumor Microenvironment

T cells are seen as the main immune cell population for tumor cell clearance. However, there are several types of T cells, and each can have distinct and even opposite roles in the tumor microenvironment. The T cells here described are divided in CD4+ T cells, which include helper T cells (Th) and regulatory T cells (Tregs); and CD8+ T cells, which are also called cytotoxic T cells (CTLs) (Figure I-2).

Th cells include different subtypes, e.g. Th1, Th2, Th17, among others, depending mainly on the released cytokines (23). Th cells are able to infiltrate the tumor microenvironment and assist in CTLs' priming, for instance (24). The role of Th cells depends on the activation performed by the presentation of tumor antigens (normally defined as neoantigens that derive from abnormal expression of proteins) by antigen presenting cells (APCs), such as dendritic cells or macrophages, detailed in the next subsection. Antigen presentation is achieved, after antigen recognition and processing, by showing the antigen associated with class II major histocompatibility molecules (MHC II) at APCs' surface. This antigen presentation by the APCs (first signal), together with co-stimulation (second signal) and cytokines release (third signal), will activate Th cells, inducing for instance IFN-γ production and consequent stimulation of CTLs.

Th cells were shown to have anti-tumor activity (25); however, due to the different subsets with distinct activities, Th cells can also assist in tumor progression. The first action is mainly performed by the Th1 subtype (Figure I-2), which releases high levels of IFN-γ and TNF-α, known to have anti-tumor properties. Namely, they induce CTLs function and inhibit angiogenesis (23). The presence of Th1 subset at the tumor site was associated with improved prognosis in gastric, colorectal, ovarian and hepatocellular cancers (26–29).

Th2 cells release IL-4, IL-6 and IL-10 and as such can have dual roles in tumor progression. However, due to the production of a highly anti-inflammatory cytokine (IL-10) that has the capacity of stimulating more pro-tumor immune cells (Figure I-2), Th2 cells were characterized as a poor prognostic predictor in pancreatic and gynecologic cancers (23).

Tregs are an immunosuppressive subset of CD4+ T cells, characterized by the expression of the transcription factor FOXP3. Tregs are necessary for the homeostasis maintenance in physiological conditions and therefore, due to their immunosuppressive phenotype, can have a pivotal role in preventing autoimmune diseases and in promoting cancer progression (reviewed in (30)). In fact, Tregs have the capacity to suppress the function of Th1 and CTLs, consequently improving tumor development (Figure I-2). This suppression can be mediated by the cytotoxic T-lymphocyte–associated antigen 4 (CTLA4) expressed at their surface (31), or by the release of immunosuppressive cytokines, namely IL-10 and TGF-β (32). As such, Tregs are associated with poor prognosis in ovarian and breast cancers, for instance (33,34). Nevertheless, some studies claim that Tregs have a protective role and lead to a favorable prognosis in colon and breast cancer (35,36).

CTLs are T cells that express the CD8 molecule, that also recognize antigens, but in this case, the antigen has to be presented by class I MHC. Normally, in the tumor microenvironment, CTLs are considered to be crucial for the elimination of tumor cells, even more than Th1 cells, due to their overwhelming cytotoxic capacity (Figure I-2) (37). CTLs eliminate tumor cells by two mechanisms – the release of perforin and granzyme B or the connection between Fas and FasL. Perforin will induce the formation of pores in the membrane of tumor cells and granzyme B will pass through those pores and act as a protease, cleaving several substrates in the cytoplasm, inducing tumor cell apoptosis (38). CTLs can also kill tumor cells by the interaction between Fas on the surface of tumor cells and FasL on the surface of CTLs. This interaction will induce a caspase cascade and lead to tumor cell apoptosis (39). Moreover, CTLs also produce IFN-γ and TNF-α that will boost cytotoxicity against the tumor cells (40). Its action can be suppressed by Tregs or tumor cells, by the escape mechanisms that are going to be further discussed in this introduction. CTLs presence in the tumor microenvironment is a good prognosis factor in breast and colorectal cancer, among others (41,42). Additionally, there is a correlation between intratumor CTLs and the reduction of tumor size (37).

Besides these immune cell populations, there are several others that can infiltrate the tumor and have different roles in the tumor progression. These other lymphocytes are B cells, NK cells, Th17, Th22, to name a few. The role of these lymphocytes in the tumor microenvironment is not going to be detailed in this introduction, since these cells are not the focus of this thesis.

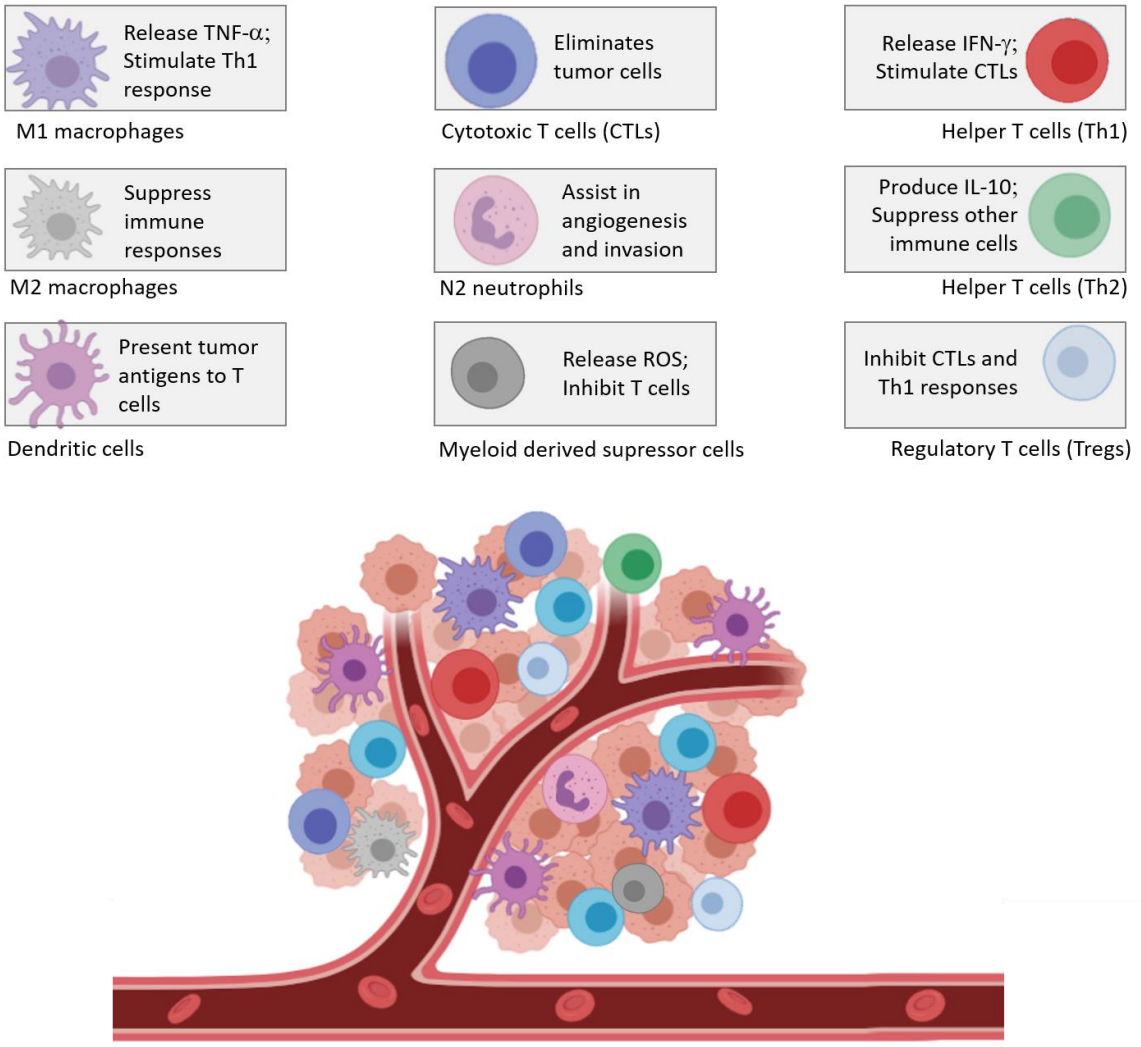


Figure I-2 **The role of some tumor infiltrating immune cells on cancer elimination/progression.** Figure executed with Biorender.

2.2. The role of Myeloid Cells in the Tumor Microenvironment

Although T cells are the ultimate effectors in tumor cell elimination, the myeloid cells can also have an important role in either tumor clearance or progression (Figure I-2). These myeloid cells comprise macrophages, dendritic cells, myeloid derived suppressor cells and neutrophils. The macrophages in a tumor microenvironment are denominated as tumor-associated macrophages (TAMs). TAMs can be divided into M1 and M2 subtypes, probably due to environmental cues (43). Briefly, M1 polarization occurs in an IFN-γ-rich environment, whereas M2 polarization should occur with stimulation of IL-4 or IL-10, for instance (44). M1 is recognized as a pro-inflammatory subtype of macrophages and upon activation can release pro-inflammatory cytokines, such as TNF-α (43). These cytokines alongside enhanced antigen presentation will lead to Th1 responses (45). On the other hand, M2 macrophages can secrete TGF-β, IL-1β, IL-6, to name a few. These released cytokines will enhance the

immunosuppressive responses in the tumor microenvironment, inhibiting the function of effector T cells. Immunosuppression can also be performed by direct contact with the employment of the immune checkpoint axis PD-1/PD-L1, which will be further explained in this introduction. Moreover, they can induce angiogenesis and tumor invasion. TAMs can also be a prognosis factor – they are associated with worst survival in breast, pancreatic and bladder cancer (46–48); and with favorable outcome in colorectal cancer (49). This dichotomy in macrophages action is important to maintain tissue homeostasis, but the mechanisms undertaken by these cells can be hijacked by the tumor cells in order to trigger more anti-inflammatory or pro-tumor responses by TAMs.

Dendritic cells (DCs) are professional APCs capable of presenting tumor antigens by class II MHC to Th cells, ultimately leading to tumor cell elimination, as explained in section 2.1. Besides antigen presentation, DCs can produce cytokines, such as IFN-γ, TNF-α and IL-12, which constitute the third signal required to activate T cell responses (50). However, the anti-tumor DCs function can be impaired in the tumor microenvironment. For instance, the presence of IL-10 in the environment has been shown to inhibit cytokine production by DCs (51). Additionally, the hypoxic conditions in the tumor microenvironment and lower pH, due to a shift in the tumor cell metabolism towards lactate production, can also limit the function of DCs (50). Instead, they can became tolerogenic and contribute to tumor tolerance.

Neutrophils are the most abundant leukocytes in circulation and are essential in host defense (52). They can also have important roles in a tumor microenvironment, which can be both anti- or pro-tumor (53). Similarly to M1/M2 macrophage polarization, neutrophils can be divided into N1 (anti-tumor) and N2 (pro-tumor). The most cancer-related subtype is the N2, which can be induced by TGF-β (53). N2 neutrophils can release reactive oxygen species (ROS), which can potentiate the inhibition of effector T cells function, cancer-promoting inflammation and cancer progression (54). In addition, they can produce VEGF, inducing angiogenesis (55), and initiate TGF-β-mediated metastasis (56). Neutrophils are recognized as a poor prognostic factor in several cancers, especially when the ratio between circulating neutrophils and lymphocytes is taken into account (57–59).

Myeloid derived suppressor cells (MDSCs) include polymorphonuclear cells (PMN-MDSCs), closely related to neutrophils, and monocytic cells (M-MDSCs), more similar to monocytes (60). These cells are normally associated with pathological conditions, such as cancer and chronic inflammation and are expanded in an IL-6 and IL-10 rich environment (61). MDSCs have a suppressive role and when activated release ROS, nitric oxide and arginase, leading to the inhibition of T cell responses (61). These cells can also release VEGF, assisting in angiogenesis, and metalloproteinases that can cleave the extracellular matrix and lead to tumor cell invasion (61).

Taking into account the variety of immune cell populations that can be find in the tumor microenvironment, and their different roles, to achieve a proper immune surveillance and

tumor cell elimination, different players and conditions have to come together in a coordinated fashion.

2.3. The Cancer Immunity Cycle

The tumor-immune interplay include a series of steps, in what was described by Chen and colleagues as the Cancer Immunity Cycle, represented in Figure I-3 (62). In the beginning, when cancer cells arise, immune cells have the potential to effectively eliminate them. For that to occur, the first step is to expose tumor antigens to the immune cells, therefore triggering an anti-tumor immune response. This antigen exhibition can be improved with the tumor cell death in a process known as immunogenic cell death, which will be detailed further on in this introduction. The released antigens are captured by antigen presenting cells (APCs), such as dendritic cells or macrophages, present in the tumor microenvironment (step 2). These antigens are processed intracellularly and presented at the APCs' surface by class I or class II MHC to T cells, mainly in the lymph nodes (step 3). Interactions are established between APCs and T cells, comprising the connection between MHC molecules in APCs and the T cell receptor (TCR) in T cells (first signal) as well as costimulatory signals, such as the contact between CD28 in the T cells and CD80/CD86 in the APCs (second signal). Additionally, for a proper T cell activation, pro-inflammatory cytokines are released by the APCs (third signal). The presentation of antigens by MHC molecules is made either to helper T cells if the presentation is performed by class II MHC or to cytotoxic T cells in the case of class I MHC presentation. Overall, the interaction between both cell types is essential for the activation of T cells and the establishment of effector T cells against specific tumor antigens. Then, the primed and activated T cells travel from the lymph nodes to the tumor microenvironment (step 4), becoming tumor infiltrated T cells (step 5). Both helper T cells (Th) and cytotoxic T cells (CTLs) recognize the tumor antigens present in the tumor microenvironment (step 6) and especially the CTLs are able to eliminate the tumor cells (step 7). The elimination of tumor cells will boost the release of more tumor antigens, restarting the cycle again.

For the Cancer Immunity Cycle to occur, leading to tumor cell elimination by the CTLs, tumors have to be recognized by the immune system. The factors in play for this recognition to occur are summarized in the Cancer Immunogram. The Cancer Immunogram, published in 2016 by Blank *et al* (63), comprises seven characteristics:

(i) tumor foreignness, which consists in the ability of cancer cells to express immunogenic antigens (such as neoantigens) that will induce T cell responses;
(ii) an host with healthy immune system - normal lymphocyte and neutrophil counts and functionalities;
(iii) immune cells able to infiltrate the tumor;
(iv) tumor cells without inhibitory immune checkpoints (that will be further characterized in this introduction), otherwise these molecules can engage with

their receptors in T cells, blocking their activity and impairing the anti-tumor response;

(v) absence of soluble inhibitors such as anti-inflammatory cytokines;

(vi) a metabolism suitable with immune cells activities, since tumor cells can shift their metabolism to aerobic glycolysis, releasing lactic acid, decreasing the pH of the microenvironment, thus impairing anti-tumor responses;

(vii) tumor sensitivity to immune effectors, for instance, to cytotoxic molecules released by CTLs.

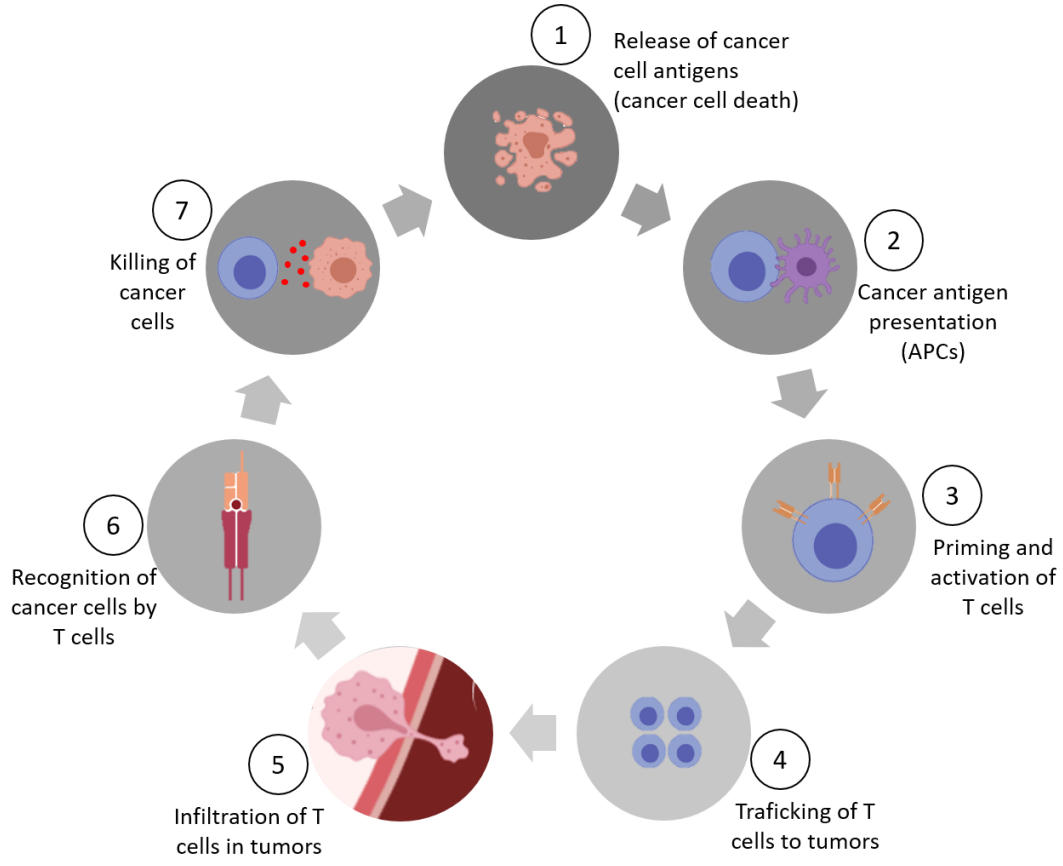


Figure I-3 **Cancer Immunity Cycle**. Adapted from Chen *et al* Immunity 2013 (62). Scheme elaborated with Biorender.

However, in cancer patients, the Cancer Immunity Cycle and consequently the Immunogram can be compromised in several ways. The release of tumor antigens (step 1) can be hampered by the inhibition of cancer cells apoptosis, for instance. Additionally, the tumor antigens can be recognized as self instead of as foreign by antigen presenting cells, leading to APCs with a tolerogenic profile. The APCs can be present in the tumor microenvironment but can be suppressed by factors released from the tumor cells, such as anti-inflammatory cytokines (64).

The interaction between APCs and T cells in the lymph nodes (step 2) can lead to poor T cell activation or to a balance shifted towards the activation of regulatory T cells instead of effector T cells. The infiltration of T cells in the tumor bed (step 5) can be inhibited by the endothelium of the blood vessels present in the tumor, for instance by altering the adhesion molecules essential for the penetration of T cells in the tumor mass (65). The recognition of tumor cells by T cells (step 6) can also be hindered either by the presence of an immunosuppressive environment that will inhibit the action of CTLs (66) or by the downregulation of class I MHC present in the tumor cells (67), leading to the withdrawal of tumor antigens. The hypoxic conditions and/or the lower pH detected in the tumor microenvironment, due to the metabolic shift observed in tumor cells, can ultimately lead to inhibition of CTLs' function, reducing their ability to eliminate target tumor cells (step 7) (68,69).

Thus, for the Cancer Immunity Cycle to occur leading to the tumor cells elimination by immune cells, the mechanisms of the effective immune response should weigh more than the immunosuppressive mechanisms, in the tumor microenvironment, in order to favor immune surveillance. Although this situation is usually observed upon the appearance of the tumor, with the time and with the selection of immune resistant tumor cells (a process known as immunoediting) the immunosuppression may become more dominant than the immune surveillance.

2.4. The Three Steps of Immunoediting

The term immunoediting was first elaborated by Dunn and colleagues (70). The immunoediting is composed of three different stages – elimination, equilibrium and escape, the three Es of immunoediting (Figure I-4). In the elimination phase, immune surveillance occurs following the Cancer Immunity Cycle described previously. If the immune system is capable of eliminating the tumor cells, there is no progression to the next stages of immunoediting. During elimination, cytotoxic T cells (CTLs) release several cytotoxic molecules, such as granzyme B and perforin. As explained above, perforin will induce the formation of pores in the membrane of tumor cells and granzyme B will pass through those pores and act as a protease, cleaving several substrates in the cytoplasm, inducing tumor cell apoptosis (38).

Nevertheless, tumors can be composed of different tumor cell variants. Some variants can evolve to produce mechanisms to remain unseen by the immune system, for instance by downregulating the expression of class I MHC. During the equilibrium phase, there is elimination by CTLs of some tumor clones, but others that are generated by genetic instability and the appearance of new mutations (one of the cancer hallmarks), evolve with the capacity to escape the immune surveillance. During this selection, an equilibrium is achieved between eliminated and immune-resistant tumor cells, enabling further tumor growth containment, but not eradication. This phase – when immune cells are able to contain the tumor, can last

undetectable for several years, since tumor may not be large enough to be perceived by imaging techniques.

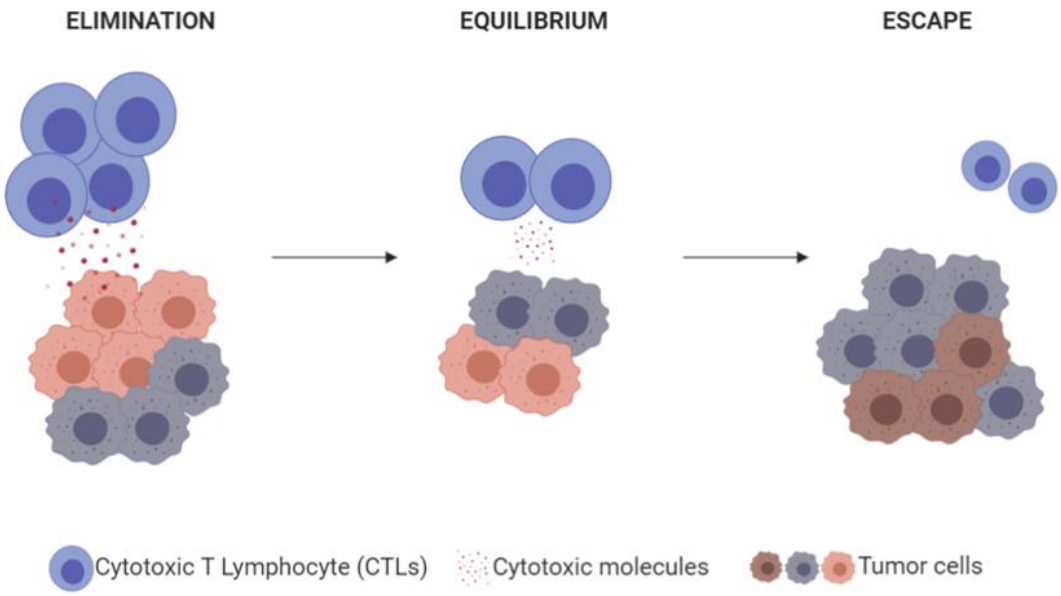


Figure I-4 **Immunoediting – elimination, equilibrium and escape**. Adapted from Dunn *et al* Nature Immunology 2002 (70). The figure was prepared with Biorender.

The third phase is the escape phase, where the tumor clones that survived the immune surveillance and acquired mechanisms to escape the immune system, are able to proliferate leading to tumor growth and eventually tumor metastization.

Combining the knowledge from Immunoediting phases with Cancer Immunity Cycle and the Cancer Immunogramm, it becomes clear that specifically the CTLs are undoubtedly necessary for tumor cells' elimination.

2.5. T cell activation

T cells, especially the CTLs, can be present in the tumor microenvironment but can be suppressed by several mechanisms, therefore possessing their cytotoxic response inhibited. So, the ability to become activated and remain active is essential for their anti-tumor capacity. Actually, the activation of CTLs is referred in the Cancer Immunogram as the ultimate effector to eliminate tumor cells (63). 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, which is upregulated in 4 hours after the stimulation, leading to high intracellular calcium levels and production of IL-2, IL-4 and TNF-α (71–73). 12 hours after the activation, CD25 (IL-2 receptor)

is upregulated, leading to the production of higher levels of IFN-γ and TNF-α (73), initiating cell proliferation and differentiation (74). HLA-DR is the last activation marker, being upregulated 24/48 hours after the activation (73,75). HLA-DR is a class II MHC molecule, normally expressed in antigen presenting cells. However, it is upregulated in activated T cells and is associated with increased IFN-γ production. It is believed that HLA-DR expression is important in the formation of T cell-T cell synapsis, leading to an amplification of IFN-γ production and other CTLs-related functions due to a rapid positive feedback (76).

3. The Role of Tumor-produced Cytokines

As it is possible to observe in the previous sections, the immune response dictated by the different immune cell populations depends not only on cell-cell contact but also on released cytokines. Cytokines are important players in a tumor microenvironment to provide the necessary growth conditions, and can also regulate cellular trafficking and different signaling pathways. These molecules can be released not only by immune cells but also by tumor cells. The role of cytokines released by the immune cells was described in the previous subsections of this introduction. Here, it will be described briefly the role of different cytokines released by the tumor cells in the tumor microenvironment. Specifically, we will focus on the cytokines that will be further analyzed in this work – IL-1β, IL-6, IL-8, IL-10, IL-17, IL-23 and TGF- β (Figure I-5).

IL-1β was shown to be upregulated in tumor cells of colon and lung cancer and melanoma (77,78), normally associated with a worse prognosis. IL-1β is capable of inducing the production of metalloproteinases, essential for the degradation of the extracellular matrix and consequent tumor invasion (79). IL-1β can also induce the expression of VEGF to stimulate angiogenesis (80) and can stimulate other cells in the tumor microenvironment to produce IL-8, IL-6 and TGF-β. This cytokine also contributes in an autocrine fashion to improve tumor cell proliferation (Figure I-5, (81)).

IL-6 is produced by tumor cells, of the breast and lung cancer and hepatocellular carcinoma, as an example (reviewed in (82)). IL-6 can control the expression of anti-apoptotic proteins (83), induce metalloproteinases secretion (84), and lead to VEGF production (85), contributing to tumor cell survival, invasion and angiogenesis (Figure I-5).

IL-8 is also able to promote angiogenic response (86) in the tumor microenvironment. Additionally, it was observed, mainly in cancer cell line models, that autocrine IL-8 action is able to induce tumor cell proliferation, migration and invasion (86–88). Moreover, it recruits neutrophils and may act indirectly on tumor metastasis (Figure I-5, (89)).

IL-10 is an immunosuppressive cytokine that can be produced by tumor cells with the main aim to limit effector anti-tumor immune response. It is also involved in resistance to apoptosis,

described in lung and breast cancer (90,91), and can promote angiogenesis (Figure I-5, (92)). IL-10 was described as a poor prognostic factor in breast cancer (93).

IL-17 has been detected in several types of cancer, including breast, prostate, gastric, colorectal and lung cancers (94–98). Similarly to the abovementioned cytokines, IL-17 can induce the expression of metalloproteinases to help in tumor cell invasion and progression (99). IL-17 stimulates regulatory T cells (Tregs), which will inhibit CTLs effector function (Figure I-5, (94)). Moreover, IL-17 levels are associated with poor disease outcome in breast cancer patients (94).

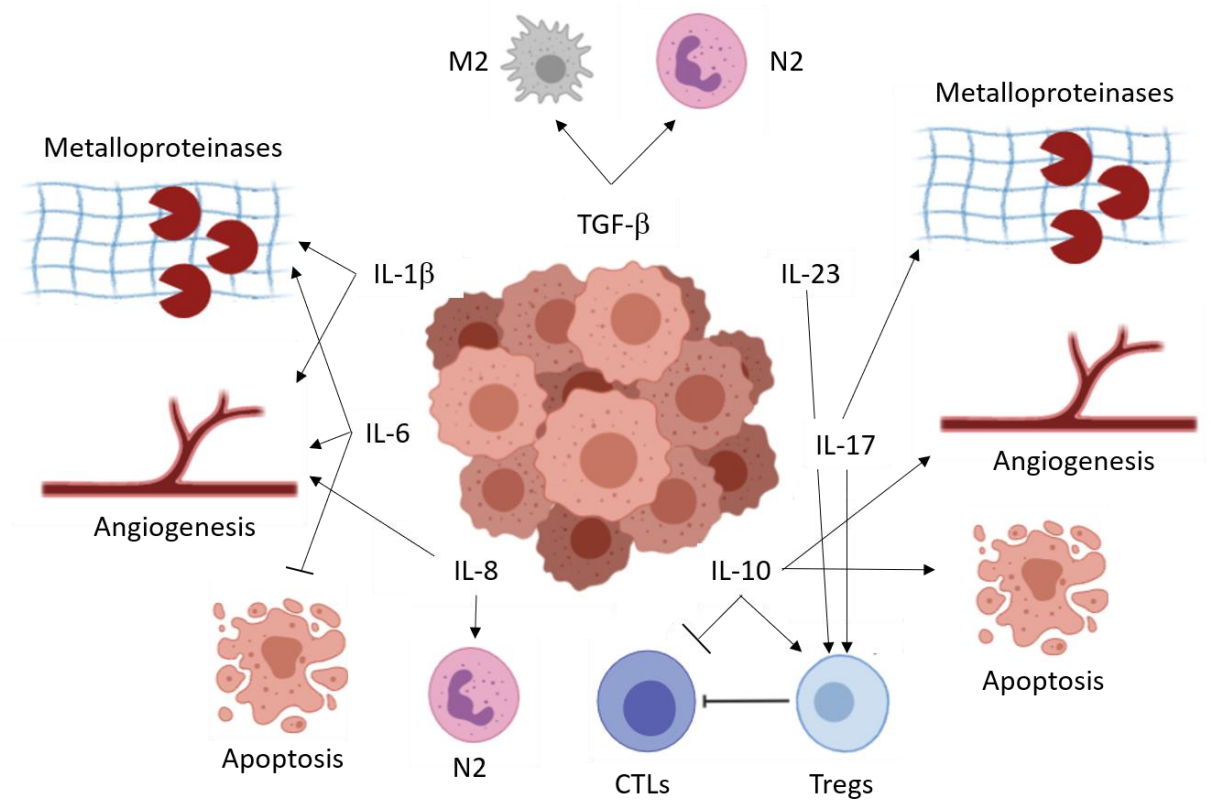


Figure I-5 **The role of tumor-produced cytokines**. Tumor cells can produce IL-1β, IL-6, IL-8, IL-10, IL-17, IL-23 and TGF-β and each have a different role in tumor progression. IL-1β induces angiogenesis and metalloproteinases production, which will degrade the extracellular matrix. IL-6 also induces angiogenesis and inhibits tumor cell apoptosis. IL-8 induces angiogenesis and N2 neutrophils. IL-10 induces angiogenesis and regulatory T cells (Tregs), inhibits tumor cell apoptosis and cytotoxic T cells (CTLs) function. IL-17 induces metalloproteinases production and Tregs' function, which consequently will inhibit CTLs cytotoxic capacity. This last function of IL-17 is shared with IL-23. TGF-β stimulate the M2 macrophages and N2 neutrophils, which have an immunosuppressive phenotype also culminating in the inhibition of CTLs cytotoxic function. This scheme was prepared with Biorender.

IL-23 is known to induce IL-17-producing helper T cells (100). IL-23 is upregulated in several types of cancer, including colon, ovarian, lung, breast cancer and melanoma (101). With *in vivo* analysis, IL-23 was shown to be associated with tumor incidence and resistance to therapy (101). As there seems to be a pathway between IL-17 and IL-23, the latter cytokine can also

indirectly reduce the cytotoxic function of CTLs, by improving IL-17 induction of Tregs (Figure I-5). Indeed in *in vivo* models, IL-23 was found to be responsible for the reduction of CTLs infiltration in tumor tissues (101).

TGF-β is one of the inductors of epithelial to mesenchymal transition. This is the process where epithelial cells, in this case the tumor cells, gain mesenchymal characteristics and consequently acquire the capacity to invade (102). TGF-β when produced by tumor cells leads to increased tumor growth (103) and immunosuppressive action on different immune cells (104), reducing immune-derived tumor cell elimination (Figure I-5). Moreover, TGF-β induces macrophages polarization towards an M2 phenotype (105) and neutrophils polarization towards an N2 phenotype (53), increasing the immunosuppressive microenvironment (Figure I-5).

4. The Chemotherapeutic Treatment Modulates Immune Features

As it is possible to observe from this introduction, the immune system and, especially, the immune cells that are capable to infiltrate the tumor, are fundamental for the tumor elimination or progression. Moreover, the presence of certain immune cells or their released molecules can influence the response to treatment – CTLs can eliminate tumor cells, improving the patients' outcome. Nevertheless, the treatment applied to the patients also influences the immune response.

One of the most studied ways by which chemotherapy boosts tumor-immune responses is by inducing the immunogenic cell death of tumor cells. During cell death, the release of damage-associated molecular patterns (DAMPs) can occur, which will trigger a "danger" state in the immune system (106). However, for these DAMPs to initiate an immune response, the dying cell has to have tumor associated antigens, like neoantigens, which normally resulted from accumulated mutations that occur in the tumorigenesis process (107).

Mechanistically, during tumor immunogenic cell death driven by chemotherapeutic agents, chaperones from the endoplasmic reticulum are transferred to the plasma membrane of the apoptotic cell, which will increase antigen uptake by dendritic cells. Additionally, the dying cells release ATP to the extracellular space, essential for dendritic cell recruitment and activation. Activated and antigen loaded dendritic cells can then trigger tumor-specific T cell responses, as explained previously. Moreover, the dying tumor cells secrete type I IFN and CXCL10, which will recruit T cells and induce the proliferation of tumor-specific T cells (reviewed in (108)).

A well-recognized example is the effect of anthracyclines, a class of chemotherapeutic agents, which have an immunostimulatory capacity, based on immunogenic cell death, thus triggering immunological responses, and furthermore eliminating immunosuppressive barriers created by a tumor (109). Doxorubicin, a type of anthracycline, was shown to promote immunogenic

cell death in mice models (110), and also was shown to induce the recruitment of myeloid cells (111). Cyclophosphamide, another chemotherapeutic drug, is able to induce Tregs depletion, favor Th1 and CTLs' response and stimulate the tumor immunogenic cell death (112–114). Paclitaxel, normally used in chemotherapeutic regimens, favors immunosurveillance, pro-inflammatory macrophages and CTLs recruitment to the tumor (115,116). Moreover, paclitaxel helps in the maturation of dendritic cells and presentation of tumor antigens (117).

Interestingly, many chemotherapeutic regimens employed nowadays are based on a combination of different drugs. This combination ideally will elicit a higher immunostimulatory response, with each drug having a different effect on the tumor immune microenvironment. For instance, a regimen composed of the abovementioned drugs should boost immunogenic cell death and depletion of immunosuppressive immune cells, such as Tregs, therefore increasing immune-mediated tumor clearance.

Additionally, besides using a combination of chemotherapeutic agents, the addition of specific immune-based therapeutic approaches would, theoretically, increase the anti-tumor responses.

5. Modulating Host Immune Features as Cancer Treatment Options

As the immune system has a pivotal role in the regulation of tumor elimination or progression, clinicians have started to take advantage of immune processes to improve cancer treatments. Thus, immunotherapy has been gaining a massive interest, especially in the last decade (118). This treatment employs different strategies to help the host immune system to work better at recognizing and destroying specific cancer cells. Since the main aim of immunotherapy is to use and potentiate the patient's own immune system, it holds the potential to become more precise, personalized and effective when compared to standard chemotherapeutic strategies. Moreover the secondary side effects should be reduced (119).

Historically, the first immunotherapies arose in the 70s/80s with two main approaches. The use of the BCG vaccine (Bacillus Calmette–Guérin) in bladder cancer patients was tested in the late 70s (120). Using a local instillation of an attenuated form of *Mycobacterium bovis* induced a local inflammatory response and the immune cells recruited could have an effect against cancer cells. Secondly, IL-2 was used as a therapy in renal carcinoma in the 80s (121). IL-2 is an inducer of T cell differentiation and proliferation, and could increase T cell-mediated tumor cell elimination.

Meanwhile, many other strategies to enhance the anti-tumor immune response, were/are being tested in clinical trials or even have been already approved. For instance, targeted antibodies, cancer vaccines, dendritic cell based vaccines, checkpoint inhibitors and cytokines. Although immunotherapy doesn´t always work for every patient, altogether it is a promising

option for cancer therapy, either to be used alone or in combination with more conventional treatments such as surgery, chemotherapy and radiation to improve their effectiveness.

Nowadays, two different types of immunotherapies have been most successfully used – the immune checkpoint blockade and the use of adoptive T cell therapy, which will be detailed afterwards.

5.1. Immune checkpoint blockade

As previously explained in this introduction, tumor cells have different mechanisms to escape the immune system. One of these mechanisms is to activate inhibitory immune checkpoints that will inhibit T cell function against tumor cells. Thus, the therapy using immune checkpoint blockade has the main goal of releasing this inhibitory connection between the tumor (or regulatory immune cells) and the effector immune cells, so that T cells can be activated and exert their anti-tumor functions.

There are several types of immune checkpoints that have been used to develop these blockade therapies, namely the programmed cell death protein 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1) that composes the PD-1/PD-L1 axis (Figure I-6). Moreover, cytotoxic T lymphocyte-associated antigen 4 (CTLA4, Figure I-6) and T cell immunoglobulin and mucin protein 3 (Tim3) are other immune checkpoints that have the potential to be targeted in the clinic.

In a tumor microenvironment, tumor cells are able to increase their PD-L1 expression to interact with PD-1 expressed on CTLs (Figure I-6, (122)), leading to the inhibition of their cytotoxic function. Additionally, antigen presenting cells (APCs) can also exhibit PD-L1 at their surface and inhibit CTLs (Figure I-6). PD-L1 expression is upregulated in several types of cancer, which is an advantage to be widely used as a therapeutic option (122). As such, there are antibodies against either PD-1 or PD-L1 with the objective to block PD-1/PD-L1 interaction and inhibit T cell anergy. Anti-PD-1 antibodies – nivolumab (from Bristol-Myers Squibb) and pembrolizumab (from MSD) are approved by the Federal Drug Administration (FDA) for different cancer types. Nivolumab is approved for bladder, lung, kidney, head and neck, colorectal and liver cancers (118). Pembrolizumab is approved for lymphoma, head and neck, lung, bladder, cervical and liver cancers (118).

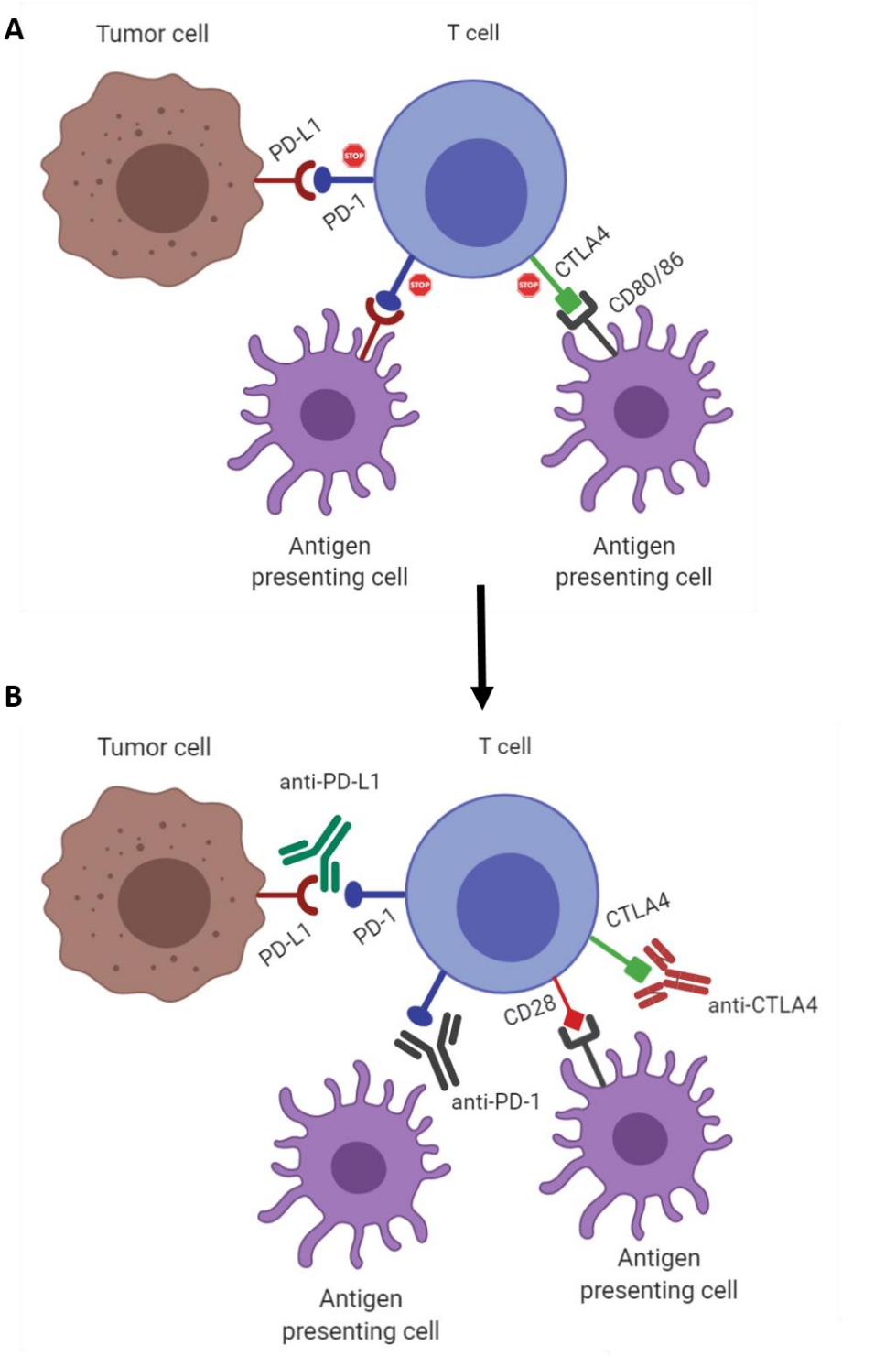


Figure I-6 **Immune checkpoint blockade therapy**. **(A)** PD-1 is normally expressed on T cells, namely on cytotoxic T cells and binds to its ligand – PD-L1 that can be expressed either on tumor cells or antigen presenting cells, such as dendritic cells. CTLA4 can also be expressed on T cells which bind to CD80/CD86 on antigen presenting cells. The immune checkpoints when connected to their ligands give an inhibitory signal to T cells, reducing its function. **(B)** With the immune checkpoint blockade therapy, antibodies against PD-1, PD-L1 or CTLA4 remove the inhibitory function of these immune checkpoints, activating T cells to perform their anti-tumor function. Figure prepared with Biorender.

Anti-PD-L1 antibodies, such as atezolizumab (from Roche), have been approved by the FDA to treat metastatic triple negative breast cancer, bladder and lung cancers (118). Nevertheless, it is important to take into account that these antibodies have a function in PD-L1+ tumors, which is not the case for all tumor types and even within the same tumor type, PD-L1 positivity is heterogeneous among patients.

CTLA4 is also an immune checkpoint commonly expressed in T cells, namely regulatory T cells. CTLA4 binds to CD80 and CD86 present at the surface of APCs (123) and this binding competes with the co-stimulatory signal between CD28 and CD80/86 (123). As such, antibodies anti-CTLA4 downregulate the suppressive function exerted on T cells, activating T cells. One of these anti-CTLA4 antibodies is ipilimumab (from Bristol-Myers Squibb), which is approved by the FDA for the treatment of melanoma, renal and colorectal carcinomas (118).

Tim3 can be expressed on the surface of T cells, either Th or CTLs and was mainly related to immunosuppression on these cells (124,125), since its ligand – Galectin9 can be overexpressed on tumor cells, namely in melanoma (126). Antibodies against Tim3 have shown an improved anti-tumor efficacy on *in vivo* models (reviewed in (127)). In a clinical setting, tumor infiltrating lymphocytes were shown to express more Tim3 than their circulating counterparts (124). CTLs expressing Tim3 are often seen as exhausted T cells (124). Nevertheless, Tim3 was also determined to be expressed on Tregs and APCs. On tumor infiltrating Tregs, Tim3 positivity is normally associated with CTLA4+ Tregs (128). Tim3 could be one of the immune checkpoints that are activated when anti-CTLA4, -PD-1 or –PD-L1 therapies are prescribed, leading to therapy failure. A combination of distinct immune checkpoint blockade could be a strategy to increase anti-tumor efficacy.

Indeed, several combined immune checkpoint blockade therapeutic options have been tested. Although immune checkpoints act to decrease CTLs activation and effector function, their mechanisms of action are different. For instance, when CTLA4 blockade is performed, tumor cells can increase their expression of PD-L1, maintaining the escape to the immune system by the PD-1/PD-L1 axis. The other way around is also possible. As such, anti-CTLA4 and anti-PD-1 have been used together in the clinic, namely nivolumab with ipilimumab, in the treatment of advanced melanoma, colorectal and renal cancers (129–131).

In addition to the combination of immune checkpoint blockade, these therapies can also be combined with chemotherapy. As previously explained, chemotherapeutic agents can improve the antigenicity of the tumor by employing tumor immunogenic cell death with the release of tumor antigens associated with improved CTLs response. Moreover, chemotherapy can also deplete suppressive immune cells, such as Tregs, also improving CTLs response (109,110,112). Indeed, the combination of chemotherapy with anti-PD-1 is approved for the treatment of lung cancer (132).

5.2. Adoptive T cell Therapy

Although immune checkpoint blockade has revolutionized cancer treatment, the patients' response rate is still very heterogeneous. Indeed, response rates vary from 10-60% and a high percentage of cancer patients experience resistance to the treatment, as well as relapse (133).

To overcome this issue, clinical studies with adoptive T cell therapy are starting to emerge (Figure I-7). This treatment uses T cells, namely CTLs, either from tumor infiltrating lymphocytes (TILs) or from the circulation. T cells are then stimulated *ex vivo* to proliferate, normally with supplemented IL-2, to perform effector functions and respond to tumor-specific cues. For this therapy to be successful normally the patients undergo a previous lymphodepletion, either by chemo or radiotherapy, followed by T cell administration (119).

To obtain CTLs from TILs, a portion of the tumor has to be resected and fragmented to a single cell suspension. The CTLs are then cultured in facilities with good manufacturing practices in order to proliferate until around 10^{10} cells are obtained (134). The *ex vivo* culture and expansion of T cells is a limiting step since it is time-consuming: approximately 6 weeks from tumor resection until CTLs infusion in the patients (134).

Besides using TILs-derived CTLs, these cells can be obtained systemically. CTLs from the peripheral blood can be placed in culture alongside APCs with the capacity to present specific tumor antigens, thus inducing tumor-specific CTLs (135). These cells will follow the same experimental steps as explained above.

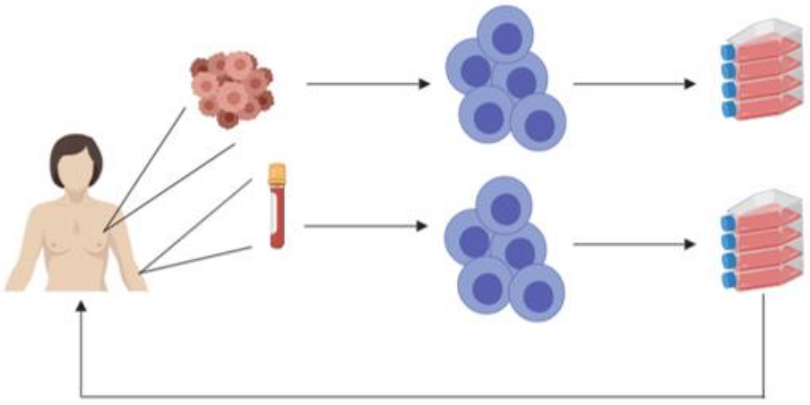


Figure I-7 **The rationale of adoptive T cell therapy**. Cytotoxic T cells (in blue) can be acquired directly from tumor infiltrating lymphocytes or from the peripheral blood. These cells are expanded *ex vivo* and reinfused in the patient. Figure performed with Biorender.

In addition, CTLs derived from the peripheral blood can be engineered to express a chimeric antigen receptor (CAR), which consists in a modified T cell receptor (TCR) to enhance the T cells specificity towards tumor antigens (136). To obtain these cells, autologous T cells are

harvested from the patient, genetically engineered, expanded in culture and then reinfused. One of the advantages of CAR T cells is that it is possible to design the CAR to recognize membrane moieties normally present in tumor cells, such as glycolipid structures, without the need of antigen presentation through the MHC, which can be downregulated in tumor cells. Thus, T cell specific tumor elimination can be enhanced, increasing the therapy response (136).

6. Breast Cancer

6.1 Breast Cancer Features

Breast cancer is the most frequent type of cancer in women, accounting for up to 2 million new cases per year worldwide (Figure I-8) (137). Interestingly, when taking into account both males and females, breast cancer is the second most frequent type of cancer (137). The estimated mortality worldwide is more than 600,000 women per year, which represents 15% of cancer-related deaths in women (Figure I-8) (137).

Breast cancer is not a single disease and can be divided into three different subtypes, based on the different molecular drivers of each subtype (Figure I-8). The most frequent subtype, which represents approximately 70% of the breast cancer patients, has an overexpression of the estrogen receptor (ER) and can also have an upregulation of the progesterone receptor (PR), hence this subtype is commonly referred to as hormone positive or ER+ breast cancer. The second subtype is characterized by an amplification of the *HER2* gene (human epidermal growth factor receptor 2) and represents approximately 15% of the breast cancer cases. HER2+ breast cancer patients can also have upregulation of the hormone receptors. The third subtype is denominated triple negative breast cancer (TNBC), because it does not possess ER, PR or HER2 and also represents approximately 15% of the breast cancer patients.

Besides the molecular-based classification, there is a histopathological classification that is correlated with the localization of the tumor. As such, breast cancer can be either ductal or lobular, depending if the tumor mass is in the milk-carrying ducts or in the milk-producing lobules (138).

Breast cancer can be further classified by the TNM staging – T refers to the tumor size, N refers to the presence of cancer cells in the axillary lymph nodes and M refers to the presence of distant metastasis (Figure I-8). T is divided into T1 – tumor size less than 2 cm, T2 – tumor dimension less than 5 cm, T3 – tumors bigger than 5 cm and T4 – tumors spread in the chest wall, the skin or both or it can represent an inflammatory tumor (T4d). N correlates with the lymph node status: N0 corresponds to absence of swollen axillary lymph nodes, N1 and N2 are related to detectable swollen and lumpy axillary lymph nodes and finally N3 is used to

describe detectable lymph nodes close to the collarbone. M is the number of distant metastasis (M) and can be either 0 (no metastasis) or 1 (presence of metastasis) (138).

There are risk factors associated with breast cancer, such as age, familial and reproductive history (139). The majority of patients have more than 50 years old when they are diagnosed with breast cancer (139). Familial history of breast cancer, specifically inherited mutations in the *BRCA1* and *BRCA2* genes is also a risk factor (139,140). Additionally, women that had an early menarche, a late menopause, have denser breasts or a long term use of oral contraceptives also have a higher risk of developing breast cancer (139,140). The lifestyle can also be considered, namely alcohol consumption, smoking and high fat diet can contribute to increase the risk of developing breast cancer (140).

Nevertheless, the first classification is clinically relevant since it will give the appropriate therapy for each patient, further detailed below. Additionally, survival is distinct between breast cancer molecular subtypes. Indeed, patients with breast cancer with a size smaller than 2 cm and without axillary lymph node involvement have a survival of 99% for ER+ tumors, 94% for HER2+ tumors and 85% for TNBC. When the disease is spread to other organs, the median overall survival for primary breast tumors of ER+ or HER2+ is 5 years, whereas for TNBC it is only 1 year (141).

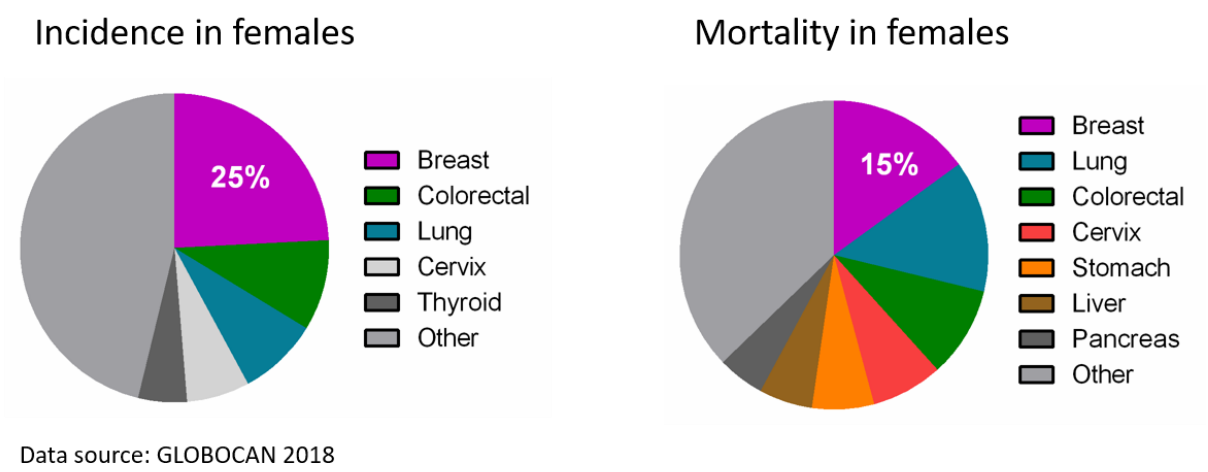


Figure I-8 **Breast Cancer - incidence and mortality in women worldwide; molecular subtypes and TNM staging**. Breast cancer occurs in approximately 25% of the world female population and represents around 15% of cancer-related deaths in women worldwide. Breast cancer can be divided in three different molecular subtypes, which are distinctively represented in the population. TNM staging is used to characterize the breast tumors accordingly to their size (T), the presence of disease in the lymph nodes (N) and the presence of distant metastasis (M). Figure elaborated with Biorender.

6.2 Breast Cancer Treatment

The treatment of breast cancer depends on the molecular subtype and if the cancer has already metastasized. For non-metastatic breast cancer, the therapy consists of a full mastectomy or a tumorectomy followed by radiotherapy. The removal of suspicious axillary lymph nodes can also be performed at surgery. The choice between a more conservative surgery or a mastectomy can derive from the diagnosis of the patient, for instance if several tumor loci are present (138). Systemic chemotherapy can be administered prior to the surgery (neoadjuvant chemotherapy) or post-surgery (adjuvant chemotherapy). In the first case, the surgery will be a conservative tumorectomy. For some patients both neo and adjuvant chemotherapies are prescribed. Briefly, neoadjuvant chemotherapy is administered in patients with inoperable or inflammatory tumors or patients that present a locally advanced disease, i.e. tumors with size larger than 2 cm and/or with disease extension to the axillary lymph node (142).

There are several chemotherapeutic regimens that can be used. However, in this introduction, the focus will be on the combined therapy composed by anthracyclines, cyclophosphamide and taxanes, since it is the one prescribed to the cohort of patients analyzed in this thesis. Anthracyclines are DNA intercalating agents that can inhibit cellular replication (141). There are several types of anthracyclines, including doxorubicin, docetaxel, epirubicin, among others. Cyclophosphamide is able to form DNA crosslinks, leading to cell apoptosis (141). Taxanes, namely paclitaxel, alters the cellular cytoskeletal properties, leading to defects in the mitosis, inhibiting the process of cell division (141). These drugs have several side effects, and the most frequent ones are weakness, edema, myalgia, anemia, neutropenia and nausea, to name a few (143).

For ER+ breast cancer patients, the gold standard treatment is the use of endocrine therapy, normally tamoxifen, for at least 5 years. Tamoxifen competes for the binding of estrogen to ER and inhibits estrogen-induced tumor growth. Besides tamoxifen, aromatase inhibitors can also be used; however, they are only effective in post-menopausal women (141). Aromatase is a crucial enzyme in the synthesis of estrogen. For pre-menopausal women, it is common to induce menopause by chemical castration, i.e. with ovarian suppressor agents (144). Tamoxifen can also be prescribed to the patient for 10 years since disease recurrence can occur decades after the first diagnosis. 10-years endocrine therapy was shown to have an improvement in breast cancer mortality; however, patients with longer endocrine therapy had a higher associated risk to develop endometrial cancer or thromboembolism (145). The decision for a 5 or 10-years treatment must rely on the clinicians that should only administer the second hypothesis in high-risk tumors. Chemotherapy is normally added to endocrine therapy in ER+ patients. The combined regimen of anthracyclines, cyclophosphamide and taxane were shown to reduce the risk of recurrence (141).

For HER2+ breast cancer patients, there is a targeted therapy that consists of an antibody against HER2+ (trastuzumab). Several clinical trials have shown that the addition of trastuzumab for 1 year to chemotherapy increases the disease-free survival and overall survival in breast cancer patients, when compared to chemotherapy alone (146). Trastuzumab can be prescribed during neoadjuvant or adjuvant regimens and the preferred combination is with chemotherapy based on anthracyclines, cyclophosphamide and taxane.

TNBC lacks a target to develop specific therapies. As such, the treatment relies mainly on chemotherapy. Again, chemotherapeutic treatment can be prescribed either in a neoadjuvant or adjuvant setting and can be composed of the same combined regimen as described above. Nevertheless, several therapeutic strategies are being developed to tackle this clinical need. Namely, some TNBC tumors have deficient mechanisms of DNA damage repair, so the use of poly (ADP-ribose) polymerase inhibitors (PARPi) and platinum agents can be considered (141). In addition, anti-VEGF drugs, namely bevacizumab, or ixabepilone that stabilizes microtubules are being tested in TNBC patients (see review (147)). Actually, olaparib (from AstraZeneca), which is a PARPi was approved by the FDA in 2018 for metastatic TNBC with *BRCA* mutation. Talazoparib (from Pfizer), another PARPi is also FDA approved for locally advanced or metastatic HER2 negative breast cancer with *BRCA* mutation.

Another promising approach is the use of PI3K inhibitors, since PI3K is associated with tumor cells growth (148). Alpelisib (from Novartis) – a PI3K inhibitor, was approved by the FDA in 2019 for ER+, HER2 negative, PI3K-mutated, advanced or metastatic breast cancer.

6.3. Immuno-Oncology in Breast Cancer

6.3.1 Predictive Biomarkers

Although breast cancer has good survival rates when compared to other tumor types, the choice between treatment regimens is sometimes challenging for the clinicians. Moreover, when patients are selected for neoadjuvant chemotherapy (NACT), more than half will not respond to this treatment (142,149), which will delay effective therapeutic options. Hence, there is an urgent unmet clinical need, which is to find predictive markers of response, especially for neoadjuvant regimes.

Though breast cancer is not considered to be very immunogenic (150), two emerging immune-related markers have been studied for the prediction of breast cancer patients' response to NACT: tumor infiltrating lymphocytes (TILs) and neutrophil-to-lymphocyte ratio (NLR). The response to NACT is evaluated after the chemotherapy when patients are subjected to surgery – if no tumor mass is present after the therapy, the patients achieved a pathological complete response (pCR). Nevertheless, tumor cells can still be present in the breast after the surgery but with a down-staging (partial response) or not (no response).

TILs are normally assessed by immunohistochemistry counterstained with hematoxylin and eosin in formalin-fixed paraffin embedded tissue samples. This assessment is semi-quantitative and can be performed either intratumor or in the stromal compartment (151). Although this method is easily applied in the daily routine, there are several antibodies that can be used to detect TILs as well as different techniques to quantify their presence. Thus, there was a need to standardize TILs assessment in breast cancer samples, which was achieved in a publication by the International TILs working group (151).

TILs were shown to have a predictive value for pCR especially in HER2+ and TNBC subtypes. Several clinical trials have supported this idea. In the GeparDuo and GeparTrio trials, 40% of the breast cancer patients from the three subtypes with samples having >60% of stromal TILs achieved pCR, compared to only 10% pCR in samples with less than 60% of stromal TILs (152). In GeparQuattro, almost 50% of the HER2+ patients had a pCR when their samples had >50% of stromal TILs, contrarily to a pCR in 31% of patients with less than 50% of stromal TILs (153). In the GeparQuinto trial, approximately 35% of the TNBC patients achieved pCR when their samples had more than 60% stromal TILs present, compared to 14% pCR in <60% stromal TILs samples (154). In GeparSixto trial, 60% of the HER2+ and TNBC patients achieved pCR if their tumor samples had more than 60% of stromal TILs present, contrarily to an almost 35% pCR rate for patients with less than 60% stromal TILs present in their samples (155). In GeparNuevo trial, higher pCR rate in TNBC patients was achieved when the patients presented higher stromal TILs (more than 60%) (156). In the NeoALLTO trial (157) the authors demonstrated that TILs are an independent prognostic factor for HER2+ patients.

Nevertheless, we have to take into account that TILs evaluation does not discriminate between the type of lymphocytes, since as it was described previously in this introduction, T lymphocytes (either the different subsets of Th, or CTLs or Tregs), B lymphocytes or NK cells can infiltrate the tumor and influence it in a different manner, as depicted in section 2.1. So their predictive value can be argued. Even infiltrating CTLs, *per se*, lack the robustness that a predictive biomarker should have, because their function can be hampered by tumor cells, through several mechanisms. For instance, by expressing inhibitory molecules like PD-L1 or by secreting immunosuppressive molecules such as IL-10 and TGF-β. Thus, it is still crucial to establish a more accurate breast cancer "immunological status" that reflects the overall strength of individual patients' anti-tumor immune response that will ultimately influence NACT efficiency.

Attempting to discriminate between TILs subset, some studies have performed immunohistochemistry with CD4 (Th and Tregs) or CD8 (CTLs) antibodies and correlate with pCR. In the three subtypes of breast cancer, a positive correlation between CD8+ TILs and pCR was found (115,158). The same correlation was found for CD4+ TILs (159). Nevertheless, high CD8 was also positively correlated with disease-free survival, but not pCR independently on the subtype of breast cancer (41). Other authors describe that a high ratio between CD8 and FOXP3 (a marker of Tregs), increases the chance of achieving a pCR in TNBC patients (160,161) and HER2+ breast cancer patients (161). In addition, another study performed in the three

breast cancer subtypes defined that high CD8 and high FOXP3 was positively correlated with pCR (162).

In spite of trying to define which type of TILs are fundamental for a good response to the treatment, the abovementioned studies lack the statistical power due to limited sample size when compared to the studies indicating TILs as a predictive factor for NACT response. Moreover, the same questions remain: although these studies are dividing some subsets of TILs, the CD4+ TILs, for instance, can be any type of helper T cells and even Tregs, which have distinct roles in tumor immunology. Moreover, the fact that CD8+ T cells (CTLs) and FOXP3+ T cells (Tregs) have the same tendency for pCR is debatable due to the fact that they have opposite roles. Hence, further studies that aim at scrutinize the TILs subsets and their role for NACT success should be performed.

The neutrophil-to-lymphocyte ratio (NLR), contrarily to the TILs, is not assessed in the tumor, but in the peripheral blood. The rationale behind this marker is that tumor-promoting inflammation can be reflected systemically and, normally, inflammation leads to an increase in neutrophil count.

In breast cancer, a high NLR is seen as a poor prognosis marker. In TNBC, it was published that patients with a NLR higher than 3, had poor prognosis (163). The same cut-off was calculated for another cohort of TNBC patients (164) and for a cohort with the three subtypes of breast cancer (165). Other authors calculated a cut-off for NLR in ER+ breast cancer patients' blood of 2.25 (166) and 1.67 (167). Additionally, in cohorts of breast cancer patients with the 3 different subtypes, the cut-off of NLR was calculated as 3.33 (168) and 2.1 (169). Meta-analysis of these studies revealed that lower NLR favors pCR (165).

Nonetheless, there is no consensus on the threshold value for the NLR that could distinguish good or poor prognosis. Moreover, the studies referred above were composed of cohorts with variable number of breast cancer patients, which can lead to the different cut-off values that were calculated.

Overall, both TILs and NRL could be markers to predict response to NACT; however, they are still not used in the clinic routine. Indeed, there are still many questions left without response – what NLR cut-off value should be used? Which patients would benefit from TILs assessment? And which would benefit with NLR analysis? Are we assessing only the chances to achieve pCR after NACT or the patients' survival?

Thus, the unmet clinical need referred in this subsection still needs to be resolved.

6.3.2. Immunotherapy

As previously mentioned, approximately half of the patients that are selected to perform NACT will not respond to this treatment (142,149). Predictive markers can help in assessing *a priori* which patients will not respond to this treatment so that other therapies can be used for non-responder patients. The problem is that there are no alternative therapies for these patients. A surgery followed by adjuvant chemotherapy could be used, but only in operable tumors; and the chances of executing a conservative surgery decreases and probably a mastectomy has to be performed.

As such, there is a lack of alternative therapeutic strategies for breast cancer patients. Again, breast cancer, compared to other types of tumors, such as melanoma, is not very immunogenic (150), which can explain the lack of success with immunotherapeutic approaches (170). For instance, the anti-PD-1/-PD-L1 therapy has a modest response in breast cancer patients (approximately 25% (171)), probably because (i) tumors could be poorly infiltrated with immune cells; (ii) PD-L1 expression is often observed in the immune components of breast tumors rather than in the tumor cells (172); and additionally (iii) PD-1 expression on tumor infiltrating T cells is highly variable between patients (170).

Nevertheless, several clinical trials are being conducted in breast cancer with the immune checkpoint blockade (Table I-1). The majority tackles TNBC patients and some test the immunotherapy in combination with chemotherapy.

Table I-1 **Ongoing clinical trials with immune checkpoint blockade therapy in breast cancer**.

Trial Name	Phase	Drug	Breast Cancer Subtype
NCT02685059	II	Anti-PD-L1 + Paclitaxel + Epirubicin + Cylophosphamide	TNBC
NCT03197389	I	Pembrolizumab	HER2/TNBC
NCT02838823	I	Toripalimab	TNBC
NCT02819518	III	Pembrolizumab + Paclitaxel or Pembrolizumab + Gemcitabine and Carboplatin or all together or without Pembrolizumab	TNBC
NCT03414684	II	Nivolumab + Carboplatin or Carboplatin alone	Metastatic TNBC
NCT03616886	I/II	Paclitaxel + Carboplatin and Durvalumab	Metastatic TNBC
NCT02752685	II	Pembrolizumab + Paclitaxel	Metastatic HER2-
NCT02999477	I	Pembrolizumab + Paclitaxel	ER+
NCT03036488	III	Pembrolizumab + Chemotherapy (Paclitaxel + Doxorubicin + Epirubicin + Cyclophosphamide + Carboplatin) or Chemotherapy alone	TNBC

NCT03409198	II	Ipilimumab + Nivolumab + Chemotherapy (Doxorubicin + Cyclophosphamide) or Chemotherapy alone	Metastatic ER+
NCT03725059	III	Pembrolizumab + Chemotherapy (Paclitaxel + Doxorubicin + Epirubicin + Cyclophosphamide) or Chemotherapy alone	HER2-
NCT02628132	I/II	Durvalumab + Paclitaxel	Metastatic TNBC
NCT02648477	II	Pembrolizumab+ Doxorubicin	Metastatic HER2-
NCT04060342	I/II	Pembrolizumab + Paclitaxel or only Paclitaxel	Metastatic TNBC
NCT02447003	II	Pembrolizumab	Metastatic TNBC
NCT02644369	II	Pembrolizumab	TNBC
NCT04191135	II/III	Pembrolizumab + Carboplatin + Gemcitabine with or without Olaparib	Metastatic TNBC
NCT03971045	II	Pembrolizumab	All
NCT04042701	I	Pembrolizumab + Trastuzumab deruxtecan	Metastatic
NCT02555657	III	Pembrolizumab or Chemotherapy	Metastatic TNBC
NCT02834013	II	Ipilimumab + Nivolumab or Nivolumab only	All

Pembrolizumab - anti-PD-1; Toripalimab - anti-PD-1; Nivolumab - anti-PD-1; Durvalumab - anti-PD-L1; Ipilimumab - anti-CTLA4; Olaparib – PARP inhibitor.

Recently, an approach employing adoptive T cell therapy was achieved successfully in a metastatic ER+ breast cancer patient (173), taking advantage of the major advances in next generation sequencing techniques. Briefly, tumor-specific mutations were assessed by whole exome sequencing and RNAseq of the tumor lesion and TILs were isolated from the tumor and cultured in the presence of IL-2. TILs that were reactive against the mutations found in the tumor (4 mutated proteins) were selected and reinfused in the patient after a proliferation phase. In addition to the TILs, the patient received IL-2 and pembrolizumab (anti-PD-1). The metastasis of the patient started to regress, and no visible lesions were observed 22 months after the TILs infusion.

This study revealed that adoptive T cell therapy is an option for breast cancer patients. However, it is necessary to take into account that this treatment is clearly very costly, due to i) the specific techniques to find tumor antigens and ii) the facilities to grow autologous cells for clinical use. Moreover, it is very demanding, time-consuming to achieve a pure population of tumor-specific T cells that represents around 10^{10} cells and still requires validation, assessment of toxicity and potential off-target effects. Thus, extensive and robust investigation in this field is still required.

In this thesis, a focus on breast cancer immune status was performed. Since, currently, breast cancer patients with locally advanced disease rely mainly on NACT, and taking into account that both TILs and NLR are debatable predictive markers, the need to find a reliable and

powerful predictive biomarker for the response of NACT will be tackled. Moreover, due to the lack of alternative treatments for breast cancer patients without response to NACT, in this thesis we developed a 3D system to test different therapeutic combinations in an effort to obtain new strategies especially for NACT non-responder patients.

Chapter II

Rationale and specific aims

Chapter II – Rationale and specific aims

Breast Cancer is the second most frequent type of cancer worldwide. However, when taking into account only the female population, breast cancer is the most frequent cancer worldwide (137). The treatment for breast cancer is effective, especially for the estrogen receptor positive and HER2 subtypes. Nonetheless, when the disease is in an advanced state, the effectiveness of the treatment is severely reduced. For inflammatory/inoperable tumors or for locally advanced disease, i.e. tumors with more than 2 cm of diameter and/or with extension to the axillary lymph node, the treatment of choice is neoadjuvant chemotherapy (NACT), independently of the breast cancer subtype. This type of chemotherapy is prescribed before the surgery and lasts for six months, allowing the reduction of the tumor mass and/or the inflammatory process, and consequently permitting a more conservative surgery. Yet, more than half of the patients do not response to this treatment, never achieving a pathological complete response (142,149). Thus, there is an urgent unmet clinical need to find new biomarkers to predict the response to NACT. This is essential in order to distinguish *a priori* the patients who will not benefit from this treatment and promptly direct them to effective treatments, reducing unnecessary chemotherapy-related toxicity. Moreover, another foremost important clinical need is the development of new therapeutic strategies to offer to the NACT non-responder patients.

Hence, the main focus of this thesis is to address both of these clinical needs and ultimately contribute to ameliorate the breast cancer prognosis and treatment. Specifically, we intended to:

1. Identify new biomarkers to predict breast cancer patients' response to neoadjuvant chemotherapy

To identify new biomarkers to predict treatment response we have established a collaboration with 3 hospitals in the Lisbon area, to obtain biopsies, blood and surgical specimens of breast cancer patients. The hospitals were Hospital CUF Descobertas, Hospital Prof Doutor Fernando Fonseca and Hospital de Vila Franca de Xira. Our approach was to focus on immune cell populations and immune-related molecules (results in Chapter IV).

Since the presence of tumor infiltrating lymphocytes (TILs) has been appointed as a predictor of response to NACT, we have deepened our analysis on TILs subtypes. Namely, cytotoxic T cells (CTLs), helper T cells (Th) and regulatory T cells (Tregs). Specifically, CTLs and Tregs have been gaining attention in the recent years because the first ones are able to eliminate tumor cells; while the last ones have the capacity to dampen the effector function of CTLs. However, the analysis of TILs is still controversial and not routinely implemented in the clinic. Indeed, due to their opposite effects (as it is the case of CTLs *vs* Tregs) they should not be analysed globally. Also the ability of tumors to engage mechanisms to escape immune surveillance and

clearance by CTLs, have to be taken in account. Hence, our hypothesis was that the presence in the tumor microenvironment of TILs, *per se*, even considering specifically the CTLs or Tregs, was not enough to predict patients response to NACT, but their activation status could be.

HLA-DR is a T cell activation marker (174), normally expressed in later stages of cell activation (73). Although HLA-DR is a well-known antigen presenting molecule, normally expressed on antigen presenting cells (such as dendritic cells), its expression on T cells has been described to be important in autoimmune diseases and viral infections (175–178). Moreover, in the context of HIV patients, it has been described that HLA-DR+ CTLs exhibit a high cytolytic potential (179,180).

Although functionally, HLA-DR is related to antigen presenting cells, the HLA-DR+ CTLs were found to also have the machinery needed for antigen processing and loading on HLA-DR molecules. Additionally, these cells were found to be able to express CD86 and CD80, which are the co-stimulatory molecules of antigen presenting cells that are necessary for the proper T cell effector function (181). Moreover, it was described that T cell-T cell synapsis occur to allow T cells to secrete IFN-γ towards each other, compelling the differentiation of more protective T cells (76). These T cell-T cell interactions and mutual antigen presentation can be essential for mounting a suitable anti-tumor response.

Taking together our hypothesis and the fact that T cell activation can be detected by HLA-DR expression, in Chapter IV, we evaluated HLA-DR expression on T cells in breast cancer samples, alongside other immune traits of the tumor microenvironment that could be related to CTLs' activation or suppression, in order to find a reliable biomarker that could predict breast cancer patients response to NACT. Moreover, a comparison between biopsies, surgical specimens and peripheral blood was conducted to assess if the immune status encountered in the tumor is systemically represented.

2. Characterize HLA-DR+ cytotoxic T cells

To further characterize HLA-DR+ cytotoxic T cells (CTLs) and test if they are active and functional, with relevant cytotoxic abilities, we have analyzed deeply this cell type on Chapter V. This aim was achieved by flow cytometry and gene expression analysis of different immune molecules that might be related with CTLs function. Moreover, an additional characterization was performed to clarify HLA-DR+ CTLs immune profile, i.e., to see if these cells belong to an effector or exhausted subtype, or to a memory class of T cells. This study is relevant to further extend the knowledge regarding activated CTLs and their subtypes, which may contribute to pave the way for future development of efficient T cell-based immunotherapies.

3. Establish a 3D *in vitro* platform of breast cancer cells and immune cells to validate HLA-DR+ cytotoxic T cells as a biomarker of response to conventional chemotherapy and explore new potential therapies.

To tackle the second main objective of this thesis – develop new therapeutic strategies for breast cancer patients without response to conventional treatment, we have established a 3D *in vitro* system based on breast cancer cell lines spheroids. A 3D system is a more physiological representation of healthy tissues and also of pathological conditions when compared to 2D culture. 2D culture is based on a monolayer of polarized cells with only one side of their surface area attached to the substrate and the remaining surface in contact with the culture medium. This type of morphology can greatly limit tissue-specific functions. On the other hand, 3D *in vitro* cell culture models offer cell-cell and cell-matrix interactions, which will influence cell structure, cell signaling, cell adhesion and mechano-sensing. Moreover, the diffusion of oxygen and nutrients is hampered to the core of the structure and produced metabolites are also poorly diffused from the core to the surface of the 3D structure. Furthermore, the addition of different cell types is possible, creating a multicellular structure (182,183). Hence, this system recapitulates key architectural and cellular features of the tumor microenvironment. As such, 3D cell models are being used as an easy to work, reliable and reproducible preclinical tool for tumor drug screening and to clarify tumorigenesis mechanisms.

For our aim, we developed a 3D structure of breast cancer cell lines in co-culture with patient-derived immune cells by using a liquid overlay technique to allow a spontaneous 3D spheroid formation. The cancer cells were co-cultured with allogenic immune cells isolated from patients' blood (PBMCs). This method was already proven to work in other reports and allogenic PBMCs have shown a capacity to produce several cytokines and eliminate target cells (184,185). The advantages of the allogenic system are the fact that they are simpler, reproducible and PBMCs derived from different patients can be used with the same system without the need of histocompatibility with the tumor cells.

To take advantage of our platform, we executed an *in vitro* approach to better clarify the role of HLA-DR+ CTLs in the response of the spheroid to conventional chemotherapeutic agents (results in Chapter VI). Also, an *in vitro* study was conducted taking in account the HLA-DR+ CTLs/NACT response status to assess if we could replicate clinical observations with the 3D platform and therefore validate *in vitro* the biomarker robustness (results in Chapter VI).

Finally, we took advantage of the model created, to test new therapeutic strategies for patients with a weak response to NACT. Namely, we tested in 3D co-cultures based on cancer cell lines and PBMCs particularly derived from NACT non-responder patients, several therapeutic agents in different combinations. For instance, we assessed the viability of cancer cells in the presence or absence of the referred PBMCs with doxorubicin (a common chemotherapeutic drug), with immune stimulus or other immunotherapeutic agents, such as the blocking antibodies against the PD-1/PD-L1 axis (Chapter VI).

Altogether, we intend to develop a 3D platform with clinical representation to test new alternative strategies to treat patients without response to NACT, improving breast cancer treatment and the patients' quality of life.

Chapter III

Materials and Methods

Chapter III - Materials and Methods

1. Patients' samples

Samples from breast cancer patients were provided by Hospital CUF Descobertas, Hospital Prof. Doutor Fernando Fonseca and Hospital de Vila Franca de Xira. Different types of samples were collected (biopsies, surgical specimens, whole blood and formalin-fixed paraffin embedded tissue) and each was differently processed, as detailed below.

1.1 Biopsies and Surgical Specimens

Biopsies, harvested before any treatment, and surgical specimens (either pre or post-treatment) were immediately collected into a saline solution and then transferred to Transfix (Cytomark), a cellular antigen stabilization solution that also fix the cells. The samples were processed within three days post collection. The processing consisted in dissociate mechanically the tissue with a BD Medicon (BD Biosciences), filter the cell suspension in a 30 μm mesh (Sysmex), centrifuge at 300g for 5 minutes and resuspend the pellet in PBS 1X. This cellular suspension was then prepared for immunophenotyping by flow cytometry (section 4 of this chapter).

1.2. Formalin-fixed paraffin embedded tissue (FFPE)

Formalin-fixed paraffin embedded (FFPE) tissue from 12 patients, whose biopsies or surgical specimens were previously immunophenotyped by flow cytometry, were chosen. 3 slices of each FFPE with 3 μm of thickness were cut in a microtome (Leica) and transferred to a water bath to attach the tissue to separate slides and left to dry at 60ºC for 1h. The slides were kept at 4ºC until the immunofluorescence protocol (section 6 of this chapter).

1.3. Blood

Approximately 10 mL of whole blood was collected in Vacutainer tubes with EDTA (BD Biosciences). An aliquot was promptly prepared for immunophenotyping by flow cytometry (section 4 of this chapter). To obtain the plasma fraction, whole blood was centrifuged at 13000 rpm for 10 min. Plasma was then frozen at -80ºC until further use for ELISA assays (section 7 of this chapter). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood, diluted in PBS 1X (1:1 ratio) and layered on top of Ficol solution (Biocol by Merck Millipore) in a 3:5 ratio (Ficol:blood). This solution was centrifuged at 2100 rpm for 30 min, without brake. After the centrifugation, the blood components were separated and the PBMCs layer isolated with a Pasteur pipette to a new falcon tube. 20 mL of PBS 1X was added and the sample centrifuged at 1300 rpm for 5 min, followed by a second wash with 10 mL of PBS 1X and centrifugation at 1000 rpm for 5 min. The number of PBMCs was counted on a Newbauer chamber and frozen in fetal bovine serum (FBS, Biowest) with 10% dimethyl

sulfoxide (DMSO, Sigma Aldrich) in a concentration of $5x10^6$ to $1x10^7$ cells per cryovial. The isolated PBMCs were maintained at -80ºC until further use.

1.4. Patients inclusion criteria and characteristics

211 patients diagnosed with breast cancer were included in this study. The inclusion criteria defined that patients should have an invasive breast tumor, have more than 18 years, understand the study in which they will participate and give informed consent. Exclusion criteria comprise patients with ductal in situ breast tumors or patients with auto-immune diseases, whose prior altered immune responses could influence the results of this study.

The breast cancer patients were divided according to the therapeutic regime: a percentage of patients were submitted to surgery followed by adjuvant chemotherapy and the second group of patients were submitted to neoadjuvant chemotherapy (NACT). This last regimen is mainly approved for patients with inoperable tumors or patients that have locally advanced disease (tumors with more than 2 cm and/or with axillary lymph node metastasis), aiming at the reduction of the tumor mass before surgery, which may allow a more conservative intervention. In general, NACT consists of 4 cycles of doxorubicin (60 mg/m^2) and cyclophosphamide (600 mg/m^2) every three weeks, followed by 12 weeks of paclitaxel (80 mg/m^2). HER2 positive patients were also treated with Trastuzumab every three weeks. Altogether, NACT treatment lasts for 6 months.

Patients' samples acquired were divided as follows (and the samples were used for distinct procedures, exemplified in Figure III-1):

- 53 biopsies, collected before the treatment, of patients that were selected for NACT (Pre-NACT)
- 46 biopsies of patients that were not submitted to NACT (no-NACT)
- 10 surgical specimens, collected after the treatment, of patients that were submitted to NACT (Post-NACT)
- 88 surgical specimens of patients that were not submitted to NACT (no-NACT)
- 31 blood samples, collected before the treatment, of patients that were submitted to NACT (Pre-NACT)
- 54 blood samples of patients that were not submitted to NACT (no-NACT)

No-NACT surgical specimens and blood samples were used in chapter IV with the objective of understanding the differences between the samples' immunophenotype when comparing the axillary lymph node invasion status or other patients' characteristics, such as age and body mass index. Furthermore, correlations between tumor immune status and circulating immune features were conducted.

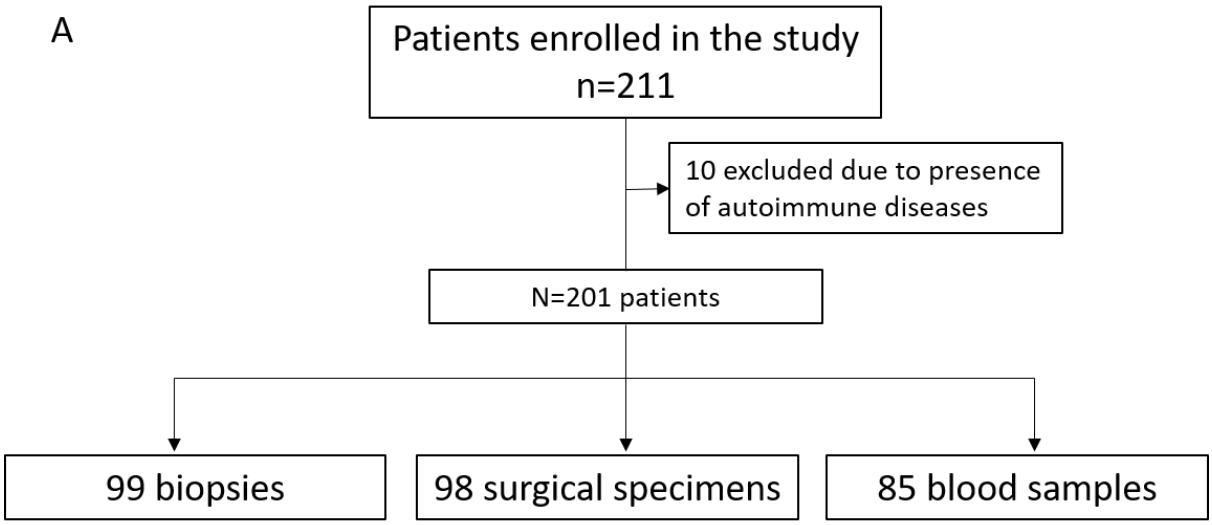
Pre-NACT biopsies and blood samples, as well as post-NACT surgical specimens, were used in chapter IV, aiming at identifying a novel reliable biomarker of patients' response to NACT. This study was conducted in two phases: pilot and validation. The cohort used in the first phase

included the first consecutive biopsies of patients selected for NACT from October 2016 to June 2018. The cohort used in the second phase included the first consecutive biopsies of patients selected for NACT, from September 2018 to July 2019.

To summarize the patients included in this study and the samples collected, a flow chart was performed (Figure III-1). The patients' clinical characteristics are described in Table III-1.

Table III-1 **Characteristics of patients enrolled in this study** (age, body mass index, and menopause). Clinical data, such as subtype of breast cancer, grade, Ki67 (related to the tumor proliferation rate), tumor dimension, node status and response to treatment are also described.

Age	Median – 59 (range: 29-87)
Body mass index (BMI)	Median – 26.02 (range: 17.04–42.19)
Post-menopause	64.61%
ER+ (PR -/+)	68.93%
HER2+ including triple positive breast cancer	16.38%
TNBC	14.69%
Grade	G1 – 19.19%
	G2 – 54.07%
	G3 – 26.74%
Ki67	Median – 20% (range: 1% - 98.40%)
Dimension (mm)	Median – 23 (range: 5-90)
Axillary lymph node invasion status	Negative – 56.36%
	Positive – 43.64%
NACT response (first cohort)	Good response – 43.33%
	Bad response – 56.67%
NACT response (second cohort)	Good response – 31.82%
	Bad response – 68.18%



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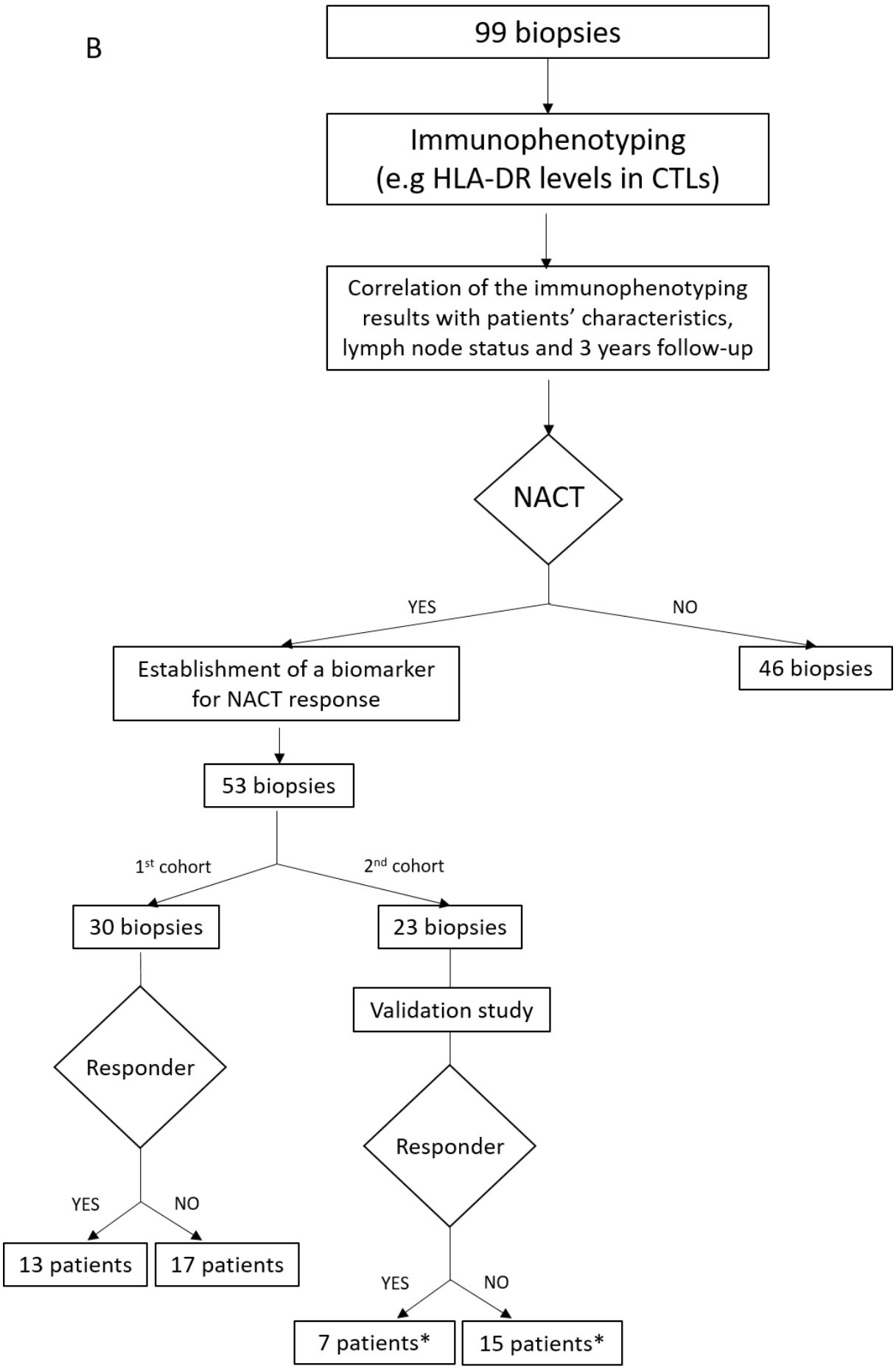
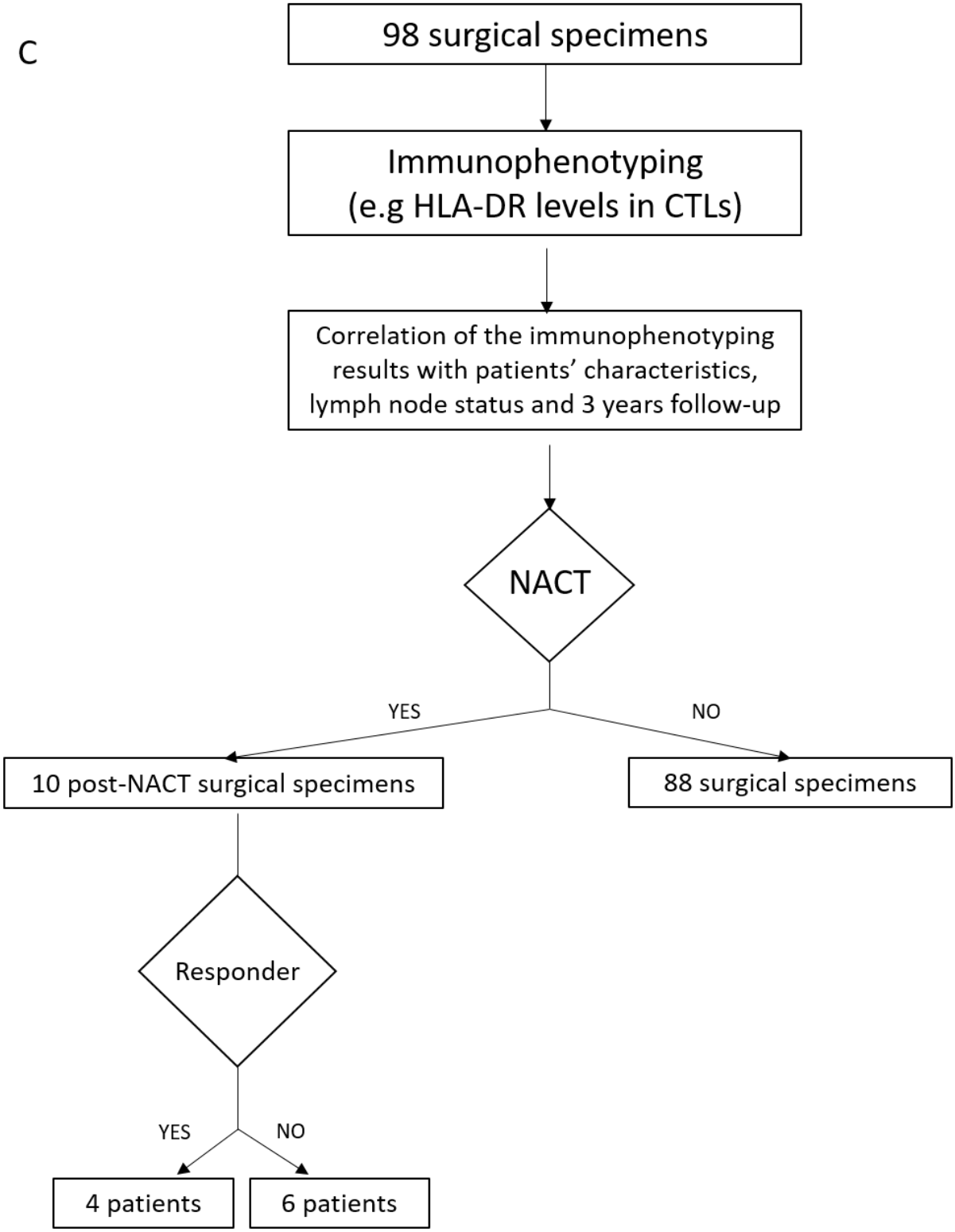

99 biopsies
Immunophenotyping
(e.g HLA-DR levels in CTLs)
Correlation of the immunophenotyping results with patients' characteristics, lymph node status and 3 years follow-up
NACT
YES
NO
Establishment of a biomarker for NACT response
46 biopsies
53 biopsies
1st cohort
2nd cohort
30 biopsies
23 biopsies
Responder
Validation study
Responder
YES
NO
YES
NO
13 patients
17 patients
7 patients*
15 patients*

C


98 surgical specimens
Immunophenotyping
(e.g HLA-DR levels in CTLs)
Correlation of the immunophenotyping results with patients' characteristics, lymph node status and 3 years follow-up
NACT
YES
NO
10 post-NACT surgical specimens
88 surgical specimens
Responder
YES
NO
4 patients
6 patients

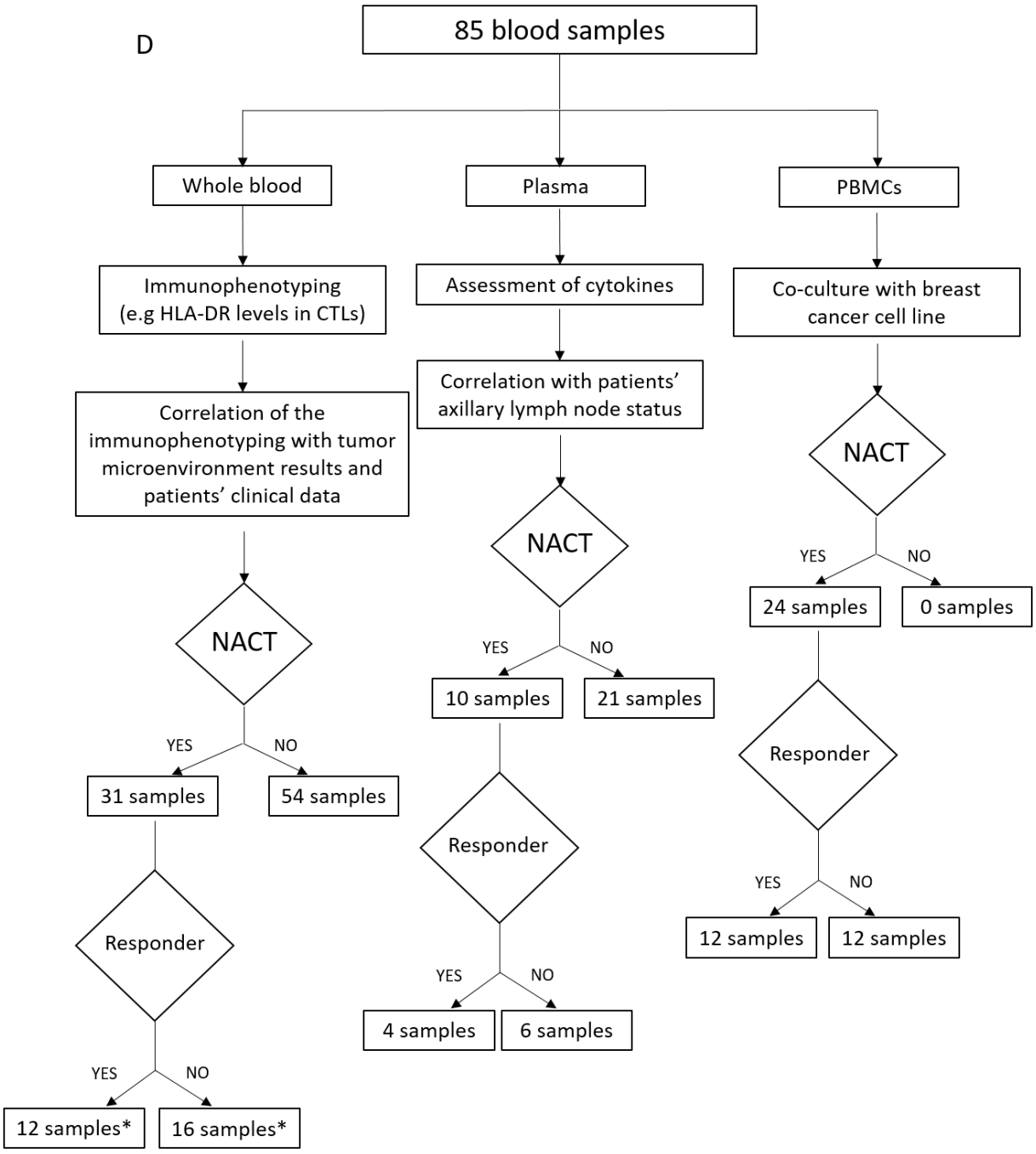


Figure III-1 **Flow chart of Breast Cancer patients enrolled in this study (A)**. **(B)** Flow chart of the biopsies, surgical specimens **(C)** and whole blood **(D)** collected for this study. *Data on the response to treatment still missing.

2. Healthy donors' samples

2.1. Blood

Whole blood from 13 healthy donors was also collected in Vaccutainer tubes with EDTA (BD Biosciences). Whole blood and plasma were obtained and processed as above described and

used in parallel with samples from breast cancer patients for comparison (Chapter IV). PBMCs were also used, but isolated from buffy coats (see section below).

2.2. Buffy coats

For some particular experiments, where a great amount of PBMCs (not necessarily from breast cancer patients) was required, buffy coats from 12 healthy donors provided by Instituto Português do Sangue e da Transplantação were used. The buffy coat was divided into three 50 mL falcons (25 mL for each), diluted in a 1:1 ratio in PBS 1X, and centrifuged at 2500 rpm for 10 min, without brake. A leukocyte enriched layer was formed and removed to a single falcon. PBS 1X was added until reach a volume of 20 mL. This solution was layered on top of 12 mL of Ficol with a Pasteur pipette and centrifuged at 2500 rpm for 20 min, without brake. The well-defined ring of PBMCs was formed, removed with a Pasteur pipette to a new falcon and washed with PBS 1X two times. Between washes the PBMCs solution was centrifuged first at 1400 rpm for 10 min with brake and after the second wash, the centrifugation step was at 800 rpm for 5 min also with brake. The number of PBMCs was counted and frozen at -80ºC, until further use, as explained above.

3. Ethics statement

This study was accepted by the Ethical committees of Hospital CUF Descobertas, Hospital Prof. Doutor Fernando Fonseca, Hospital de Vila Franca de Xira and NOVA Medical School, Faculdade de Ciências Médicas da Universidade Nova de Lisboa. Furthermore, the study was also accepted by Comissão Nacional de Protecção de Dados (CNPD).

Participants were recruited on a voluntary basis and written informed consent was obtained. Blood, biopsies and surgical specimens were collected during the patients' clinical routine and were provided to the lab only when the patients' diagnosis was not jeopardized.

Sample processing was only performed at CEDOC/NOVA Medical School, according to the Declaration of Helsinki. Moreover, it complied with national legislation for the scientific use of human biological samples (Law n. 12/2005 and Decree-Law n. 131/2014).

Clinical data from patients were managed in compliance with the EU and Portuguese legislation. Data were anonymized and stored securely in accordance with the EU's Data Protection Directive (Directive 95/46/EC) and Portuguese Legislation (Law n. 67/98).

4. Flow cytometry

4.1. Staining

Antibody staining for flow cytometry was performed in biopsies/surgical samples, as well as in whole blood samples. Briefly, after obtaining a cell suspension from biopsies/surgical samples collected in saline solution, staining with BD Horizon^{TM} Fixable Viability Stain 450 (BD Biosciences) was used to select live cells, for 20 min in the dark at 4ºC. Followed by a wash step with PBS 1X and centrifugation at 300g for 5 min. Samples kept in Transfix were not stained with this viability dye. After this step, a cocktail of mouse anti-human monoclonal fluorescent antibodies (mAbs) was added and the mixture kept in the dark for 15 min at room temperature. In the case of whole blood, the staining protocol was similar but an additional step of red blood cell lysis was performed, with RBC lysis buffer (Biolegend), in the dark for 20 min at 4ºC. A wash step was performed by adding 1 mL of PBS 1X and centrifuged at 300g for 5 min. After the cell surface staining, whenever there were intracellular markers to be analyzed, cells were fixed and permeabilized with Fix/Perm kit (eBioscience) for 30 min at room temperature. Intracellular mAbs were added for 30 min, followed by a wash step by adding 1 mL of PBS 1X and centrifuged at 300g for 5 min. The samples were fixed with FlowFix (Polysciences). Data were acquired in BD FACS Canto II with FACSDiva Software v8.0.1 (BD Biosciences) and the results were analyzed using FlowJo software v10.

The mAbs used were: anti-CD45-PercP (clone HI30), anti-CD3-PercP (HIT3a), anti-CD3-APC (UCHT1), anti-CD19-PE (HIB19), anti-CD15-PE (HI98), anti-CD161-FITC (HP-3G10), anti-CD4-FITC (OKT4), anti-CD8-PE (HIT8a), anti-CD8-Pacific Blue (SK1), anti-HLA-DR-APC (L243), anti-CD127-PE-Cy7 (A019D5), anti-CD1c-APC-Cy7 (L161), anti-CD163-PE (GHI/61), anti-CD206-APC-Cy7 (15–2), anti-PD-1-FITC (EH12.2H7), anti-PD-L1-APC (29E2A3), anti-CTLA4-PE (L3D10), anti-CD69-APC-Cy7 (FN50), anti-Tim3-APC (F38-2E2), anti-IL-8-APC (E8N1), anti-IFN-γ-PE (4S.B3), anti-IFN-γ-APC-Cy7 (4S.B3), anti-Granzyme B-FITC (QA16A02), anti-IL-1β-FITC (JK1B-1), anti-IL-2-PE-Cy7 (MQ1-17H12), anti-IL-2-PE (MQ1-17H12), anti-CD11b (ICRF44), anti-IL-6-APC (MQ2-13A5), anti-IL-17-FITC (BL168), anti-IL-23/IL-12-PE (C11.5), anti-TGF-β-APC (TW4-6H10), anti-CD69-PercP (FN50), anti-CD39-BV421 (A1), anti-Tim3-APC-Cy7 (F38-2E2), anti-Ki67-PE (Ki-67), all from Biolegend; anti-IDO-PE (eyedio), anti-IL-10-FITC (BT-10), anti-CD103-PE-Cy7 (B-Ly7) from eBioscience; anti-CD25-PE (MEM-181) and anti-CD11b-FITC (LT11) from ImmunoTools.

4.2. Gating strategy

Using the mAbs previously enumerated, the immune populations were defined as follows: cytotoxic T lymphocytes as CD45+/CD3+/CD8+; helper T lymphocytes as CD45+/CD3+/CD4+; regulatory T lymphocytes as CD45+/CD3+/CD4+/CD25^{high}/CD127^{low}; B lymphocytes as CD45+/CD19+; NK cells as CD45+/CD161+; M2 macrophages as CD45+/CD11b+/

CD163+/CD206+; M1 macrophages as CD45+/CD11b+/CD163 negative/CD206 negative; dendritic cells as CD45+/CD1c+; and neutrophils as CD45+/CD15+.

The gating strategy is shown in figure III-2-4. The immune populations are represented hereafter as a percentage of single cells (Figure III-2). For other immune markers, namely HLA-DR, PD-1, PD-L1, CTLA4, Tim3, CD69, IL-1β, IL-2, IL-6, IL-17, IL-23/IL-12, IFN-γ, Granzyme B, TGF-β, IL-8 and IL-10, we wanted to evaluate their expression level and thus a quantification was performed using the median fluorescent intensity (MFI). More specifically, the ratio between the MFI of the positive population and the MFI of the negative population was calculated to minimize the influence of potential fluctuations in the auto-fluorescence of the samples and the voltages of the flow cytometer. The negative population was superimposed with the unstained control. Unstained controls were mainly used due to limitations in sample size. Fluorescence minus one (FMO) controls were also used for some samples and similar results were obtained, thereby validating our analysis.

T-distributed stochastic neighbor embedding (t-SNE) analysis was performed (section 4 of Chapter V) with an R plugin in FlowJo. The samples from patients' biopsies were concatenated in a single file in FlowJo and the t-SNE analysis was performed in the gate of CTLs in this concatenated file. The objective of this analysis was to reduce the complex dimensionality of different patients' tumor infiltrating CTLs and observe the distinct markers expressed in both HLA-DR+ and HLA-DR negative CTLs.

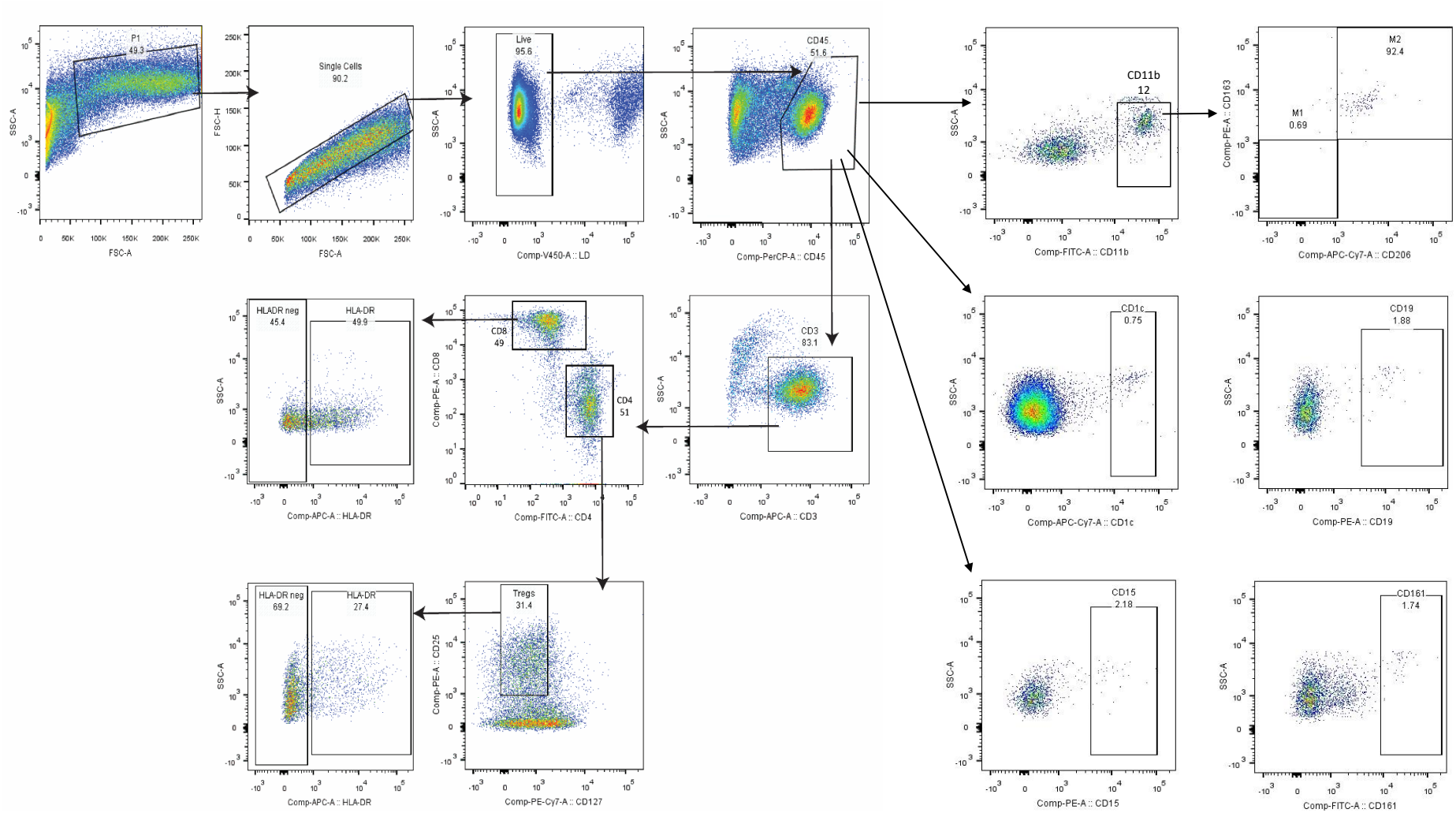


Figure III-2 **Gating strategy used to select different immune populations**.

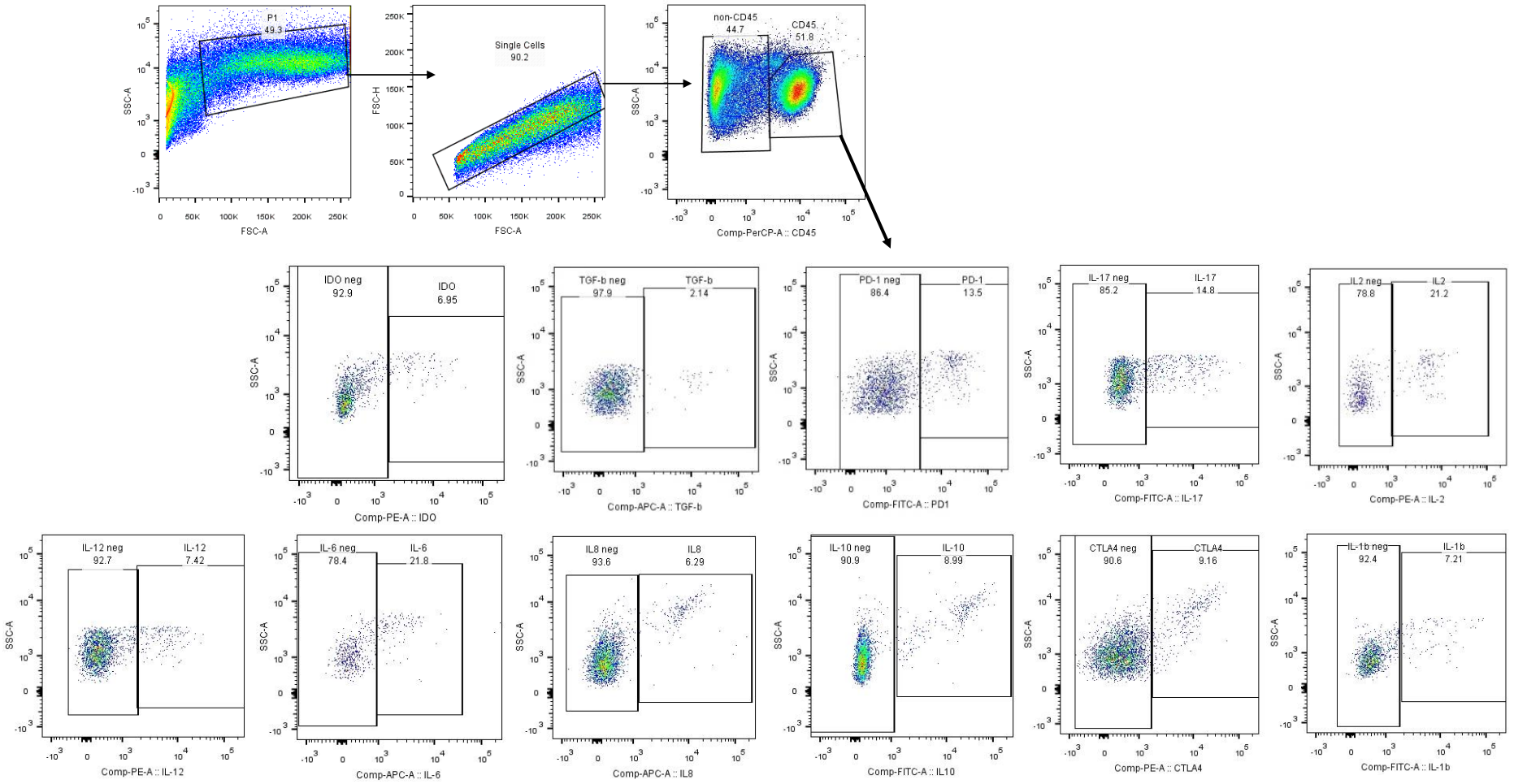


Figure III-3 **Gating strategy used to select the expression of different markers inside the CD45+ population**.

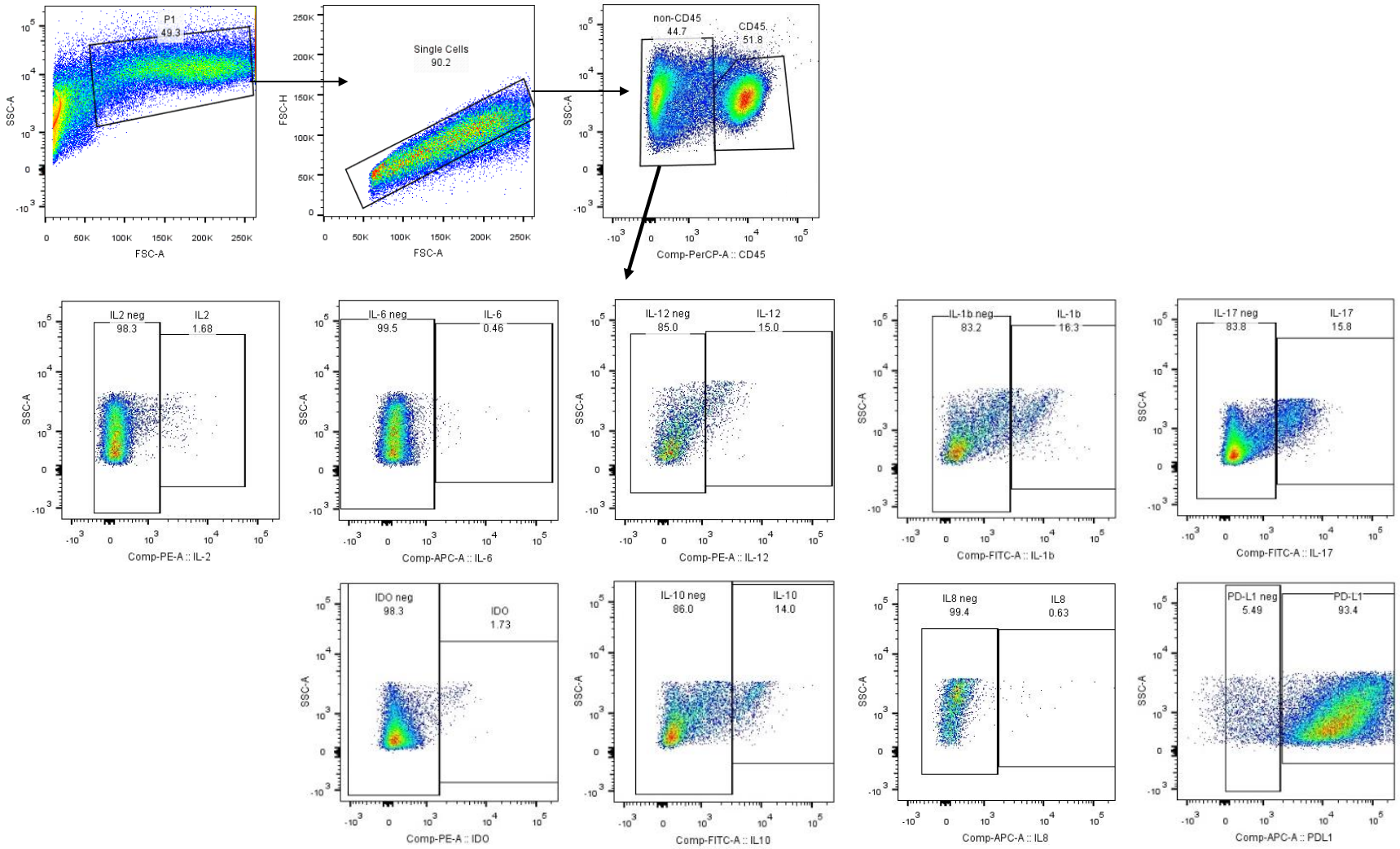


Figure III-4 **Gating strategy used to select the expression of different markers inside the CD45 negative population**.

5. Fluorescence activated cell sorting (FACS)

Fluorescence activated cell sorting (FACS) was performed in BD FACS Aria III for two different experiments. Namely, for the analysis of gene expression on HLA-DR+ cytotoxic T cells (CTLs) and HLA-DR negative CTLs by qRT-PCR (chapter V); and to evaluate the anti-tumor activity of HLA-DR+ cytotoxic T lymphocytes through a cytotoxicity assay (chapter VI).

In the first case, PBMCs from buffy coats of healthy donors were stained with BD Horizon™ Fixable Viability Stain 450 (BD Biosciences), to select live cells, for 20 min in the dark at 4ºC. Followed by a wash step with PBS 1X and centrifugation at 300g for 5 min. Cell surface staining was executed for 15 min at room temperature with the mAbs anti-CD3-PercP, anti-CD8-PE and anti-HLA-DR-APC, and washed again with PBS 1X. Cells were sorted into two populations - CD3+/CD8+/HLA-DR+ and CD3+/CD8+/HLA-DR negative with an efficiency of 98% (Figure III-5). The populations were sorted directly to a lysis buffer (from RNeasy Micro Kit, Qiagen) with 1% β-mercaptoethanol (Sigma Aldrich) for further processing for qRT-PCR analysis (see section 9 of this chapter).

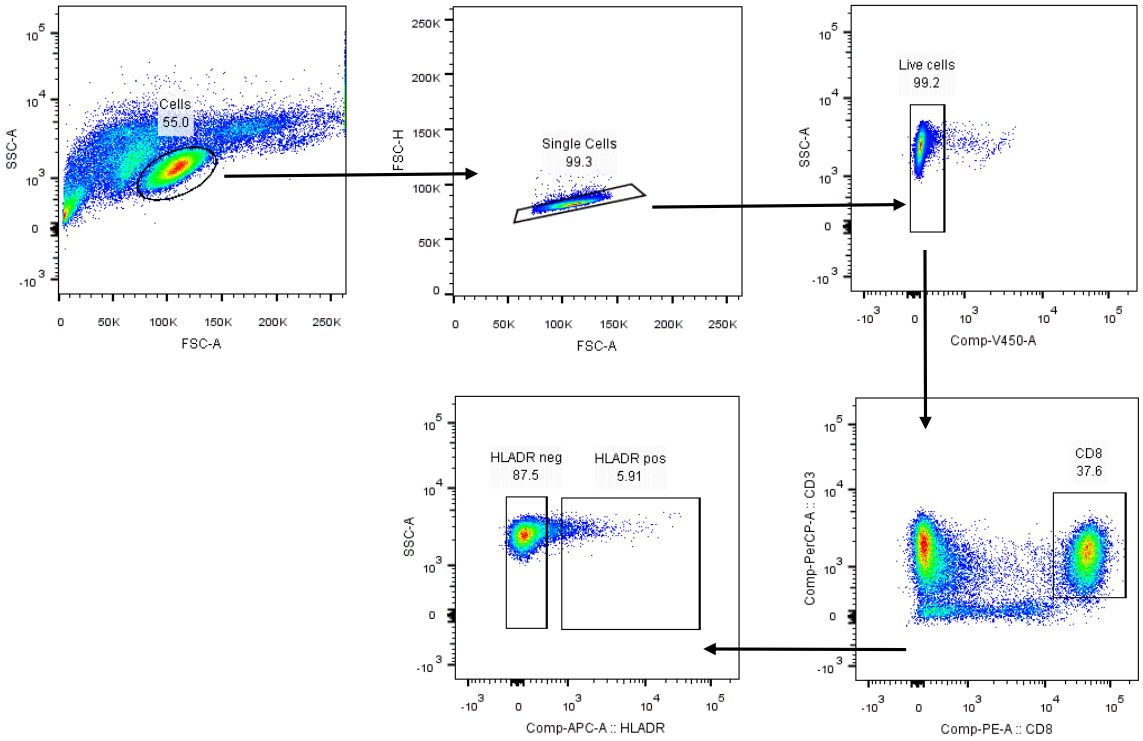


Figure III-5 **Gating strategy for sorting CD8+/HLA-DR positive and CD8+/HLA-DR negative populations**.

For the second case, PBMCs from buffy coats of healthy donors were also used. The staining was performed as above, but with anti-CD45-PercP instead of anti-CD3-PercP. The stained cell

suspension was resuspended in PBS 1X with 2% FBS and 10% Penicillin/Streptomycin and filtered through a 0.2 μm mesh (Sysmex). In this case, cells were sorted in three distinct populations: CD45+/CD8+/HLA-DR+, CD45+/CD8+/HLA-DR negative and CD45+/CD8 negative, with an efficiency between 90 and 95% (Figure III-6). Cells were collected in RPMI-1640 (Gibco) supplemented with 10% Penicillin/Streptomycin (GE Healthcare) and 10% FBS.

In both cases, cell debris, aggregates and dead cells were removed (Figure III-5-6). Cells were separated with a maximum of stringency to avoid cell population cross-contamination.

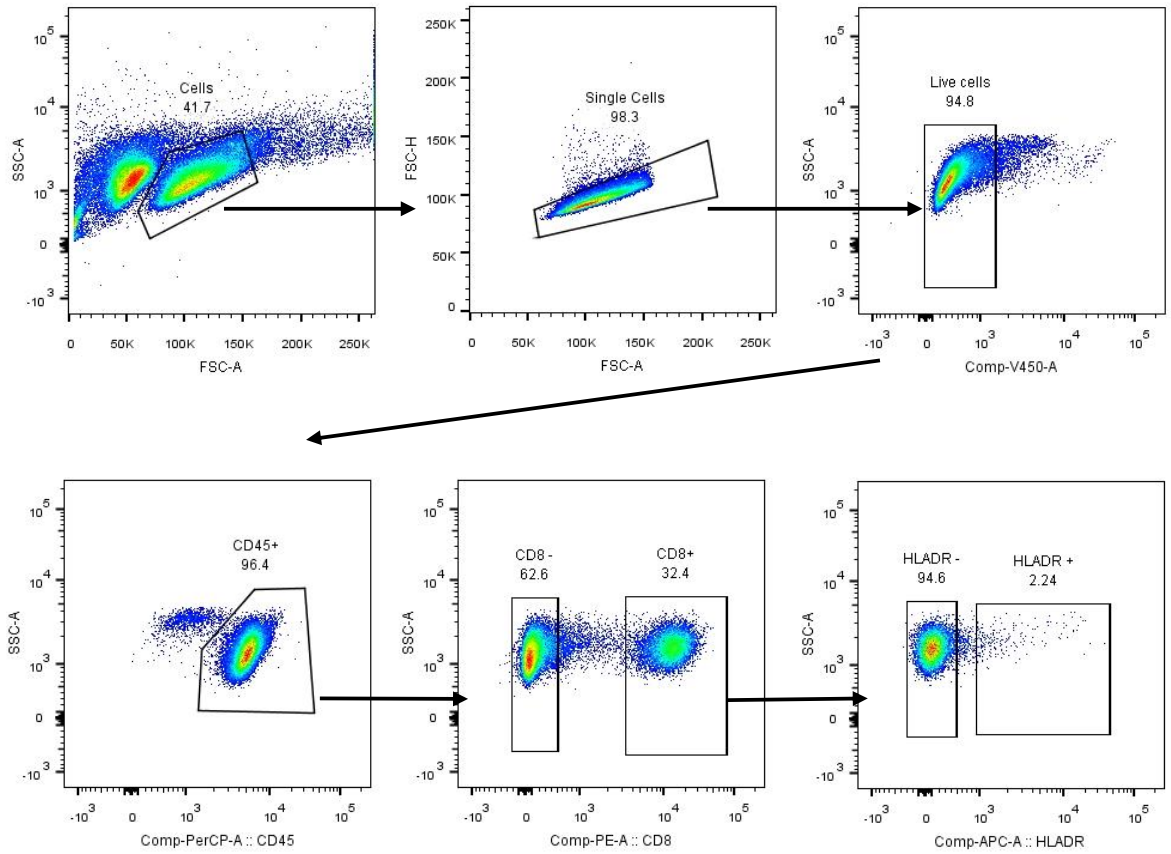


Figure III-6 **Sorting strategy used to collect three different populations – CD45+/CD8-, CD45+/CD8+/HLA-DR+ and CD45+/CD8+/HLA-DR- from buffy coats of healthy donors.**

6. Immunofluorescence

In order to evaluate the localization of some studied molecules in the tumor microenvironment, immunofluorescence was performed in selected FFPE samples, matched with the samples previously analyzed by flow cytometry. To start, the paraffin was removed in a xylene bath (10 min) and passed through a gradient of alcohol (100, 96, and 70%) and dH_2O, 30 seconds each. Antigen retrieval was performed by placing the slides in a container with 1 mM EDTA pH 9 + 0.05% Tween 20 (Sigma Aldrich) and heated in a microwave at 900W

for 30 min. During this time, new solution was added to the container if evaporation was observed. After the antigen retrieval, the tissue was permeabilized with PBS 1X + 0.3% Triton X-100 (ACROS Organics) for 30 min with shaking. Then, the slides were placed in a wet chamber and the tissue boundaries were marked with a hydrophobic pen (DAKO). Blocking was performed with PBS 1X + 0.1% Triton X-100 + 1% bovine serum albumin (BSA, Sigma Aldrich) + 1.5% goat serum (Sigma Aldrich) for 5h. The primary antibody was added at a concentration of 1:100 in blocking solution overnight at 4ºC. The primary antibodies used were mouse monoclonal anti-human CD8 (32-M4) from Santa Cruz Biotechnology and rabbit polyclonal anti-human HLA-DRA from Sigma Aldrich.

On the next day, 4 rounds of washing with PBS 1X + 0.1% Triton X-100 was executed with shaking (10 min each). The secondary antibody was applied at a concentration of 1:500 in blocking solution for 2h in the dark at room temperature. The secondary antibodies used were goat anti-mouse Alexa 488 and anti-rabbit Alexa 568. 3 more rounds of washing with PBS 1X + 0.1% Triton X-100 were executed with shaking (10 min each). Counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI; 0.001 mg/mL in PBS 1X, Sigma Aldrich) for 10 min with shaking. At least 5 rounds of washing with PBS 1X were executed with shaking, 10 min each. The slides were mounted in Fluorescent Mounting Media (DAKO). Images were acquired in a confocal microscope (LSM710, Zeiss) and analyzed with Fiji software (186).

7. ELISA

ELISA was performed to quantify cytokines in patients' plasma and in the supernatants of cell culture (described in section 11 of this chapter). For the first case, plasma was isolated from whole blood as described above (section 1.3 of this chapter) and the levels of IL-10 and IFN-γ were analyzed. For the second case, the cell culture supernatant was harvested and centrifuged at 2000 rpm for 10 min to exclude cell debris and frozen at -20ºC. Only IFN-γ was quantified in the cell culture supernatant. The IL-10 kit (Immunotools) and the IFN-γ kit (Biolegend) were used according to the manufacturer's instructions.

For IFN-γ detection, a 96 well plate was coated with the capture antibody in coating buffer (1L of dH_20 with 8.4g $NaHCO_3$ and 3.56g Na_2CO_3 pH 9.5) overnight at 4ºC. The next day, the plate was washed 4 times in PBS + 0.05% Tween-20 (wash buffer). Blocking with PBS 1X + 1% BSA was performed for 1h with shaking. The plate was washed again 4 times. 100 μL of the standards and of the samples were added to each well and incubated for 2h with shaking, followed by another 4 cycles of washing. Detection antibody was added and the plate incubated for 1h with shaking. Another similar round of washing was performed. Avidin-HRP (horseradish peroxidase) was added and incubated for 30 min with shaking. This time, the wash cycles were performed 5 times, waiting 1 minute between washes. TMB substrate (Biolegend) was added to the plate and incubated in the dark for 30 min, followed by the addition of the stop solution (Biolegend). The absorbance was read at 450 nm and 570 nm

(Tecan Infinite F200 PRO). The concentration was calculated taken into account the standard curve values and the difference between the absorbance at 450 and 570 nm.

For IL-10 detection, a similar protocol was used, but with a different coating buffer (only PBS 1X) and blocking solution (PBS 1X + 2% BSA + 0.05% Tween-20). The incubation with the detection antibody was performed for 2h, instead of 1h as above.

8. Gene expression

To get more insights into the differences between CD8+ HLA-DR+ and CD8+ HLA-DR negative cells, quantitative real-time PCR (qRT-PCR) of several genes was performed in both populations sorted from healthy donors' PBMCs. After sorting, these cells were collected directly to a lysis buffer supplemented with 1% β-mercaptoethanol to isolate total RNA using the RNeasy Micro Kit from Qiagen. The lysates were transferred to QIAshredder spin column (Qiagen) and centrifuged for 2 min at full speed. 70% ethanol was added to the homogenized lysate, passed through RNeasy MinElute® spin column and centrifuged at 10000 rpm for 30 seconds. The spin column membrane was washed and centrifuged again at the same speed and time. DNase I was added to the membrane for 15 min and centrifuged again at 10000 rpm for 30 seconds. Another washing step was performed followed by the addition of 80% ethanol to the membrane and centrifuged at 10000 rpm for 2 minutes. Another centrifugation at full speed for 5 minutes was carried out to ensure that no ethanol or buffer remained. In the end, 14 μL of RNA-free water was added to the membrane and centrifuged at 10000 rpm for 1 min. RNA quantification was performed in a Nanodrop (Thermo Scientific).

The RNA was reverse transcribed to cDNA using the Transcriptor High Fidelity cDNA synthesis kit (Roche), according to the manufacturer's instructions. Briefly, to 500 ng of RNA, oligo(dT) primers were added and kept at 65ºC for 10 min, followed by the addition of reverse transcriptase and two heating cycles, first at 55ºC for 30 min and then at 85ºC for 5 min.

Quantitative real-time PCR (qRT-PCR) was performed with several primers, described in Table III-2, using Roche LightCycler 480 and FastStart Essential DNA Green Master Mix (Roche). Cyclic conditions were: 95ºC for 10min, followed by 45 amplification cycles, each consisting of 10 sec at 95ºC, 10 sec at 56ºC, and 20 sec at 72ºC, and finally a melting step of 10 sec at 95ºC, 60 sec at 65ºC, and 1 sec at 97ºC.

The relative mRNA levels were normalized against the housekeeping gene RPL13A expression and calculated by the 2(-ΔΔCt) method (187).

Table III-2 **Genes assessed with qRT-PCR and the primers used.**

Gene	Forward primer	Reverse primer
TNFα	AGATGATCTGACTGCCTGGG	CTGCTGCACTTTGGAGTGAT
Granzyme B	GGGGGACCCAGAGATTAAAA	CCATTGTTTGGTCCATAGGAG
Perforin	GCAATGTGCATGTGTCTGTG	GGGAGTGTGTACCACATGGA
Eomes	CAGCACCACCTCTACGAACA	CGCCACCAAACTGAGATGAT
Tbet	CACCTGTTGTGGTCCAAGTTT	AACATCCTGTAGTGGCTGGTG
RPL13A	TGCGTCTGAAGCCTACAAGA	TCCGTAGCCTCATGAGCTGTT

9. 2D cell cultures

9.1. Ex vivo stimulation assay

To evaluate whether the immune cells of breast cancer patients are able to become activated upon stimulation, PBMCs isolated from patients and healthy donors (as described in sections 1.3 and 2.2 of this chapter) were stimulated *ex vivo* by plating $1x10^5$ cells per well in a 96 well plate with U bottom (Sigma Aldrich). Stimulation was performed during 4h (or overnight for HLA-DR expression analysis) with 35 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) and 1 µg/mL of ionomycin (Merck Millipore). To assess intracellular cytokine levels, Brefeldin A (Biolegend) was added for 4h to stop the extracellular transport of these molecules. Cells were collected and stained with mAbs and analyzed by flow cytometry.

9.2. Breast cancer cell lines

In vitro assays were conducted with three different breast cancer cell lines: Hs 578T, MCF-7 and MDA-MB-231. The first and the third cell lines are triple negative breast cancer; while the second one is positive for estrogen receptor. They were all cultured in DMEM (Biowest) supplemented with 10% FBS and 1% Penicillin/Streptomycin. For Hs 578T, insulin (Sigma) was also added at 0.005%. The cells were maintained in monolayer, either in 6 well plates or T75 flasks in a humidified environment (37ºC, 5% CO_2) until they reached a confluency of 80-90%. Cell passage was performed by detaching the cells with TrypLE (Gibco).

9.3. Establishment of 2D co-culture

To determine if the activation of cytotoxic T cells by tumor cells was dependent on the cell-to-cell contact or if released soluble factors are sufficient, PBMCs from breast cancer patients were cultured with Hs 578T cell line or only the cell line supernatant. Hs 578T cells were maintained for 4 days in DMEM until they reached confluency. The cells were harvested and their supernatant was further centrifuged at 2000 rpm for 10 min to eliminate cellular debris and possible cellular antigens. For the co-culture, PBMCs were plated on a 96 well plate with U bottom in 4 different conditions: in monoculture (negative control), with the addition of the

supernatant from Hs 578T culture, with a canonical stimulus of PMA and ionomycin (positive control), and in co-culture on a ratio of 20:1 (PBMCs:Hs 578T). PBMCs were collected 48h after, stained with mAbs and analyzed by flow cytometry.

10. 3D cell cultures

10.1. Establishment of 3D co-cultures

3D cell culture was executed with a scaffold-free technique in low adherence. To achieve low adherence, autoclaved 1.5% of agarose (Invitrogen) in dH_2O was placed in a 96 well plate (50 μL per well) and let to cool down for 20 min. 1000 MCF-7 cells or 1000 MDA-MB-231 in 200 μL of supplemented DMEM were placed on top of the agarose. The next day, 100 μL of the medium was replaced by 100 μL of fresh medium. Following the first day, the medium was changed every other day (Figure III-7).

In the case of MCF-7, the spheroid was fully formed at day 3, similar to what has been reported (188). Then, 100 μL of medium was removed and 100 μL of allogenic patients' PBMCs suspension (containing 1000 cells) in supplemented RPMI was added, to achieve a 1:1 ratio of PBMCs to the cell line (Figure III-7). PBMCs, obtained as described in section 1.3 of this chapter, were added either unstimulated or previously stimulated with PMA/ionomycin or by T cell receptor (TCR) engagement with co-stimulatory signal. For this TCR stimulation, 5 μg/mL of mouse anti-human anti-CD3 (Biolegend) and 1 μg/mL of mouse anti-human anti-CD28 (Biolegend) antibodies were added to the PBMCs suspension. The crosslinking antibody rat anti-mouse IgG1 (Biolegend) was added two minutes after at a concentration of 5 μg/mL.

In order to confirm that PBMCs penetrate the spheroid, in a few experiments, these cells were previously stained with 5 μM CellTracker™ Orange CMTMR (Invitrogen), for 30 min in unsupplemented RPMI at 37ºC, followed by the replacement with fresh supplemented RPMI. Tumor cells were stained with 5 μM Cell Trace CFSE proliferation kit (Invitrogen). Briefly, cells in suspension in PBS 1X were stained for 20 min at 37ºC, followed by the addition of supplemented medium and incubation at 37ºC for 5 min. Cells were centrifuged at 1100 rpm for 5 min and resuspended in supplemented DMEM. The capacity of PBMCs to invade the spheroid was assessed 24h after in a Zeiss Axiovert 40 microscope and by confocal microscopy (LSM710, Zeiss). Image analysis was performed with Fiji.

In the case of the MDA-MB-231 cell line, the spheroids were only fully formed on day 6, and PBMCs were added, as explained above, on this day (Figure III-7).

Two days after the co-culture (day 5 for MCF-7 spheroids and day 8 for MDA-MB-231 spheroids), doxorubicin (Sigma Aldrich) was added, either at 0.1 μg/mL or 0.5 μg/mL. DMSO was used as negative control. 1 μg/mL of anti-PD-1 and anti-PD-L1 were added to the spheroids either alone or in combination with doxorubicin.

48h later (day 7 for MCF-7 spheroids and day 10 for MDA-MB-231 spheroids), the spheroids were removed from the plate and the supernatant frozen at -20ºC after centrifugation at 2000 rpm for 10 min. Spheroids were dissociated by pipetting and stained with a Live/Dead probe to evaluate, by flow cytometry, the anti-tumor abilities of patients PBMCS (i.e. the percentage of viable cancer cells) in the co-culture. From the supernatants, IFN-γ was quantified by ELISA.

Spheroids' area was also quantified on the last day of co-culture by photographing at least 3 wells per condition in a bright field (Zeiss Axiovert 40) and measuring spheroid area with Fiji software, by applying an automatic threshold.

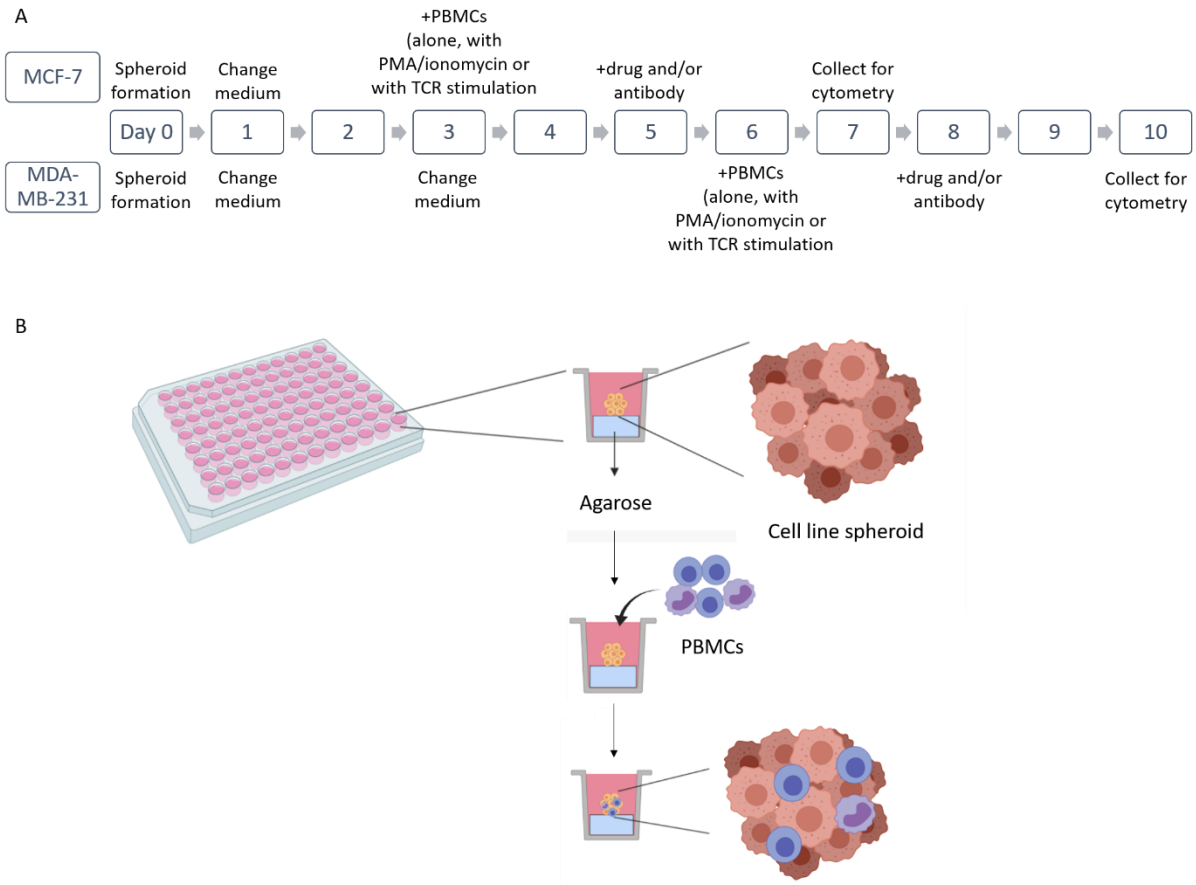


Figure III-7 **3D co-culture of breast cancer cell lines and patient-derived immune cells**. **(A)** Scheme of the protocol for the 3D *in vitro* spheroids of MCF-7 and MDA-MB-231 cell lines in co-culture with patient-derived peripheral blood mononuclear cells (PBMCs). **(B)** Representation of the 3D culture in 96 well plates and the infiltration of the PBMCs.

10.2. Cytotoxicity assay

To compare the cytotoxic capacity of HLA-DR+ cytotoxic T cells (CTLs) and HLA-DR negative CTLs, the MCF-7 3D spheroid co-culture was used. Buffy coats from healthy donors were obtained and PBMCs were isolated as above described (section 2.2 of this chapter). Stimulation was performed with 35 ng/mL of PMA and 1 µg/mL of ionomycin for 48h in 6 well plates with supplemented RPMI. After the stimulation, cells were collected and sorted (described in section 5 of this chapter). Sorted populations were cultured with the MCF-7 spheroids (day 3) in a 1:1 ratio in the following conditions:

- MCF-7 and HLA-DR+ CTLs
- MCF-7 and HLA-DR+ CTLs plus CTLs negative fraction
- MCF-7 and HLA-DR negative CTLs
- MCF-7 and HLA-DR negative CTLs plus CTLs negative fraction
- MCF-7 and CTLs negative fraction

The co-culture was maintained for 4 days, after which the spheroids were collected and stained with Live/Dead to assess MCF-7 viability by flow cytometry.

11. Statistical analysis

Statistical analysis was performed in GraphPad Prism v6 and SPSS v25 (IBM). Statistical significance was considered for $p<0.05$. A comparison between samples was performed by a nonparametric Mann-Whitney test and correlations were calculated with Spearman r-test. For the comparison of the immunological parameters regarding patients' age, histological grade, tumor dimension, breast cancer subtype and body mass index, a two-way ANOVA, either with Turkey's multiple comparisons (for 3 groups) or Sidak's multiple comparisons (2 groups) was performed.

To assess the biomarker performance, ROC curves were performed to assign a threshold to stratify NACT-responders from non-responders. This analysis was executed in both cohorts separately and for HLA-DR expressing CTLs and Tregs. These immune markers were chosen since they were significantly different between responders and non-responders to NACT. The determined area under the curve (AUC), sensitivity and specificity were taken into account. The cut-off point for the HLA-DR expression level in CTLs was determined by ROC curve analysis, considering the expression value that corresponded to the maximum sensitivity and specificity. Univariate and multivariate logistic regressions were executed taking into account both cohorts in the same analysis. The variables used for the logistic regressions were chosen to represent the patients' clinical data and also to include the immune parameters determined in the tumor microenvironment. Population size for the validation study was calculated with a 95% confidence interval taken into consideration the number of breast cancer patients

selected for NACT in each hospital per year. Results for the biomarker study were reported according to the Recommendations for Tumor Marker Prognostic Studies (REMARK).

Progression free survival and overall survival were analyzed by a Kaplan Meier curve with a log-rank test and hazard ratio analysis.

Paired t-test was used for the analysis of the *in vitro* 2D co-culture assays; whereas nonparametric Mann-Whitney was used for the analysis of the 3D co-culture assays.

Chapter IV

Cytotoxic T cells expressing HLA-DR: a new biomarker for breast cancer aggressiveness and response to neoadjuvant chemotherapy

Chapter IV - Cytotoxic T cells expressing HLA-DR: a new biomarker for breast cancer aggressiveness and response to neoadjuvant chemotherapy

Breast cancer remains the leading cause of cancer-related deaths among women worldwide. Increasing evidence suggests that anti-cancer chemotherapy is influenced by the immune system (189). Indeed, tumors can be heavily infiltrated by immune cells, in particular, tumor-infiltrating lymphocytes (TILs), which have been associated with good prognosis in various cancers, including breast cancer (189). Several reports have been advocating that TILs, especially cytotoxic T lymphocytes (CTLs) due to their anti-tumor cytotoxic activity, could serve as a robust marker for predicting treatment response. However, the data is still conflicting and clinical use of TILs as a predictive biomarker is yet to occur. Moreover, tumor cells display several mechanisms of escape to the immune system, leading to tumor progression and resistance to chemotherapy. In order to tackle this question, we pursued a thorough characterization of the immune cells present in the tumor microenvironment to better understand the crosstalk between both cell types and provide an improved biomarker to predict response to chemotherapy.

1. HLA-DR-expressing T lymphocyte populations distinguish breast cancer axillary lymph node invasion status

To get insight into the composition and the role of the immune infiltrate in breast cancer, we used a flow cytometry multi-panel and analyzed several immune populations, including B lymphocytes, NK cells, T lymphocytes (divided in T helper cells (Th), cytotoxic T cells (CTLs) and regulatory T cells (Tregs)), neutrophils, dendritic cells, M1 and M2 macrophages. These immune populations were quantified in breast cancer patients' biopsies and surgical specimens, assessed either pre- or post-treatment (see flow chart on section 1.4 of Chapter III).

To better understand the relationship between the different immune populations and the progression of the disease, we started by dividing the patients according to the axillary lymph node invasion status (Figure IV-1). Patients that have disease extended into the axillary lymph node, normally have more advanced and aggressive breast cancer when compared to patients without axillary lymph node metastasis (190). We observed that, although there was high heterogeneity in the degree of immune infiltration between both groups of patients, the average value of the frequency of T lymphocytes, B lymphocytes, NK cells, neutrophils, dendritic cells, M1 and M2 macrophages infiltrating the tumor, assessed by flow cytometry, was similar between them (Figure IV-1A and B, (191)). Additionally, the average value of the frequency of the distinct T lymphocyte populations – helper (Th), cytotoxic (CTLs) and regulatory (Tregs) – were also similar between these two groups (Figure IV-1C, (191)).

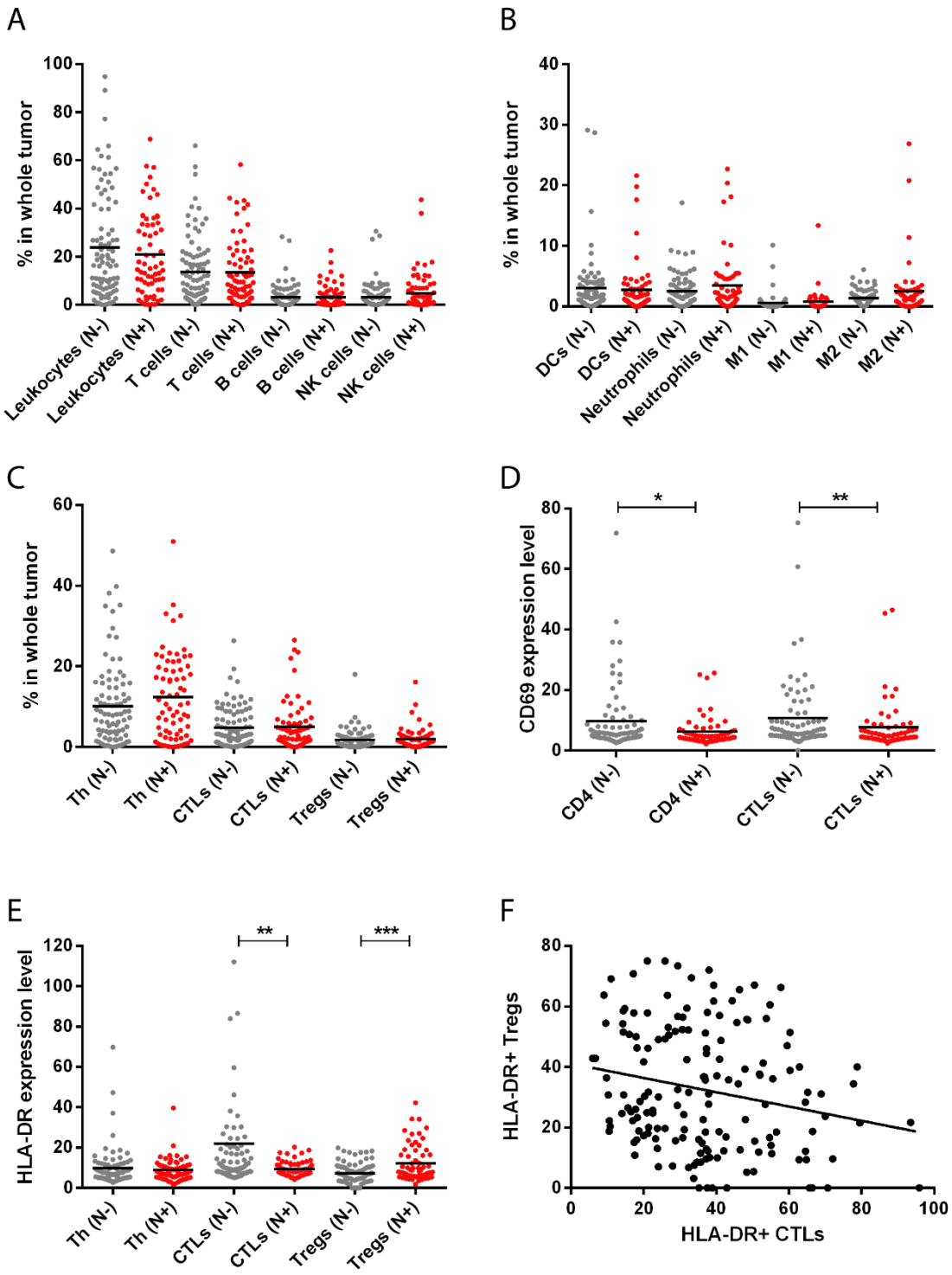


Figure IV-1 **HLA-DR-expressing T cells differentiate patients with axillary lymph node metastasis from patients without axillary lymph node involvement.** Comparison between patients without axillary lymph node metastasis (N-, grey dots, n=93) and patients with axillary lymph node metastasis (N+, red dots, n=72) regarding **(A)** percentage of leukocytes, T cells, B cells and NK cells in whole tumor; **(B)** percentage of dendritic cells (DCs), neutrophils, M1 and M2 macrophages in whole tumor; **(C)** helper T cells (Th), cytotoxic T cells (CTLs) and regulatory T cells (Tregs) in whole tumor. The percentage of each population was obtained by flow cytometry analysis with respect to the gate of single cells. **(D)**

CD69 expression level in both CD4+ T cells (both Th and Tregs population) and CTLs; **(E)** HLA-DR expression level in Th, CTLs and Tregs. The expression level is the median fluorescent intensity of positive population normalized to the negative population. **(F)** Correlation between the percentage of HLA-DR+ Tregs and HLA-DR+ CTLs (Spearman r=-0.2, p=0.01, n=158). *p<0.05, **p<0.01, ***p<0.001.

Then, we considered the activation state of T lymphocyte populations, by the analysis of the expression of established T cell activation markers, CD69 and HLA-DR. CD69 is an early, transiently expressed activation marker, whereas HLA-DR appears later in the activation process but it remains in the cell surface throughout activation (75,192). Interestingly, this analysis revealed significant differences between patients without axillary lymph node invasion and patients with axillary lymph node invasion, especially regarding the expression of HLA-DR. Namely, HLA-DR expression level was higher in CTLs (p=0.003) and lower in Tregs (p=0.0007) in patients without axillary lymph node metastasis when compared to patients with axillary lymph node metastasis (Figure IV-1E). Furthermore, the expression of HLA-DR in CTLs negatively correlated with the expression of this marker in Tregs (r=-0.2, p=0.01, Figure IV-1F).

The analysis of CD69 expression in T lymphocyte populations also revealed a difference between both groups of patients – higher CD69 in CD4+ T cells (both Th and Tregs, p=0.01) and in CTLs (p=0.005) in patients without axillary lymph node metastasis (Figure IV-1D).

These results suggested that more than simply the presence and/or the quantity of immune cells within the tumor immune microenvironment, the quality (i.e. the activation state) of certain immune cells, namely CTLs and Tregs, might be relevant for cancer progression and aggressiveness (191).

Although we did not discriminate between Th subsets (e.g. Th1, Th2, Th17), that might influence cancer differently, since CD69 expression level in CD4+ T cells had the same tendency as CD69 expression level in CTLs, we could assume that helper T cells present are of a Th1 subtype (Figure IV-1D). Nevertheless, further analysis with specific antibodies against the Th subsets should be performed.

To assess the localization of HLA-DR+ CTLs we have performed immunofluorescence in formalin-fixed paraffin embedded (FFPE) tissue of breast cancer patients divided according to the axillary lymph node invasion status (Figure IV-2). Interestingly, in patients without axillary lymph node metastasis, a co-localization between CD8 (CTLs) and HLA-DR is visible in intratumor regions, whereas the co-localization is not observed outside the tumor margins. In patients with axillary lymph node metastasis, although there are CTLs both inside the tumor structure and outside, there is no co-localization with HLA-DR. Cells positive for HLA-DR can be other subtypes of T cells, antigen presenting cells or even tumor cells. This result points out that activation of CTLs occurs inside the tumor structure, where the tumor antigens are visible and where CTLs can exert their cytotoxic functions (191).

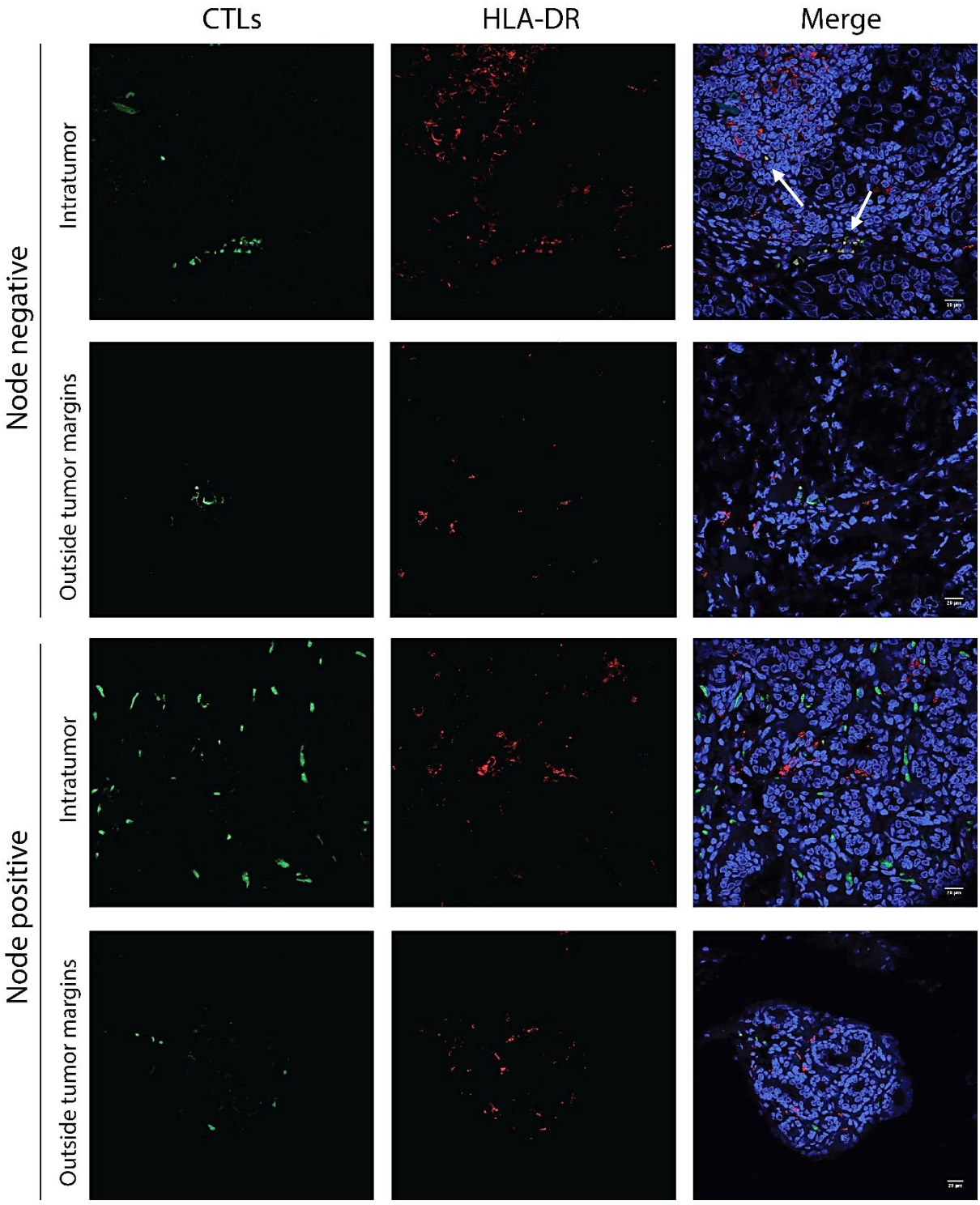


Figure IV-2 **HLA-DR+ cytotoxic T cells are located inside tumor structures in patients without axillary lymph node metastasis.** Representative images of immunofluorescence experiments (n=6) performed in slices of paraffin tissue of surgical breast cancer samples of patients without axillary lymph node metastasis (Node negative) and with axillary lymph node metastasis (Node positive) for CTLs (green, left panel) and HLA-DR (red, middle panel). Nuclei are stained in blue with DAPI and the three stainings were merged (right panel). Images from the tumor structures and from the tumor-surrounding tissue were acquired. White arrows point to co-localization. Scale bars: 20 µm.

2. HLA-DR-expressing cytotoxic T cells are similar when patients' age and body mass index, or tumor histological grade, dimension and breast cancer subtype are taken into consideration

Besides comparing the immune features of breast tumors between patients with or without axillary lymph node metastasis, we performed the same comparisons between patients regarding their age (<50 years old and >50 years old, Figure IV-3), the breast cancer subtype (ER+, HER2+ or TNBC, Figure IV-4), their tumor histological grade (G1, G2 or G3, Figure IV-5), their body mass index (normal (18.5–24.9), overweight (25.0–29.9) or obese (>30), Figure IV-6) and their tumor dimension (<20 mm or ≥20 mm, Figure IV-7). We have performed these analyses since those categories can be correlated with prognosis. For instance, it is well known that patients with worst prognosis are younger (pre-menopause) women, obese, with tumors with higher histological grade and larger than 2 cm (reviewed in 5,2).

The main differences found were: patients with less than 50 years old have a higher percentage of T cells infiltrating the tumor ($p<0.001$), either Th ($p<0.001$) or CTLs ($p<0.05$, Figure IV-3); HER2+ breast cancer samples have a higher percentage of Th cells when comparing to TNBC ($p<0.05$, Figure IV-4); and tumor samples with grade 1 have less infiltrating leukocytes in general than tumors with grade 2 or 3 ($p<0.05$, Figure IV-5).

Interestingly, HLA-DR-expressing T cell populations were not significantly different between these groups, highlighting the fact that this trace has potential to be used to predict breast cancer aggressiveness independently of breast cancer subtype, grade, tumor dimension and patient's age or body mass index.

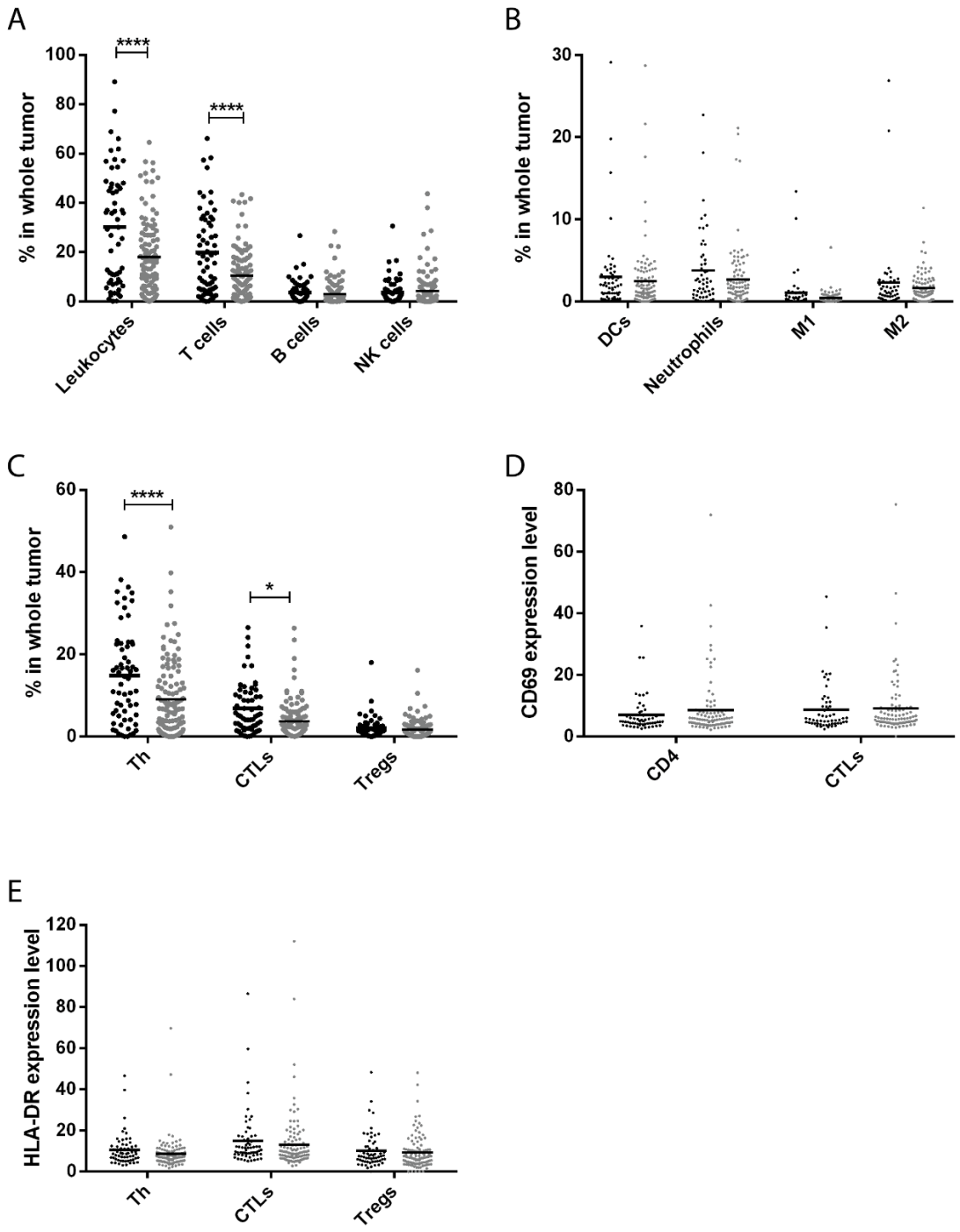


Figure IV-3 **HLA-DR-expressing T cells are not different between patients segregated by age.** Comparison between patients with less than 50 years old (black dots, n=63) and patients with more than 50 years old (grey dots, n=115) regarding **(A)** percentage of leukocytes, T cells, B cells and NK cells in whole tumor; **(B)** percentage of dendritic cells (DCs), neutrophils, M1 and M2 macrophages in whole tumor; **(C)** helper T cells (Th), cytotoxic T cells (CTLs) and regulatory T cells (Tregs) in whole tumor. The percentage of each population was obtained by flow cytometry analysis with respect to the gate of single cells. **(D)** CD69 expression level in both CD4+ T cells (both Th and Tregs) and CTLs; **(E)** HLA-DR expression level in Th, CTLs and Tregs. The expression level is the median fluorescent intensity of positive population normalized to the negative population. Data is represented with the mean value. *p<0.05, ****p<0.0001.

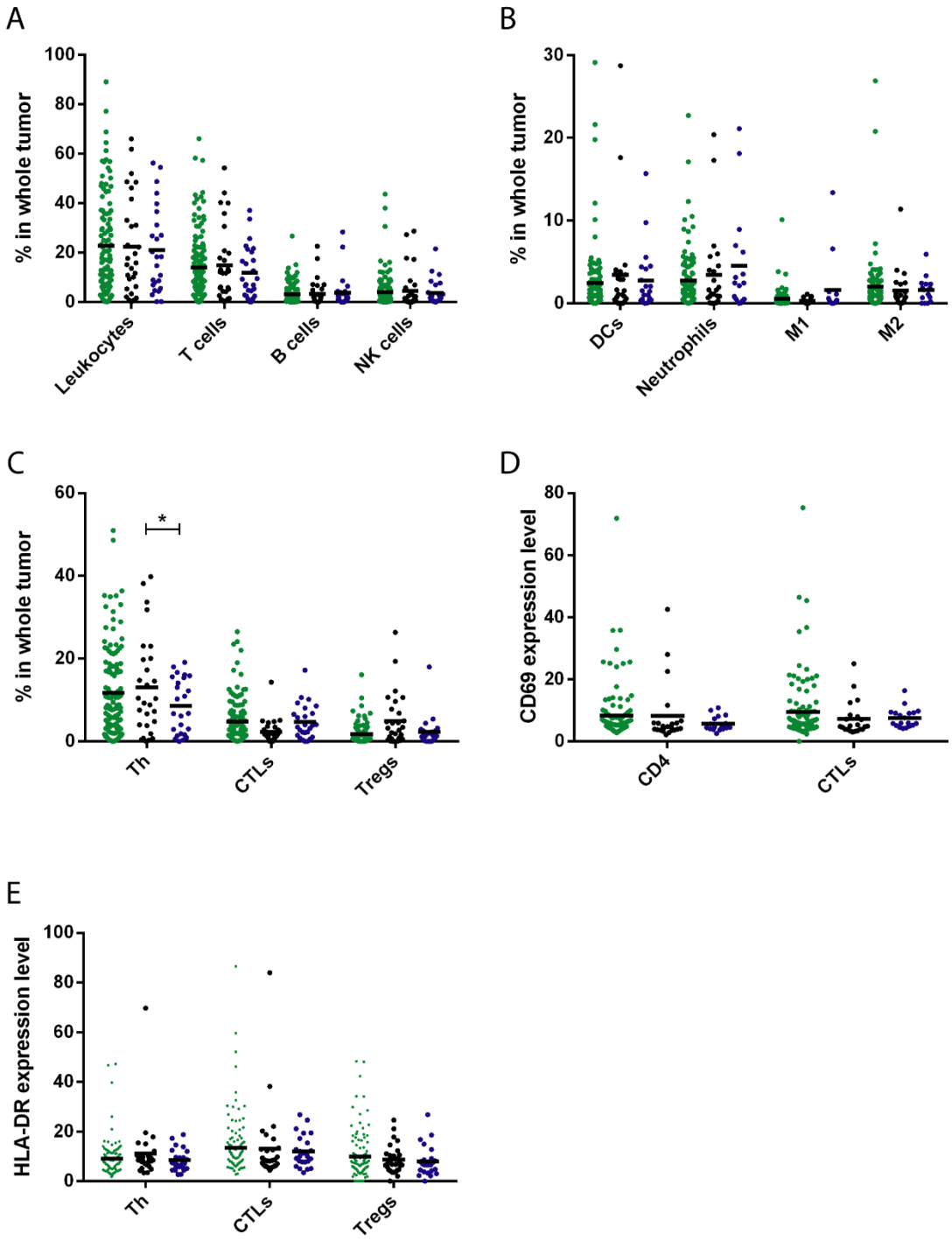


Figure IV-4 **HLA-DR-expressing T cells are not different between patients with distinct breast cancer subtypes.** Comparison between patients with estrogen receptor positive breast cancer (green dots, n=122), patients with HER2+ breast cancer (black dots, n=29) and patients with triple negative breast cancer (blue dots, n=26) regarding **(A)** percentage of leukocytes, T cells, B cells and NK cells in whole tumor; **(B)** percentage of dendritic cells (DCs), neutrophils, M1 and M2 macrophages in whole tumor; **(C)** helper T cells (Th), cytotoxic T cells (CTLs) and regulatory T cells (Tregs) in whole tumor. The percentage of each population was obtained by flow cytometry analysis with respect to the gate of single cells. **(D)** CD69 expression level in both CD4+ T cells (both Th and Tregs) and CTLs; **(E)** HLA-DR expression level in Th, CTLs and Tregs. The expression level is the median fluorescent intensity of positive population normalized to the negative population. Data is represented with the mean value. *$p<0.05$.

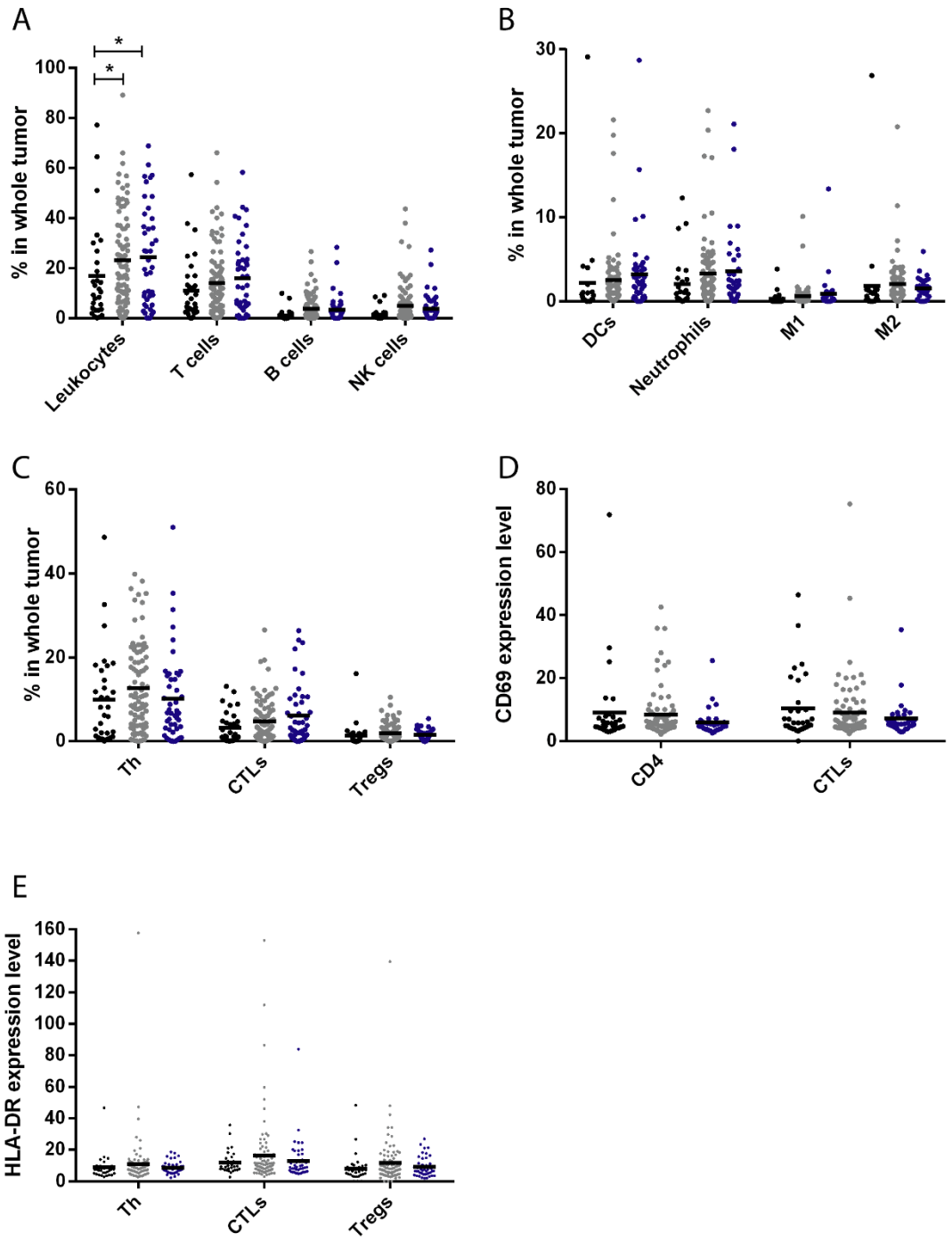


Figure IV-5 **HLA-DR-expressing T cells are not different between tumors with distinct histological grade.** Comparison between patients grade 1 breast cancer (black dots, n=33), patients with grade 2 breast cancer (grey dots, n=93) and patients with grade 3 breast cancer (blue dots, n=46) regarding **(A)** percentage of leukocytes, T cells, B cells and NK cells in whole tumor; **(B)** percentage of dendritic cells (DCs), neutrophils, M1 and M2 macrophages in whole tumor; **(C)** helper T cells (Th), cytotoxic T cells (CTLs) and regulatory T cells (Tregs) in the whole tumor. The percentage of each population was obtained by flow cytometry analysis with respect to the gate of single cells. **(D)** CD69 expression level in both CD4+ T cells (both Th and Tregs) and CTLs; **(E)** HLA-DR expression level in Th, CTLs and Tregs. The expression level is the median fluorescent intensity of positive population normalized to the negative population. Data is represented with the mean value. *p<0.05.

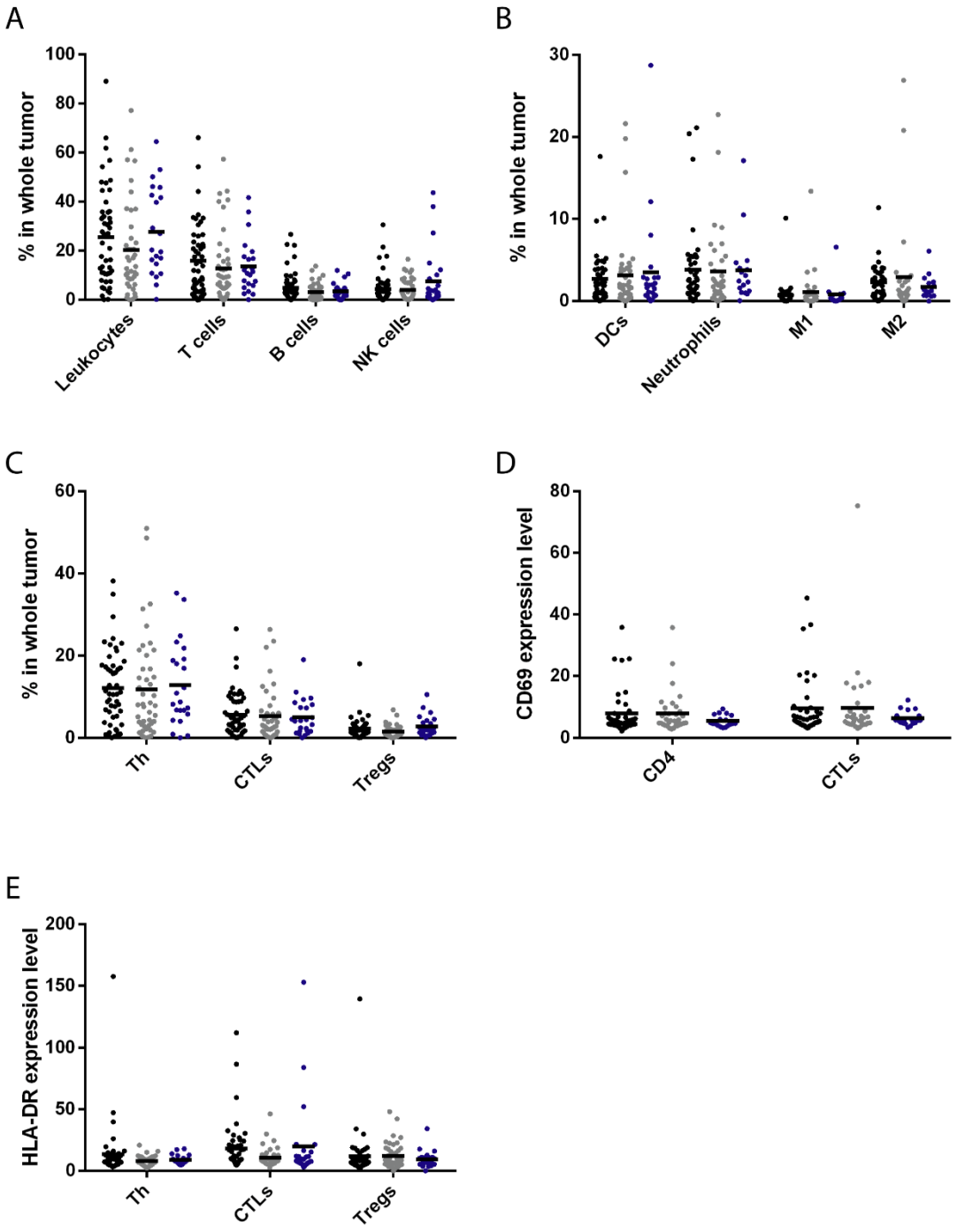


Figure IV-6 **HLA-DR-expressing T cells are not different when patients are segregated according to their body mass index.** Comparison between patients with normal body mass index (BMI from 18.5 to 24.9, black dots, n=51), overweight patients (BMI from 25 to 29.9, grey dots, n=45) and obese patients (BMI higher than 30, blue dots, n=23) regarding **(A)** percentage of leukocytes, T cells, B cells and NK cells in whole tumor; **(B)** percentage of dendritic cells (DCs), neutrophils, M1 and M2 macrophages in whole tumor; **(C)** helper T cells (Th), cytotoxic T cells (CTLs) and regulatory T cells (Tregs) in whole tumor. The percentage of each population was obtained by flow cytometry analysis with respect to the gate of single cells. **(D)** CD69 expression level in both CD4+ T cells (both Th and Tregs) and CTLs; **(E)** HLA-DR expression level in Th, CTLs and Tregs. The expression level is the median fluorescent intensity of the positive population normalized to the negative population. Data is represented with the mean value.

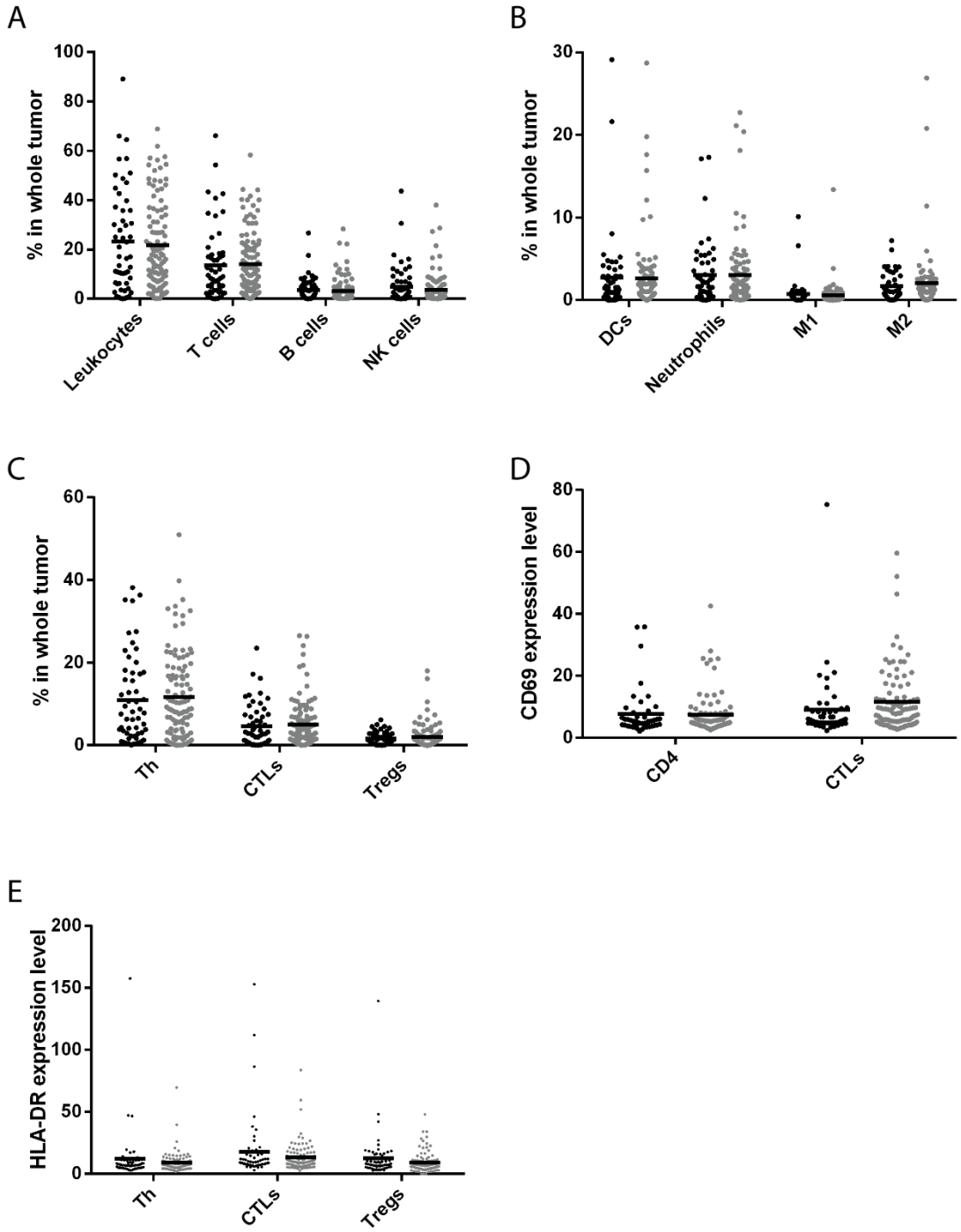


Figure IV-7 **HLA-DR-expressing T cells are not different when patients are segregated according to tumor size.** Comparison between patients with tumors with less than 20 mm (black dots, n=82) and patients with tumors with more than 20 mm (grey dots, n=110) regarding **(A)** percentage of leukocytes, T cells, B cells and NK cells in whole tumor; **(B)** percentage of dendritic cells (DCs), neutrophils, M1 and M2 macrophages in whole tumor; **(C)** helper T cells (Th), cytotoxic T cells (CTLs) and regulatory T cells (Tregs) in whole tumor. The percentage of each population was obtained by flow cytometry analysis with respect to the gate of single cells. **(D)** CD69 expression level in both CD4+ T cells (both Th and Tregs) and CTLs; **(E)** HLA-DR expression level in Th, CTLs and Tregs. The expression level is the median fluorescent intensity of the positive population normalized to the negative population. Data is represented with the mean value.

3. HLA-DR-expressing cytotoxic T cells are associated with patients' response to neoadjuvant chemotherapy

Considering that the HLA-DR expression in T lymphocyte populations reflects the breast cancer aggressiveness, by distinguishing the axillary lymph node invasion status, we asked if this trait could also be useful to predict patients' response to neoadjuvant chemotherapy (NACT). As previously referred, this treatment is used for inflammatory or inoperable tumors or patients that have locally advanced disease (tumors with more than 2 cm and/or with axillary lymph node metastasis). However, less than 50% of the patients have a good response to this treatment (142,149). As such, it is of foremost importance to be able to detect, prior to treatment implementation, which patients will not respond to NACT, to promptly transfer them to alternative therapies.

For this reason, we have analyzed the first consecutive biopsies of patients selected to perform NACT. These biopsies (from 30 patients) were collected from October 2016 to June 2018 and were included in the first cohort for the biomarker study. They were analyzed by flow cytometry, with the same antibody multi-panel as above. Additionally, an immunofluorescence protocol from FFPE tissue of biopsies of some of these patients was performed.

The 30 patients were divided into responders and non-responders, according to their radiological and pathological outcomes. NACT-responders were classified as patients that achieved a pathological complete response (pCR, n=6) or that had a pathological partial response with less than 10% of the initial tumor still present after treatment and without axillary lymph node involvement (n=7). NACT non-responders included patients that still maintained more than 50% of the initial tumor mass after treatment (n=6), or patients that developed brain, liver and/or lung metastasis during NACT (n=3) or patients that had an early relapse after NACT (n=8).

From the NACT-responders, 38.46% of the patients had HER2+ breast cancer, 30.79% had ER+ breast cancer and 30.79% had TNBC. In the case of NACT non-responders, 41.18% had ER+ breast cancer, 17.64% had HER2+ breast cancer and 41.18% had TNBC.

The frequency of distinct immune cell populations – B lymphocytes, T lymphocytes and NK cells (Figure IV-8A), dendritic cells, neutrophils, M1 and M2 macrophages (Figure IV-8B) and the three T cell subsets – Th, CTLs and Tregs (Figure IV-8C), were then compared between NACT-responders and non-responders. No significant difference was observed in the quantity of these cells between both groups (191).

The CD69 expression level was also calculated, but no difference was observed between NACT-responders and non-responders (Figure IV-8D). Interestingly, patients from the first group showed a higher level of HLA-DR in CTLs ($p<0.0001$) and lower level of HLA-DR in Tregs

(p=0.009) when compared to the patients from the second group (Figure IV-8E), suggesting that the level of HLA-DR in both populations of T lymphocytes influences NACT response (191).

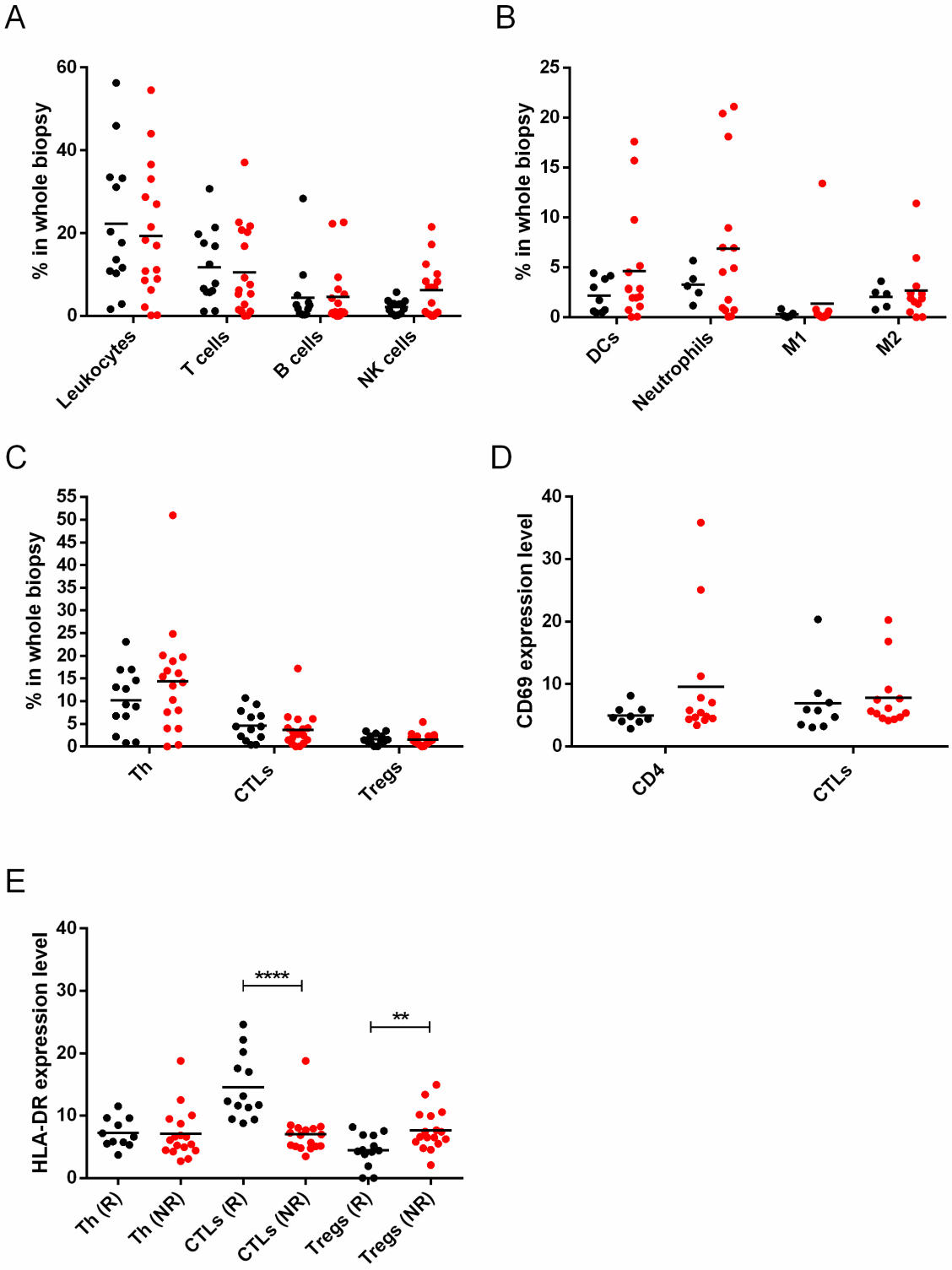


Figure IV-8 **The HLA-DR level in cytotoxic T cells and regulatory T cells is associated with patients' response to neoadjuvant chemotherapy. (A)** Percentage of leukocytes, T cells, B cells and NK cells in biopsies from breast cancer patients that responded to NACT (R, black dots, n=13) and that had no response (NR, red dots, n=17). **(B)** Percentage of dendritic cells (DCs), Neutrophils, M1 and M2

macrophages in the same patient's biopsies. **(C)** Percentage of helper T cells (Th), cytotoxic T cells (CTLs) and regulatory T cells (Tregs) in the same patient's biopsies. The percentage of each population was obtained by flow cytometry analysis with respect to the gate of single cells. Level of CD69 expression in CD4 T cells (both Th and Tregs) and CTLs **(D)** and HLA-DR expression level in Th, CTLs and Tregs **(E)**, expressed as the median fluorescent intensity of positive population normalized relatively to negative population. Data is represented with the mean value. **p<0.01, ****p<0.0001.

In order to verify the predictive value of HLA-DR expression in CTLs and Tregs for NACT response, we plotted the levels of HLA-DR+ CTLs and HLA-DR+ Tregs, of the total 30 biopsies in one single column, regardless of the response (Figure IV-9A). Excitingly, even with a small sample size, a robust separation of responders (black dots) and non-responders (red dots) was observed, especially for the expression level of HLA-DR in CTLs.

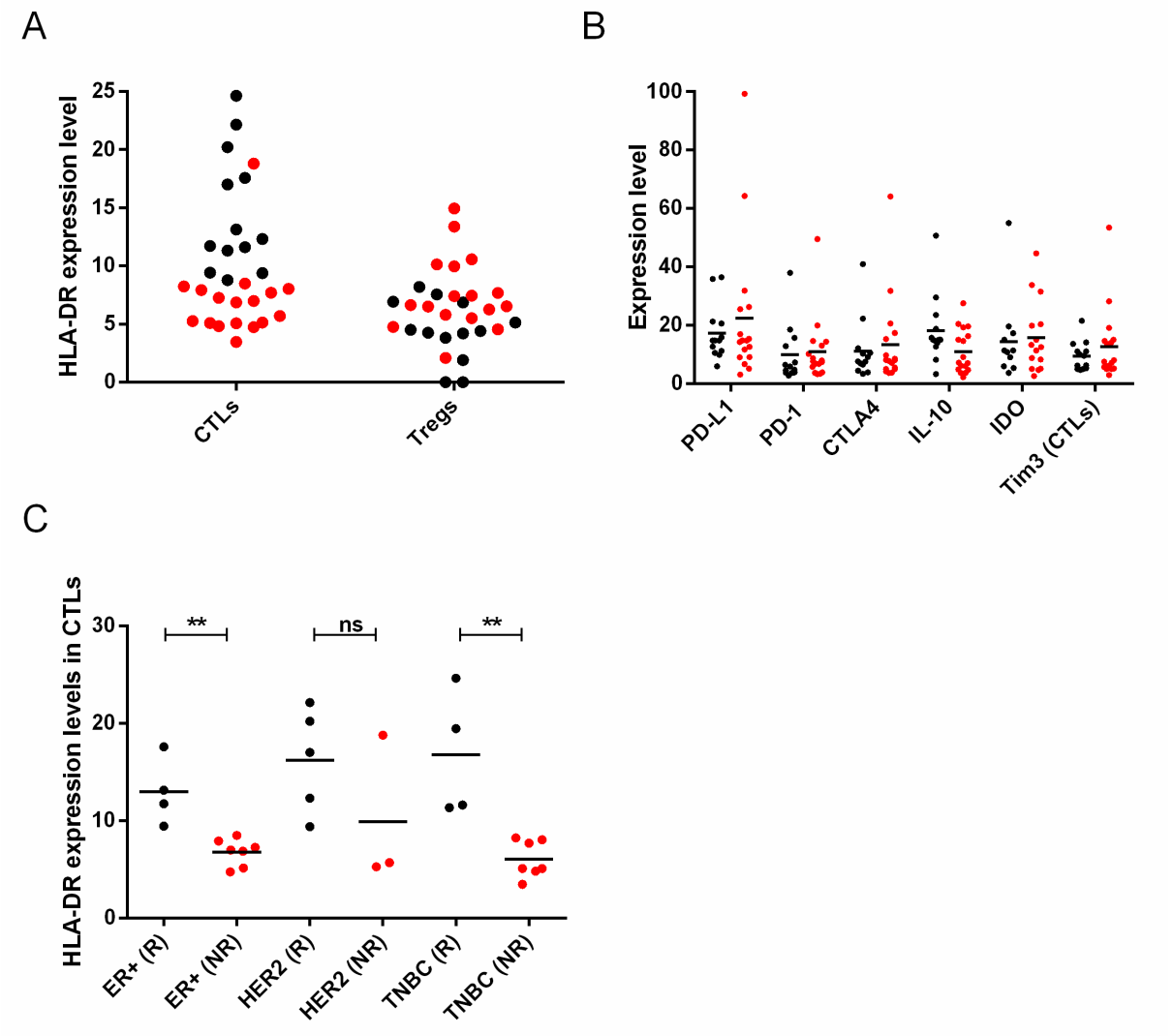


Figure IV-9 **The HLA-DR level, especially in cytotoxic T cells, is associated with patients' response to neoadjuvant chemotherapy, independently of the breast cancer subtype. (A)** HLA-DR expression in CTLs and Tregs with data for responders (black dots, n=13) and non-responders (red dots, n=17)

plotted in a single column. **(B)** The same analysis as in (A) was performed for other markers that theoretically could be used in the clinic: PD-L1, PD-1, CTLA4, IL-10, IDO, and Tim3+ CTLs. **(C)** HLA-DR expression level in CTLs of NACT-responders and non-responders divided by the subtype of breast cancer – estrogen receptor positive (ER+), HER2 positive or triple negative (TNBC). Data is represented with mean value. ns – non-statistical, **p<0.01.

ROC curve analysis was performed, leading to a statistically valid cut-off point for the HLA-DR expression level in CTLs (8.943 - value above which patients are NACT-responders) and in Tregs (5.655 - value beneath which patients are NACT-responders) (Table IV-1). For HLA-DR-expressing CTLs, the area under the ROC curve was 0.959, the sensitivity was 94.12% and the specificity was 100%. For HLA-DR-expressing Tregs, the area under the ROC curve was 0.849, the sensitivity was 81.25% and the specificity was 75%. These results highlighted that, mainly, HLA-DR-expressing CTLs evaluated in biopsies could predict response to NACT with accuracy (191). To clarify the few borderline cases, the analysis of HLA-DR+ Tregs can also be useful, since there is a negative correlation between both markers (Figure IV-1F).

Table IV-1 **ROC curve parameters for HLA-DR+ cytotoxic T cells (CTLs) and HLA-DR+ regulatory T cells (Tregs).**

	HLA-DR+ CTLs	HLA-DR+ Tregs
Threshold Value	8.943	5.655
AUC	0.959	0.849
Sensitivity	94.12%	81.25%
Specificity	100%	75%

Other immune signatures of cancer immune status, namely the expression of PD-1, PD-L1, cytotoxic T lymphocyte-associated protein 4 (CTLA4), T cell immunoglobulin and mucin domain 3 (Tim3) by CTLs, IDO and IL-10, that potentially could be used as biomarkers of NACT response were analyzed (Figure IV-9B). Although differences could be observed between biopsies of NACT-responders and non-responders, none of them was statistically significant, as was HLA-DR in CTLs. Therefore, we did not observe the segregation of patients according to the level of expression of any of these molecules (Figure IV-9B) pointing the previous immune feature as a better biomarker of NACT response (191).

Then, to emphasize the potential predictive value of HLA-DR expression level in CTLs we analyzed this trace for the three subtypes of breast cancer - HER2+, ER+ and TNBC, separately (Figure IV-9C). Notably, HLA-DR expression level in CTLs can segregate NACT-responders from NACT non-responders, independently of breast cancer subtype (191).

Besides the frequency and the type of immune cells in the tumor tissue, their location could also be important to predict patients' clinical outcome (194). Therefore, we have assessed, by

immunofluorescence, selected FFPE tissue samples matched with the previously analyzed by flow cytometry, and evaluated where the HLA-DR+ CTLs are mainly located. We observed that in biopsies of patients with good response to NACT (Figure IV-10), the location of these HLA-DR-expressing CTLs was mainly present in intraepithelial tumor structures. Representative images in Figure IV-10 show a co-localization of anti-CD8 (CTLs) and anti-HLA-DR within the tumor, while no co-localization between these two markers was observed in the tumor surrounding normal tissue. Indeed, outside the tumor margins, CTLs were found but they do not seem to be activated, as the HLA-DR staining was more spread throughout the tissue.

These results suggest that CTLs located in intraepithelial tumor structures are more activated than the CTLs found at the tumor surrounding normal tissue, probably due to high proximity with tumor antigens and tumor-released soluble factors, therefore suggesting that these CTLs should be the ones with anti-tumor cytotoxic activity.

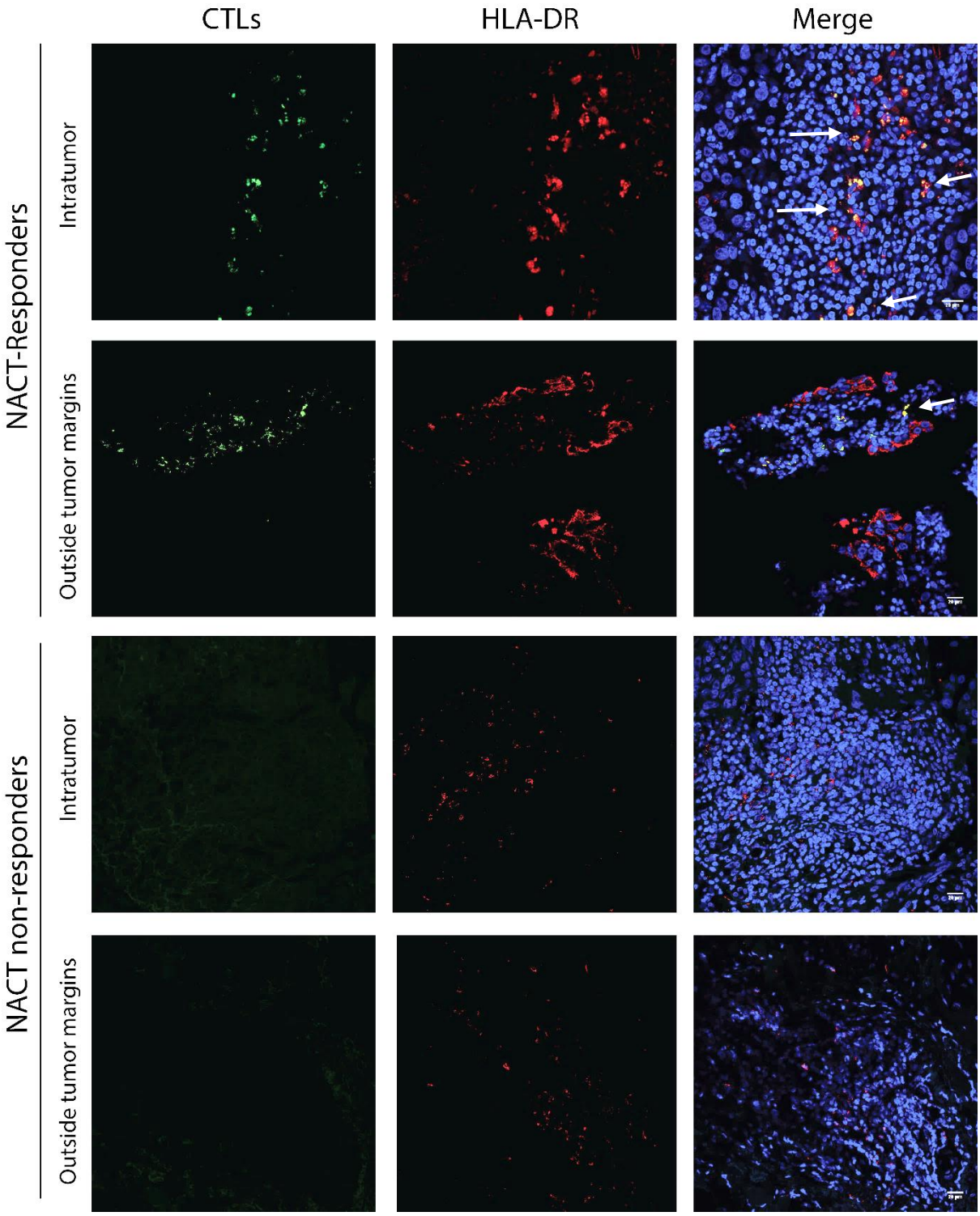


Figure IV-10 **HLA-DR+ cytotoxic T cells are located inside tumor structures in patients with response to neoadjuvant chemotherapy**. Representative images of immunofluorescence experiments (n=6) performed in slices of paraffin tissue of breast cancer biopsies of patients that responded to NACT and of NACT non-responders' patients for CTLs (green, left panel) and HLA-DR (red, middle panel). Nuclei are stained in blue with DAPI and the three stainings were merged (right panel). Images from the tumor structures and from the tumor-surrounding tissue were acquired. White arrows point to co-localization. Scale bars: 20 µm.

4. Cytotoxic T cells with high levels of HLA-DR are present in post-NACT surgical specimens of NACT-responders

It has been reported that chemotherapeutic agents are able to alter the immune context of breast cancer, usually boosting anti-tumor CTLs' activity (195). Thus, post-NACT samples (not necessarily matched with the previous biopsies) were also analyzed by flow cytometry. These specimens also revealed that non-responders have infiltrating CTLs with low levels of HLA-DR (p=0.009) and Tregs with high levels of HLA-DR (although not significant in this case) when compared to responders (Figure IV-11). This observation suggested that in a tumor immune microenvironment prior enriched in poorly immune-competent cells, NACT is not sufficient to modulate this background in a way that favors response. This further supports the idea that the level of HLA-DR in CTLs could predict breast cancer patients' response to NACT (191).

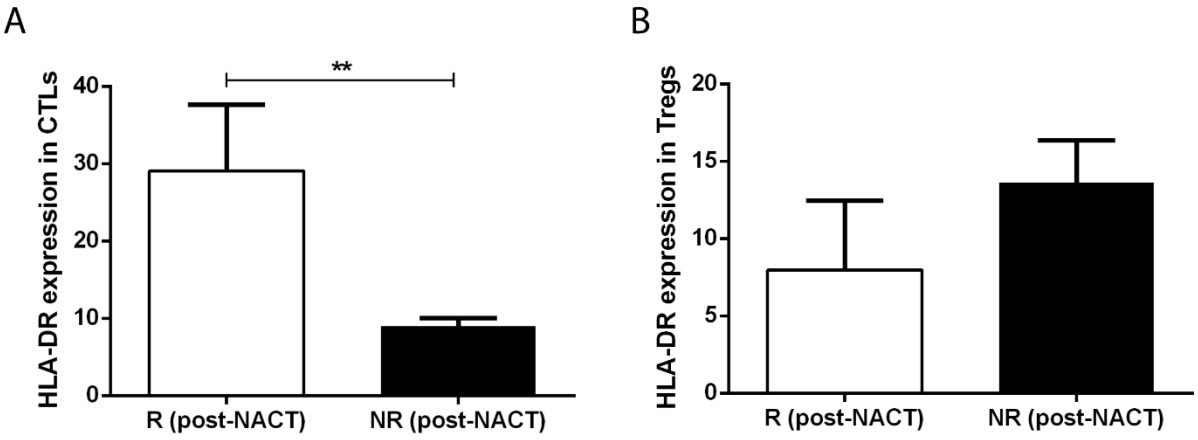


Figure IV-11 **HLA-DR-expressing cytotoxic T cells are higher in tumors from NACT-responders, after the treatment.** Level of HLA-DR in CTLs **(A)** and Tregs **(B)**, assessed by flow cytometry, in responders (R, white bars, n=4, mean ± SEM) and non-responders (NR, black bars, n=6, mean ± SEM), expressed as the median fluorescent intensity of positive population normalized to the negative population. **p<0.01.

5. Validation of HLA-DR-expressing cytotoxic T cells as a predictor of neoadjuvant chemotherapy response

In order to establish HLA-DR-expressing cytotoxic T cells as a biomarker of breast cancer patients' response to NACT, we are conducting a validation prospective study, in a second independent cohort of breast cancer patients (Saraiva *et al*, manuscript in preparation). The samples from these patients have been supplied by Hospital CUF Descobertas and Hospital Vila Franca de Xira. The validation study is being performed taking into consideration the REMARK criteria - reporting recommendations for tumor marker prognostic studies (196). This

cohort included biopsies of patients selected to perform NACT from September 2018 onwards (23 patients, of which, only 22 have already finished NACT). Following the REMARK criteria, we have calculated the population size of the validation cohort, taking into consideration the number of breast cancer patients selected for NACT per year in both hospitals. In each hospital, approximately 20% of the breast cancer cases are selected for NACT, and both have approximately 150 breast cancer patients per year. Therefore, the population size, with a 95% confidence interval and 5% margin of error is 53 patients. To achieve this number we are still enrolling patients.

So far, we have divided the patients we already know the outcome, into NACT-responders and non-responders according to the same criteria as in the first cohort. 7 patients responded to NACT and from these, three patients achieved a pathological complete response. The non-responder patients (n=15), included patients that developed bone metastasis (n=2) or that still had more than 50% of the initial tumor after NACT, or presented axillary lymph node metastasis at the time of the surgery (n=13).

From the NACT-responders, 43% of the patients had HER2+ breast cancer and 57% had TNBC. In the case of NACT non-responders, 47% had ER+ breast cancer, 40% had HER2+ breast cancer and 13% had TNBC.

As previously, we started by analyzing the percentage of the T lymphocyte subpopulations, namely CTLs and Tregs (Figure IV-12A), which were not significantly different between NACT-responders and non-responders. Expectedly, the expression level of HLA-DR in CTLs was again statistical significant between both groups (p=0.03, Figure IV-12B). As before, data regarding HLA-DR expression level in CTLs was represented for NACT-responders and non-responders in a single column (Figure IV-12C) and segregation between responders and non-responders is still observed, even if not so conclusive as in the previous study, due to the reduced sample size.

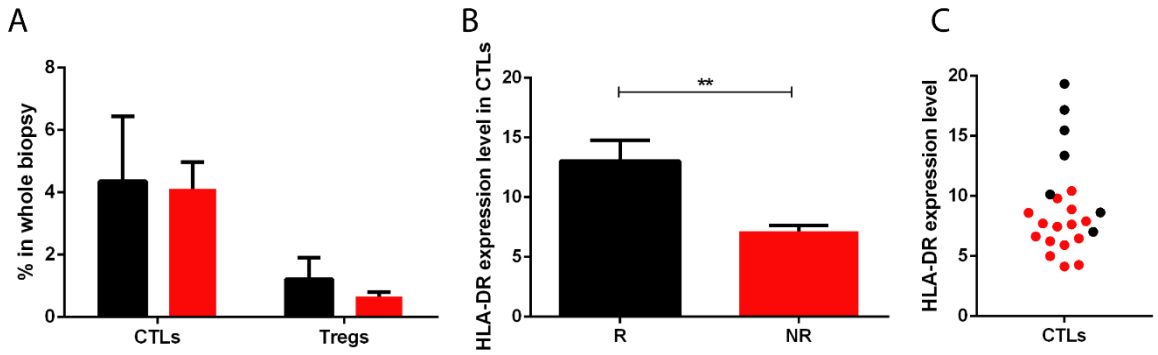


Figure IV-12 **HLA-DR-expressing cytotoxic T cells also predict response to neoadjuvant chemotherapy in the validation cohort**. **(A)** Percentage in the whole biopsy of cytotoxic T cells (CTLs) and regulatory T cells (Tregs) between patients with response to NACT (black bars, n=7, mean ± SEM) and patients without response to NACT (red bars, n=15, mean ± SEM). This percentage is calculated by flow cytometry relative to the gate of the single cells. **(B)** HLA-DR expression level in CTLs between NACT-responders (R, black bar, n=7, mean ± SEM) and NACT non-responders (NR, red bar, n=15, mean ± SEM). **(C)** Same analysis as in (B) but represented in the same column, where each dot represents a

patient – NACT-responders are represented as black dots, while NACT non-responders are represented as red dots. The expression level is calculated by flow cytometry and represents the median fluorescent intensity of the positive population normalized to the negative population. **p<0.01.

With the aim of validating the results from the first cohort of patients, we used the parameters calculated with the ROC curve (Table IV-1) to predict which patients would respond and which would not respond to NACT. From the 22 patients, 4 did not fit into the prediction. Namely, 2 patients who responded to NACT had an HLA-DR expression level in CTLs of 7 and 8.61 (slightly below the threshold value of 8.943 calculated in the ROC curve of the first cohort). Moreover, two patients without response to NACT presented tumor infiltrating CTLs with HLA-DR expression above the threshold value - 9.81 and 10.43.

To analyze the differences between cohorts, a new ROC curve was executed for the second cohort (Table IV-2). The parameters for this ROC curve were an area under the curve (AUC) of 0.89, with 80% sensitivity and 85.71% specificity (Table IV-2). The parameters were only slightly different from the ones obtained for the ROC curve of the first cohort, namely the cut-off point for the HLA-DR expression level in CTLs was calculated as 8.63 (value above which patients are NACT-responders) which was close to the previous one (8.943).

Table IV-2 **ROC curve parameters for HLA-DR+ cytotoxic T cells (CTLs) in the second independent cohort of breast cancer patients submitted to neoadjuvant chemotherapy**.

	HLA-DR+ CTLs
Threshold Value	<8.63
AUC	0.89
Sensitivity	80%
Specificity	85.71%

Although we could not predict the outcome of all patients enrolled in the second cohort, the majority (18 out of 22) responded as expected according to our model. Moreover, the cut-off points calculated by both ROC curves are very similar (8.943 and 8.63, respectively). Thus, in general, these results corroborate the previous study, highlighting the potential use of HLA-DR-expressing CTLs at the biopsy level to predict response to neoadjuvant chemotherapy. Nevertheless, it is important to note that the validation study is still ongoing and that the ROC curve parameters of the second cohort, namely the sensitivity and even the threshold value, are possibly going to be improved.

Following the REMARK criteria, and using both cohorts together, we executed univariate (Table IV-3) and multivariate analysis (Figure IV-13, Table IV-4). Performing univariate and multivariate analysis in each cohort separately would be unfeasible due to the sample size of each one. Univariate analyses were employed to demonstrate the relationship between the marker and the patients' outcome. Besides analyzing this relationship for HLA-DR expression level in CTLs, we performed univariate analysis for other immunological parameters as well as

patients' clinical data. With the multivariate analysis we were able to construct a model to assess if any other variable had an influence on the biomarker – HLA-DR expression level in CTLs.

Table IV-3 **Univariate analysis for the biomarker HLA-DR expression level in CTLs**. HLA-DR expression level in CTLs and other immunological markers and clinical parameters were also analyzed by univariate analysis. p-value, odds ratio and the confidence interval of the odds ratio are represented.

Parameter	p-value	Odds Ratio	95% Confidence Interval
HLA-DR expression level in CTLs	0.001	1.778	1.262-2.504
Immune cells (CD45)	0.827	1.004	0.967-1.042
T cells (CD3)	0.553	0.986	0.94-1.034
B cells (CD19)	0.786	0.987	0.896-1.087
NK cells (CD161)	0.094	0.839	0.683-1.031
T helper cells (CD4)	0.309	0.972	0.919-1.027
CTLs (CD8)	0.644	1.035	0.894-1.199
Tregs ($CD25^{high}/CD127^{low}$)	0.299	1.277	0.805-2.028
M1 macrophages	0.701	0.569	0.032-10.068
M2 macrophages	0.707	0.93	0.635-1.36
Neutrophils (CD15)	0.283	0.893	0.727-1.098
HLA-DR expression level in Tregs	0.188	0.910	0.791-1.07
PD-L1	0.24	1.012	0.992-1.033
IL-10	0.107	1.039	0.992-1.089
Age	0.723	0.992	0.948-1.038
Body Mass Index	0.827	1.014	0.895-1.149
Tumor dimension	0.305	1.019	0.983-1.058
Node invasion status	0.162	0.412	0.119-1.426
Ki67 (%)	0.141	1.017	0.994-1.041
Grade	0.593	0.615	0.104-3.658

Through the univariate analysis, it is possible to observe that the HLA-DR expression level in CTLs is significantly different between NACT-responders and non-responders (p=0.001, Table IV-3). Moreover, the odds ratio for this variable is greater than 1, highlighting the tendency to a favorable outcome. Most of the variables used in this method had an odds ratio close to 1, revealing that they do not possess enough strength to employ a tendency for NACT-response. Nevertheless, it is possible to observe that for instance the node status, i.e. the presence of axillary lymph node metastasis in the patient, has an odds ratio inferior to 1, suggesting that patients with positive lymph node involvement have a tendency for worse response to NACT. The presence of Tregs, on the opposite, have a tendency to favor NACT-response. This result seems counterintuitive but again reveals that lymphocytes can be present in the tumor microenvironment but their activity can be hampered by tumor cells. Nonetheless, none of these variables had statistical significance, suggesting that they do not influence NACT response.

The multivariate analysis was done with selected clinical characteristics of the patients and selected immunological markers based on previously published biomarker studies for the response to neoadjuvant chemotherapy. Namely, the presence of tumor infiltrating lymphocytes, such as different subsets of T cells (197), or the age at diagnosis may have predictive potential. To these published parameters, we added the breast cancer subtype and other variables that we found to be correlated with HLA-DR expression level in CTLs such as HLA-DR expression level on Tregs (Figure IV-1F) or PD-L1 expression on tumor cells (Figure IV-17). A forest plot summarizing the odds ratio and 95% confidence interval of the different variables measured by the multivariate analysis is shown in Figure VI-13.

Table IV-4 **Multivariate analysis for the biomarker HLA-DR expression level in CTLs and other clinical parameters, as age, subtype of breast cancer, and percentage of different immune cell populations**. p-value, odds ratio and the confidence interval of the odds ratio are represented.

Parameter	p-value	Odds Ratio	95% Confidence Interval
HLA-DR expression level in CTLs	0.007	1.726	1.163-2.562
Age	0.393	0.958	0.867-1.058
Subtype	0.513	1.469	0.464-4.648
HLA-DR expression level in Tregs	0.335	0.894	0.712-1.123
PD-L1	0.481	1.023	0.96-1.091
CD4	0.472	0.95	0.825-1.093
CD8	0.404	1.138	0.84-1.543

Interestingly, the HLA-DR expression level in CTLs is the only statistically significant variable, even when taking into account all the other parameters (p=0.007). Moreover, the odds ratio

continues to be higher than 1, namely it is 1.726, demonstrating a favorable action towards response to NACT. The biomarker here proposed (HLA-DR expression level in CTLs) can predict response to NACT in breast cancer patients even when the patients' age, the breast cancer subtype, and other immunological parameters of the tumor, such as HLA-DR expression level in Tregs, PD-L1 expression in tumor cells, percentage of infiltrating CD4+ and CD8+ T cells are taken into account.

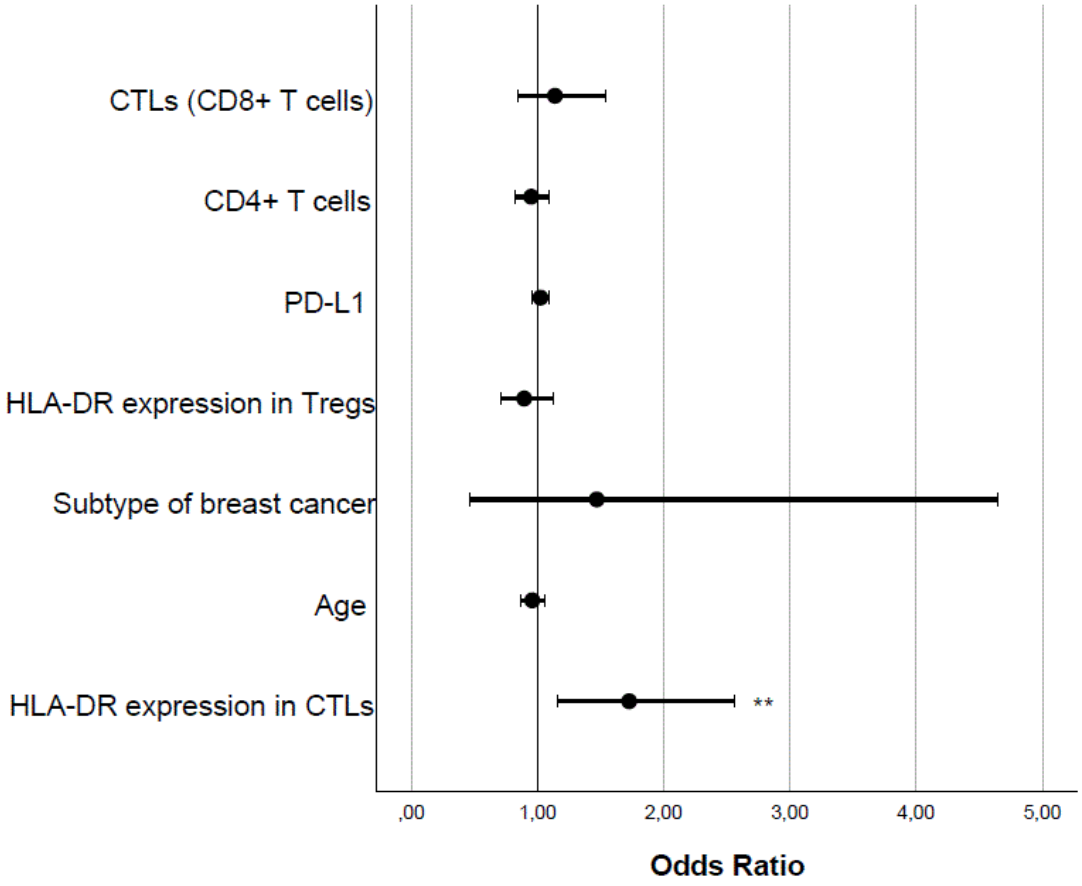


Figure IV-13 **Forest plot of the effect of different immune markers and patients' clinical data on the response to neoadjuvant chemotherapy**. Odds ratio with a 95% confidence interval are shown and were calculated by a multivariate logistic regression. **p<0.01.

Besides performing the multivariate analysis with chosen variables based not only on the literature but also on the correlations between the biomarker and other immunological variables, we also performed a stepwise multivariate analysis (Table IV-5). This stepwise analysis is an automatic method that relies mainly on the statistical significance. Interestingly, even with an automatic approach, the HLA-DR expression level in CTLs was the only significant variable to predict NACT response (p=0.008, odds ratio of 2.119), even when taking into account the other variables, namely the breast cancer subtype, the tumor dimension and the percentage of tumor infiltrating CTLs.

Table IV-5 **Stepwise multivariate analysis that included the biomarker HLA-DR expression level in CTLs, the percentage of CTLs, tumor dimension and breast cancer subtype.** p-value, odds ratio and the confidence interval of the odds ratio are represented.

Parameter	p-value	Odds Ratio	95% Confidence Interval
HLA-DR expression level in CTLs	0.008	2.119	1.220-3.682
Subtype	0.245	0.120	0.003-4.261
Tumor dimension	0.05	1.098	1.001-1.205
CD8	0.07	1.332	0.983-1.805

To understand the correlation between the expression level of HLA-DR in CTLs and the probability to respond to NACT, we executed a probability curve (Figure IV-14). As it is possible to observe, an HLA-DR expression level in CTLs above 10 will lead to an approximately 50% of probability of response. Accordingly, patients with tumor infiltrating CTLs with an HLA-DR expression level above 15 will have approximately 90% of probability to respond to NACT. Additionally, this probability curve was performed dividing the patients by the breast cancer subtype and age. Interestingly, no significant differences in the curve were detected (results not shown).

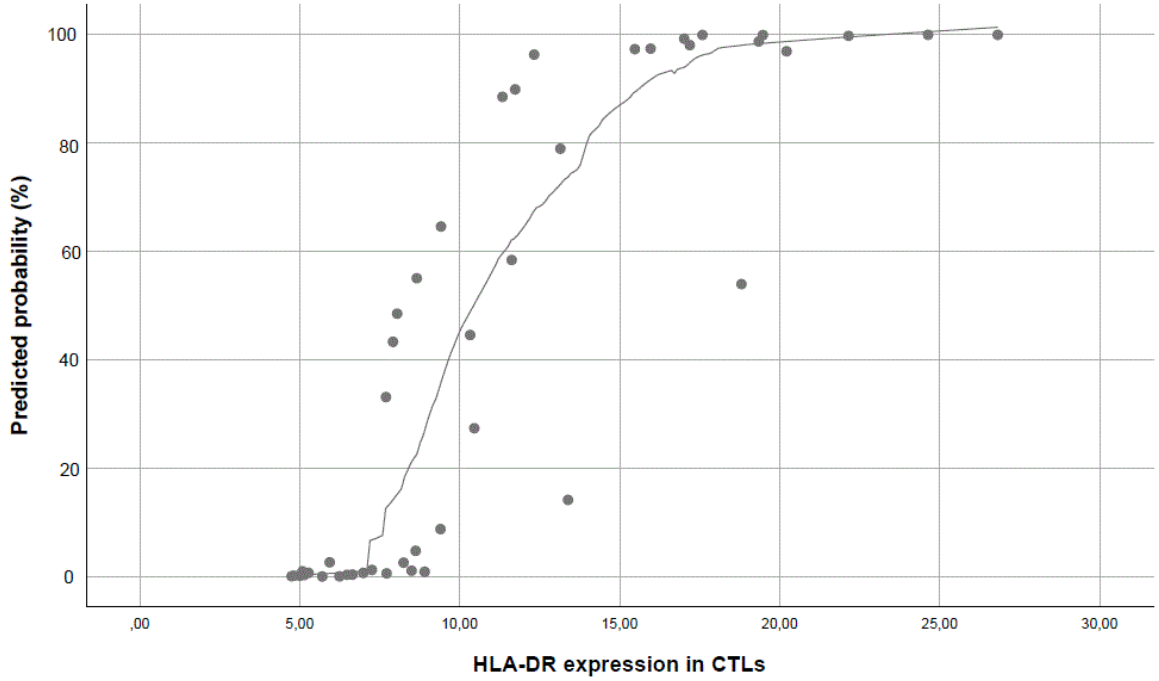


Figure IV-14 **Probability of response to NACT according to the HLA-DR-expression level in tumor infiltrating CTLs.**

6. High expression levels of HLA-DR in tumor infiltrating cytotoxic T cells is associated with progression-free survival

Besides analyzing HLA-DR+ CTLs in biopsies of patients that were selected to perform NACT, we also analyzed this immune-related parameter in biopsies and surgical specimens of patients not selected for NACT (which were directed to other therapeutic settings, such as surgery followed by adjuvant chemotherapy). Considering these patients altogether we conducted a 3-year follow-up evaluation and divided them according to the expression level of HLA-DR in CTLs (Figure IV-15). Namely, patients were divided in HLA-DR low in CTLs (value below the threshold calculated in the ROC curve of the first cohort) and in HLA-DR high in CTLs (value above the threshold calculated in the first cohort) and a progression-free survival curve was executed. Interestingly, we observed that patients with HLA-DR low in CTLs start to progress earlier than patients with HLA-DR high in CTLs. More specifically, the hazard ratio is 0.26 (95% confidence interval 0.1-1.04) for patients with tumors infiltrated with CTLs with low levels of HLA-DR and the hazard ratio for patients with tumors bearing HLA-DR high CTLs is 3.88 (95% confidence interval 0.96-10.28).

Although the difference between the curves was not significant yet (p=0.059), it shows a clear tendency, highlighting the fact that the presence of tumor infiltrating activated CTLs with high levels of HLA-DR not only allows a good response to NACT, but can also lead to better progression-free survival (Saraiva *et al*, manuscript in preparation).

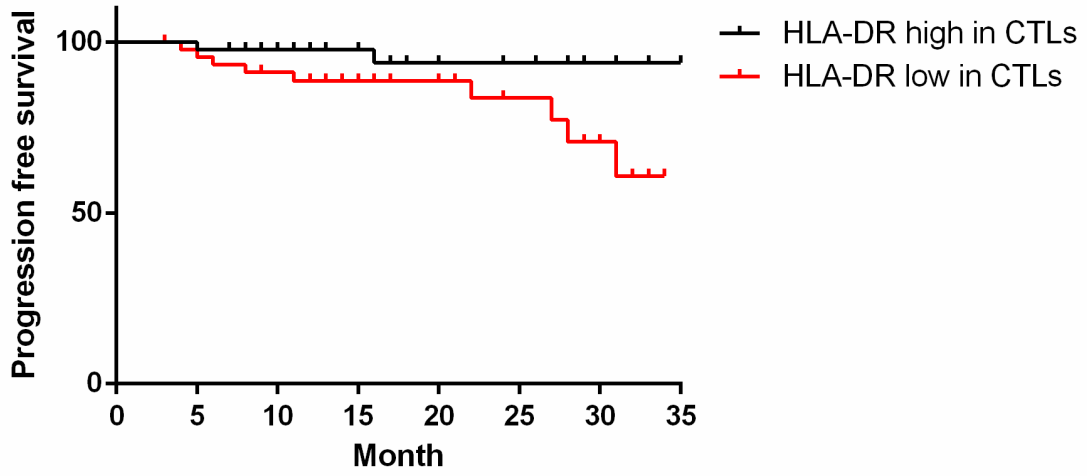


Figure IV-15 **Progression-free survival of breast cancer patients**. The patients were divided according to the HLA-DR level in intratumor cytotoxic T cells (CTLs). Specifically, two groups were created: high HLA-DR expression level in CTLs (black line, n=43) and low HLA-DR expression level in CTLs (red line, n=46). The division between groups was performed according to the threshold value calculated in the ROC curve of the first cohort.

Additionally, we performed an overall survival curve (Figure IV-16) also using data from the 3-years follow-up study. In this case, it is possible to observe that there is no difference between

patients with tumor infiltrating CTLs with high levels of HLA-DR *vs* patients with tumor infiltrating CTLs with low levels of HLA-DR. Actually, only one patient from the first category and two patients from the second category did not survive during this period. This observation is in agreement with the survival rates in breast cancer in the first years following treatment (137). For a better understanding of the overall survival, a 5 or 10 years follow-up evaluation should be performed.

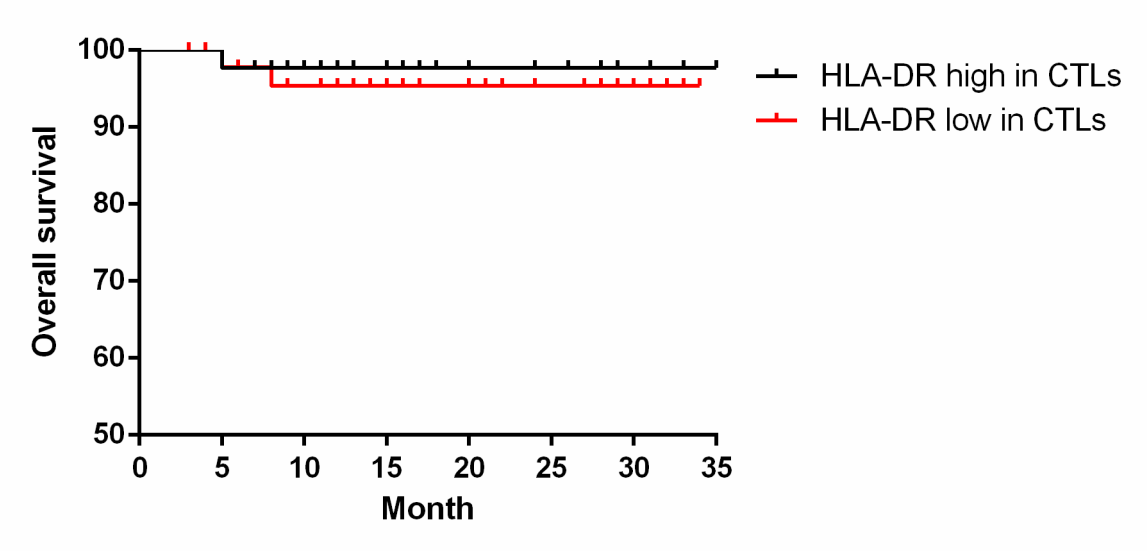


Figure IV-16 **Overall survival of breast cancer patients**. The patients were divided according to the HLA-DR level in intratumor cytotoxic T cells (CTLs). Specifically, two groups were created: high HLA-DR expression level in CTLs (black line, n=43) and low HLA-DR expression level in CTLs (red line, n=46). The division between groups was performed according to the threshold value calculated in the ROC curve of the first cohort.

7. HLA-DR expression level in cytotoxic T cells negatively correlates with immunosuppressive and pro-tumor features of the tumor microenvironment

An anti-tumor or a pro-tumor immune response is elicited not only by the immune cells within the tumor and their bio-effector molecules, but also by tumor antigens and the expression of cytokines, chemokines and other immune mediators released by the cancer tissue. Thus, the stimulation of anti-tumor CTLs' activity should, at least in part, rely on the molecules present in the tumor milieu. Interestingly, we have observed, by flow cytometry analysis of breast tumors, that CTLs expressing HLA-DR were inversely correlated with immunosuppressive activated Tregs (also expressing HLA-DR, Figure IV-1F). Moreover, they were also negatively correlated with the expression of molecules from the non-immune compartment that squelch the anti-tumor immune program and/or enhance growth and survival of cancer cells (Figure IV-17). Namely, HLA-DR expression level in CTLs was negatively correlated with the expression level of TGF-β ($r=-0.34$, $p<0.01$), which may promote the metastatic profile; PD-L1 ($r=-0.36$, $p<0.0001$), that inhibits activation of CTLs (198,199); IL-6 ($r=-0.43$, $p<0.001$), IL-8 ($r=-0.36$,

p<0.01) and IL-1β (r=-0.41, p<0.001) that are inflammatory cytokines that may act as growth factors to sustain cancer cell proliferation and invasion (81,82,200). It was also negatively correlated with IL-23/IL-12 (r=-0.31, p<0.01) that is pro-inflammatory but can also impair CTLs' activity through the activation of Tregs (101) (Figure IV-17).

	CTLs HLA-DR	Tregs HLA-DR	PD-L1	TGF-β	IL-6	IL-1β	IL-8	IL-23	IL-10	IL-17
CTLs HLA-DR	1	*-0.2*	*-0.36*	*-0.34*	-0.43	-0.41	*-0.36*	*-0.31*	0	-0.16
Tregs HLA-DR	*-0.2*	1	-0.17	-0.05	*0.4*	0.41	*0.31*	0.39	*0.43*	*0.32*
PD-L1	*-0.36*	-0.17	1	-0.05	0	0.09	-0.07	0.03	0.03	0.05
TGF-β	*-0.34*	-0.05	-0.05	1	*0.52*	*0.22*	*0.53*	*0.39*	0.2	*0.3*
IL-6	-0.43	*0.4*	0	*0.52*	1	*0.25*	*0.53*	0.34	*0.21*	*0.23*
IL-1β	-0.41	0.41	0.09	*0.22*	*0.25*	1	0.12	0.36	*0.75*	*0.86*
IL-8	*-0.36*	*0.31*	-0.07	*0.53*	*0.53*	0.12	1	0.3	*0.23*	0.17
IL-23	*-0.31*	0.39	0.03	*0.39*	0.34	0.36	0.3	1	*0.44*	*0.42*
IL-10	0	*0.43*	0.03	0.2	*0.21*	*0.75*	*0.23*	*0.44*	1	*0.75*
IL-17	-0.16	*0.32*	0.05	*0.3*	*0.23*	*0.86*	0.17	*0.42*	*0.75*	1

Spearman correlation

-0.5 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1

Figure IV-17 **HLA-DR-expressing T cells correlate with immunosuppressive and pro-tumor molecules released by tumor cells to the tumor microenvironment**. Heat map of Spearman correlations between the expression level of HLA-DR in CTLs and the expression level of HLA-DR in Tregs; and between the expression level of HLA-DR in both of these cells and the expression level of pro-tumor or immunosuppressive molecules from the tumor environment, namely PD-L1, TGF-β, IL-6, IL-1β, IL-8, IL-23/IL-12, IL-10 and IL-17. The expression level of each molecule was assessed by flow cytometry and represents the median fluorescent intensity of the positive population normalized to the negative population. The heat map is represented as a gradient from blue (negative correlations) to red (positive correlations). Numbers in italic represent statistical significance of p<0.01, numbers underlined represent p<0.001 and numbers in italic and underlined represent p<0.0001.

On the other hand, the expression level of HLA-DR in Tregs was positively correlated with the expression of these pro-tumor molecules - IL-1β (r=0.41, p<0.001), IL-23/IL-12 (r=0.39, p<0.01), IL-6 (r=0.4, p<0.01) and IL-8 (r=0.31, p<0.01) – and, additionally, IL-10 (r=0.43, p<0.0001) and IL-17 (r=0.32, p<0.01), which could also undermine the activation of CTLs and lead to tumor progression (94,201) (Figure IV-17).

When the same analysis was performed with CTLs or Tregs without accounting for HLA-DR expression, there was no significant correlation between these cells and these molecules of the tumor milieu, highlighting the importance of T lymphocytes activation and how tumor cells influence it, by secreting different molecules in the tumor microenvironment (191).

8. Circulating cytotoxic T cells maintain HLA-DR expression of tumor infiltrating cytotoxic T cells in breast cancer patients

Envisioning the potential use of HLA-DR-expressing CTLs as a biomarker to predict breast cancer response to NACT, we asked if this immune trait was reflected systemically. If HLA-DR-expressing CTLs are detected in the whole blood of breast cancer patients and if its analysis reflects the response to NACT, it would be a major advantage to be able to perform a liquid biopsy to detect the biomarker and conduct clinical decisions.

To verify an association between the tumor immune microenvironment and peripheral blood, blood samples were collected from 43 matched patients, prior to treatment implementation, and immunophenotyped by flow cytometry. Intriguingly, we observed that the expression of HLA-DR in tumor infiltrating CTLs correlated with HLA-DR expression in circulating CTLs (r=0.43, p=0.004, Figure IV-18A, (191)). Moreover, the HLA-DR level in circulating CTLs also distinguished NACT-responders from non-responders (p=0.02, Figure IV-18B). HLA-DR expression level in systemic CTLs of healthy donors was also analyzed. Healthy donors have less HLA-DR expression level in CTLs when compared to NACT-responders and more compared to NACT non-responders.

As we did for the analysis of HLA-DR+ CTLs in biopsies, we have plotted the value of HLA-DR expression of both responders and non-responders in the same column (Figure IV-18C). Here, the segregation of the patients was not as good as in the case where this feature was analyzed in the biopsy. A ROC curve analysis was also performed for the expression of HLA-DR in systemic CTLs (Table IV-6) and we obtained a threshold value of 16.08, meaning that below this value patients would be considered NACT non-responders. The area under the ROC curve was 0.76 and the analysis was executed with 86.67% sensitivity and 63.64% of sensibility (Table IV-6).

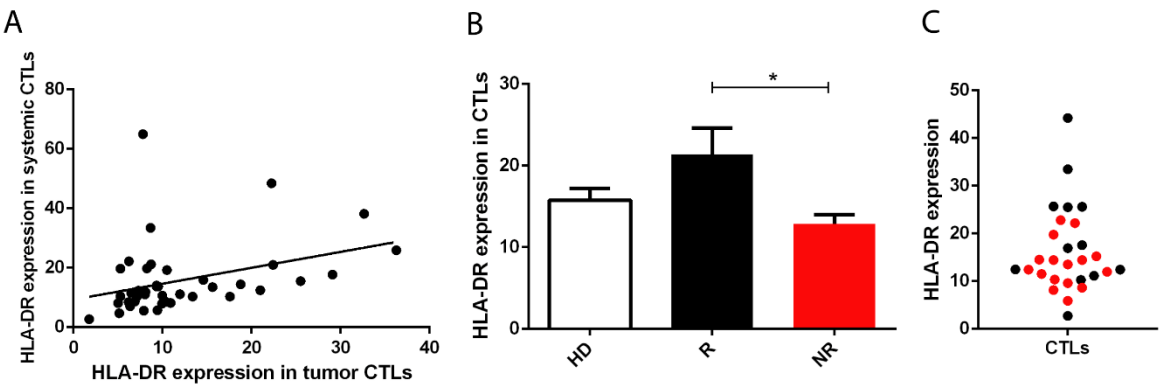


Figure IV-18 **Systemic HLA-DR-expressing cytotoxic T cells maintain the profile of intratumor HLA-DR-expressing CTLs and can correlate with response to neoadjuvant chemotherapy**. **(A)** Correlation between expression of HLA-DR in systemic CTLs and tumor infiltrating CTLs (Spearman r=0.43, p=0.004, n=43). **(B)** HLA-DR expression in systemic CTLs in healthy donors (HD, white bar, n=13, mean ± SEM),

NACT-responders (R, black bar, n=12, mean ± SEM) and non-responders (NR, red bar, n=16, mean ± SEM). **(C)** HLA-DR expression level in circulating CTLs of NACT-responders (black dots, n=12) and NACT non-responders (red dots, n=16). The expression level is the median fluorescent intensity of the positive population normalized to the negative population. *p<0.05.

Table IV-6 **ROC curve parameters for HLA-DR-expressing circulating cytotoxic T cells (CTLs) in breast cancer patients submitted to neoadjuvant chemotherapy**.

	HLA-DR+ CTLs
Threshold Value	<16.08
AUC	0.76
Sensitivity	86.67%
Specificity	63.64%

Thus, although there is a correlation between the expression of HLA-DR in CTLs from tumor and from circulation, and systemic CTLs can infer response to NACT, the analysis of HLA-DR-expressing CTLs at the biopsy level should not be discarded, since the ROC curve obtained for systemic HLA-DR-expressing CTLs revealed that this potential biomarker has a weaker performance when assessed in blood (Saraiva *et al*, manuscript in preparation).

From the whole blood of breast cancer patients, we also collected plasma to assess the circulating levels of the cytokines IFN-γ and IL-10 (Figure IV-19). These cytokines were chosen because IFN-γ reflects an activation status of CTLs, whereas IL-10 reflects an anti-inflammatory environment and for instance the activation status of Tregs. Besides dividing the patients in NACT-responders and NACT non-responders, we also divided them according to the axillary lymph node invasion status, as a readout of the results in section 1 of this chapter.

Patients without axillary lymph node invasion, as well as NACT-responders, had higher levels of IFN-γ (p=0.004, p=0.01, respectively, Figure IV-19A and C) and lower levels of circulating IL-10 (p=0.02, non-statistical, respectively, Figure IV-19B and D). These results reflect the fact that NACT-responders and patients without axillary lymph node metastasis have a higher expression level of HLA-DR in CTLs when compared to either NACT non-responders or patients with axillary lymph node metastasis (191).

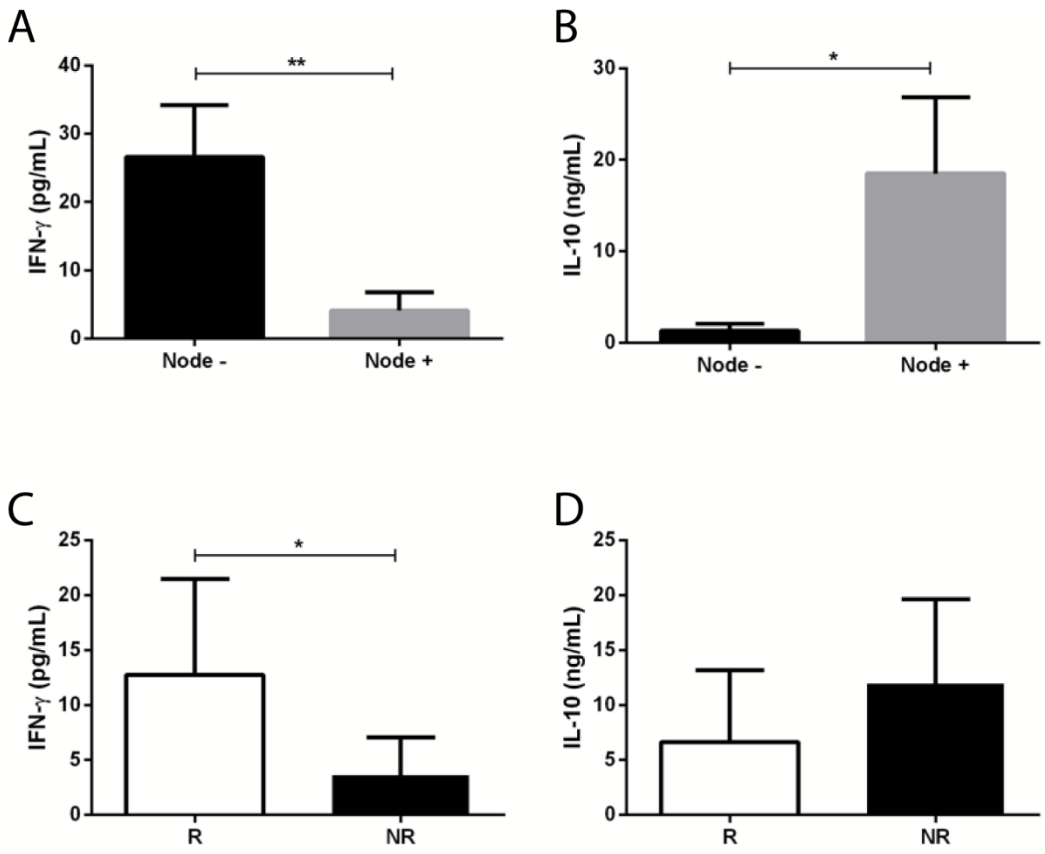


Figure IV-19 **The circulating cytokines correlate with the HLA-DR-expressing T cells and can distinguish the axillary lymph node invasion status and response to treatment**. **(A)** IFN-γ and **(B)** IL-10 levels assessed by ELISA in plasma of patients without axillary lymph node metastasis (Node -, black bars, n=9, mean ± SEM) and with axillary lymph node metastasis (Node +, grey bars, n=12, mean ± SEM). IFN-γ **(C)** and IL-10 **(D)** levels assessed by ELISA in plasma from responders (R, white bars, n=4, mean ± SEM) and non-responders (NR, black bars, n=6, mean ± SEM). *p<0.05, **p<0.01.

Altogether, the results from this chapter point out that the presence of CTLs and other immune cells in the tumor microenvironment is not sufficient to predict response to treatment. Tumor cells can escape the immune system by turning themselves invisible to the immune surveillance or by releasing factors that can diminish the immune response. The activation status of the CTLs, revealed by the expression of HLA-DR at their surface, is a more reliable biomarker to predict tumor aggressiveness but more importantly patients' response to treatment, since it can reflect the tumor-immune crosstalk.

Chapter V

HLA-DR+ cytotoxic T cells exhibit effector memory T cell markers

Chapter V - HLA-DR+ cytotoxic T cells exhibit effector memory T cell markers

After demonstrating that the presence of HLA-DR-expressing cytotoxic T cells (CTLs) in the biopsies is required for neoadjuvant chemotherapy (NACT) success and therefore predicts breast cancer patients' response to NACT, we tried to further characterize this T cell subset. By deepening the knowledge about these cells, in the future, we could use this information to better manipulate patients' immune system in order to boost anti-tumor immune responses.

1. Patient-derived immune cells are able to increase HLA-DR when stimulated *ex vivo*

In selected patients, especially patients that were designated to perform neoadjuvant chemotherapy (NACT), peripheral blood mononuclear cells (PBMCs) were isolated from their whole blood. After the end of the treatment, patients were divided into NACT-responders and non-responders. The PBMCs isolated were cultured under a canonical stimulus of PMA/ionomycin to clarify if they could become activated *ex vivo*. This activation would be essential to conduct several functional experiments with immune cells isolated from the patients' blood, since the material provided by core biopsy or by surgery is limited.

PBMCs isolated from buffy coats of healthy donors were used as a control. After culture with stimulation, the PBMCs were collected and stained for flow cytometry to detect the expression of HLA-DR in CTLs and of the cytokines IFN-γ and IL-10 (Figure V-1), known to be key anti-tumor and pro-tumor cytokines, respectively.

Interestingly, even with the same stimulation protocol, the expression of HLA-DR in CTLs was higher in PBMCs of NACT-responders regarding PBMCs of non-responders ($p=0.02$) and healthy donors ($p=0.01$, Figure V-1A), demonstrating a hardwired capacity of the immune cells isolated from NACT-responders to become even more activated. Although NACT non-responders have lower levels of HLA-DR in CTLs (section 3 of Chapter IV), when the PBMCs of these patients were stimulated *ex vivo*, they were able to increase HLA-DR level in CTLs even further when compared to PBMCs from healthy donors ($p=0.03$, Figure V-1A). This result suggests that CTLs from NACT non-responders still have the capacity to become activated and their low activation (low HLA-DR levels) in NACT non-responders' biopsies should be due to the immunosuppressive microenvironment dictated by tumor cells (191).

Also, stimulated PBMCs of NACT-responders have increased levels of IFN-γ in CTLs when compared to CTLs of PBMCs from NACT non-responders ($p=0.01$) and from healthy donors (non-statistical, Figure V-1B). Additionally, stimulated PBMCs of NACT-responders have lower levels of IL-10, produced by Th cells and/or Tregs (Figure V-1C) than stimulated PBMCs of non-responders ($p=0.01$). As previously observed in the plasma of patients (section 8 of Chapter IV), the production of these cytokines is closely related to the activation of CTLs/Tregs, i.e.

more IFN-γ expressed in CTLs of NACT-responders' PBMCs and more IL-10 expressed in Th/Tregs of NACT non-responders' PBMCs, upon *ex vivo* stimulation (191).

Altogether, these observations further support that the tumor immune features are maintained systemically. Moreover, the fact that PBMCs of NACT non-responder patients, especially the CTLs, have the ability to become activated, is a major advantage to perform further functional assays.

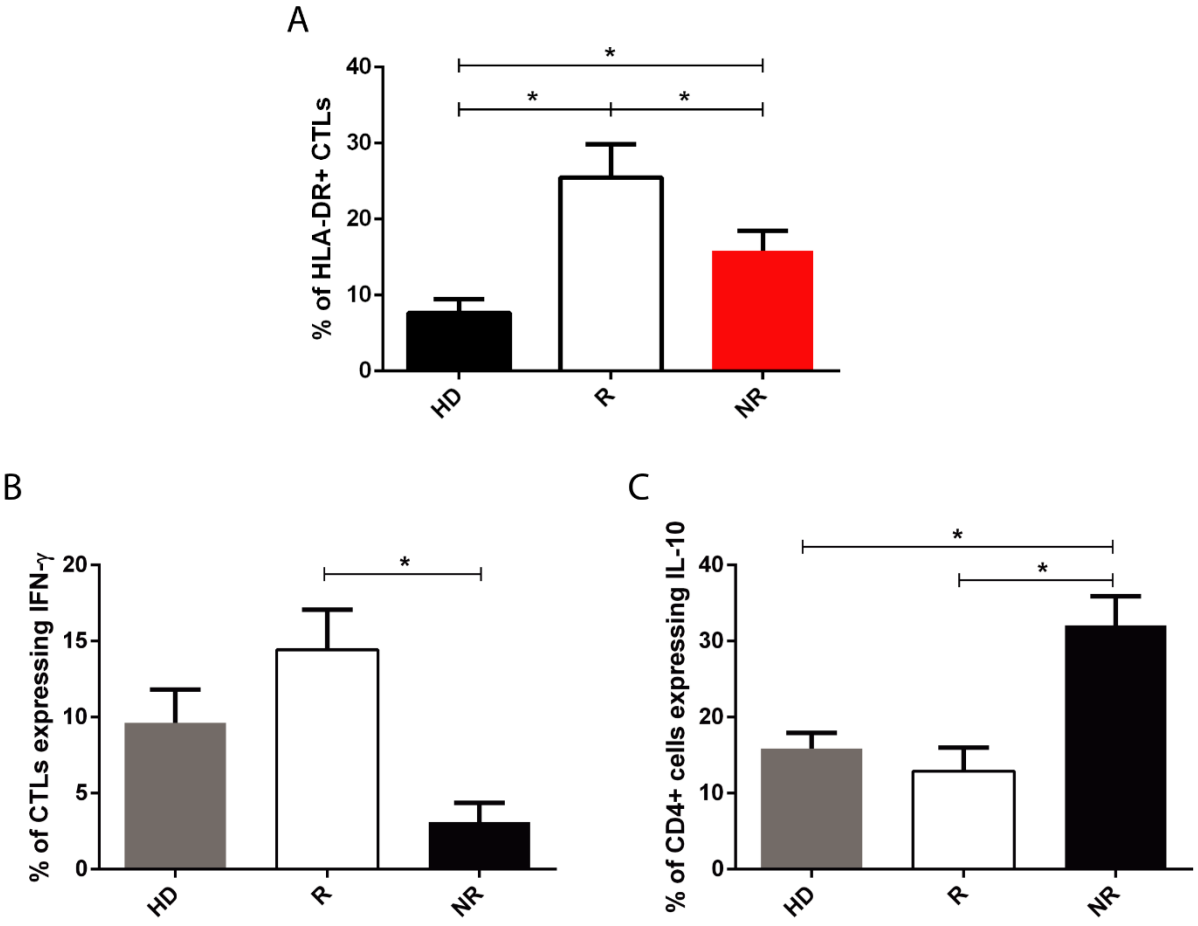


Figure V-1 **Stimulated PBMCs from NACT-responders produce more IFN-γ and less IL-10. (A)** Percentage of HLA-DR positive cytotoxic T cells (CTLs), assessed by flow cytometry, of *ex vivo* stimulated PBMCs isolated from responders (R, white bar, n=5), from non-responders (NR, red bar, n=5) and from healthy donors (HD, black bar, n=5). **(B)** Percentage of CTLs expressing IFN-γ and **(C)** percentage of CD4+ T cells (either Th or Tregs) expressing IL-10, assessed by flow cytometry, in stimulated PBMCs isolated from responders (R, white bars, n=4), from non-responders (NR, black bars, n=5) and from healthy donors (HD, grey bars, n=5). *p<0.05.

2. HLA-DR expression increases in cytotoxic T lymphocytes without cell-to-cell contact

To assess if HLA-DR expression in cytotoxic T lymphocytes (CTLs) resulted from cellular contact with tumor cells or is mainly induced by cytokines in the environment, as it is suggested by the negative correlations between HLA-DR+ CTLs and cytokines released by tumor cells (section 7 of Chapter IV), we did a 2D co-culture assay (Figure V-2). In this co-culture, we tested isolated PBMCs from distinct patients, independently of their response to NACT, and cultured them in different conditions: with Hs 578T breast cancer cell line in a 20:1 proportion, with only the supernatant of the cell line, with a canonical stimulus of PMA/ionomycin (positive control), or in monoculture (negative control). The co-cultures were maintained during 48h, followed by PBMCs harvesting and staining for flow cytometry.

Curiously, the supernatant of the cell line alone induced the expression of HLA-DR in CTLs ($p=0.006$), similarly to the result obtained in the co-culture ($p=0.003$, Figure V-2), where tumor cells were in direct contact with the immune cells. These results highlight the fact that cytokines from the tumor milieu are able to influence the activation state of CTLs, without the requirement of contact with the tumor cells (191).

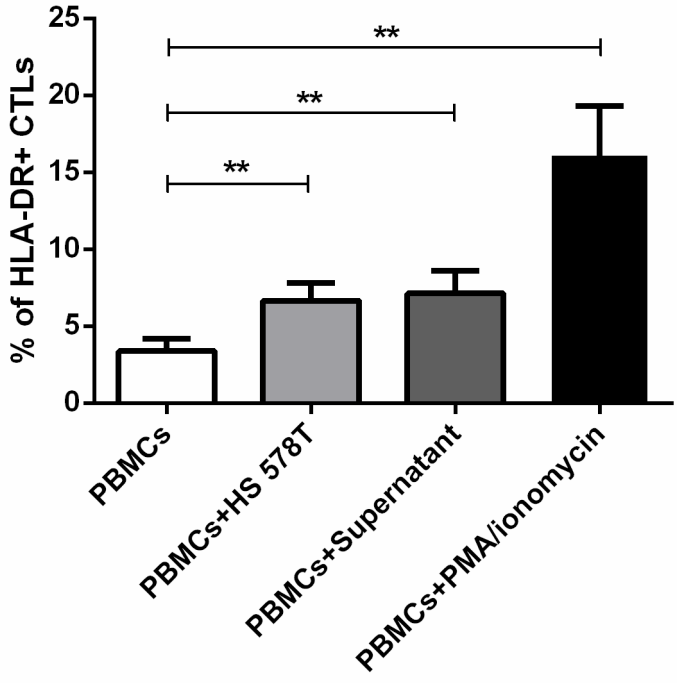


Figure V-2 **HLA-DR increases in CTLs without cell-to-cell contact with tumor cells.** Percentage of HLA-DR+ cytotoxic T cells (CTLs), assessed by flow cytometry, in the following conditions: PBMCs in mono-culture, co-culture of PBMCs with HS 578T cell line, PBMCs with the cell line supernatant and PBMCs with the canonical stimulus (PMA/ionomycin). **$p<0.01$, $n=6$.

3. HLA-DR+ cytotoxic T cells have increased expression level of effector immune response-related molecules

Since HLA-DR is a recognized marker of T cell activation, we set out to analyze if HLA-DR+ CTLs produced more molecules related to their activation, when compared to HLA-DR negative CTLs (Figure V-3).

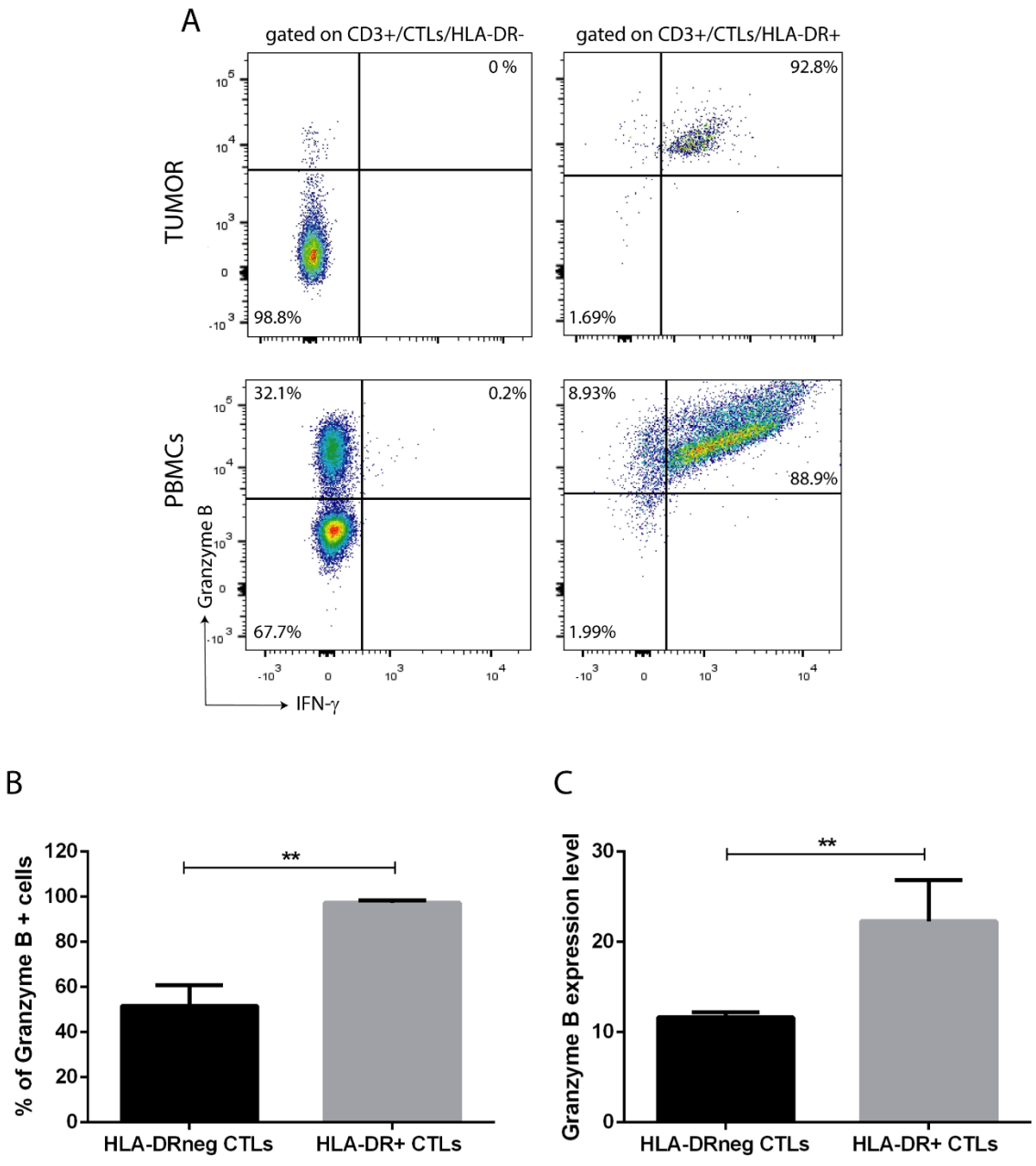


Figure V-3 **HLA-DR+ cytotoxic T cells express IFN-γ and Granzyme B**. **(A)** Representation of a flow cytometry analysis of Granzyme B and IFN-γ expression in HLA-DR+ cytotoxic T cells (CTLs) and in HLA-DR negative CTLs from pre-treatment tumor samples and from PBMCs isolated from patients. **(B)** Percentage of Granzyme B in HLA-DR negative CTLs (black bar, n=6) and HLA-DR+ CTLs (grey bar, n=6) from PBMCs isolated from patients' whole blood. **(C)** Granzyme B expression level, calculated as the

ratio between the median fluorescent intensity of the positive and negative population, in HLA-DR negative CTLs (black bar, n=6) and HLA-DR+ CTLs (grey bar, n=6) from patients' PBMCs. **p<0.01.

To achieve this task, we have analyzed by flow cytometry, specifically in those cells, the expression of effector immune response-related molecules, such as IFN-γ and Granzyme B, which are the most important players of activated CTLs. This analysis was performed in HLA-DR+ and HLA-DR negative CTLs from tumors and from PBMCs of breast cancer patients (Figure V-3, (191)). Interestingly, in tumor infiltrating cells, only HLA-DR+ CTLs expressed high levels of IFN-γ and Granzyme B (Figure V-3A), substantiating their immune-competent profile. Regarding circulating cells, HLA-DR+ CTLs express significantly more IFN-γ and Granzyme B than HLA-DR negative CTLs (Figure V-3A). In fact, even if HLA-DR negative CTLs from PBMCs had a higher level of Granzyme B expression when compared to HLA-DR negative CTLs from the tumor, the percentage of HLA-DR+ CTLs from PBMCs that express Granzyme B, and the corresponding median fluorescent intensity, is significantly higher than in HLA-DR negative CTLs from PBMCs (p=0.002, Figure V-3B-C).

After corroborating that HLA-DR+ CTLs produced more cytotoxicity-related molecules, such as IFN-γ and Granzyme B, by flow cytometry analysis, we set out to analyze other molecules produced by these cells by qRT-PCR. For that, we have used isolated PBMCs from buffy coats of healthy donors and stimulated them in culture with PMA/ionomycin, to increase the levels of HLA-DR in CTLs. After the stimulation, these cells were collected and sorted, by fluorescence activated cell sorter, in two populations – HLA-DR+ CTLs and HLA-DR negative CTLs. Both populations were sorted directly to lysis buffer. Then, the RNA was extracted and transformed in cDNA for gene expression analysis of *Granzyme B*, *Perforin*, *TNF-α*, *Eomes* and *Tbet* (Figure V-4). HLA-DR+ CTLs expressed higher levels of the cytolytic proteins *Granzyme B* (p=0.02) and *Perforin* (p=0.03); *Eomes* (p=0.03) and the inflammatory cytokine *TNF-α* (p=0.03), which also has anti-tumor properties, in comparison with HLA-DR negative CTLs (Figure V-4, (191)). On the other hand, the expression of *Tbet* was lower in HLA-DR+ CTLs when compared to HLA-DR negative CTLs (p=0.01). Eomes is a transcription factor involved in the differentiation of CTLs, increases upon CTLs activation, regulates IFN-γ response and cytotoxicity (202,203). Tbet is also a transcription factor required for the development of effector cells and regulates CTLs' response to virus infection (204,205).

Although so far the results point out that HLA-DR+ CTLs are effector T cells that produce cytotoxicity-related molecules, normally an effector T cell has a $Tbet^{high}/Eomes^{high}$ phenotype (206). Besides effector T cells, there are also exhausted T cells, central memory, effector memory, tissue resident memory T cells, and other subtypes that are going to be characterized in the next subsection. A $Tbet^{low}/Eomes^{high}$ profile is a characteristic of central memory, effector memory, exhausted T cells and terminally differentiated effector T cells which are active and functional, but lost the capacity to proliferate (206). To better understand the

profile of HLA-DR+ CTLs, we analyzed several surface markers that could distinguish between the subtypes of cytotoxic T cells.

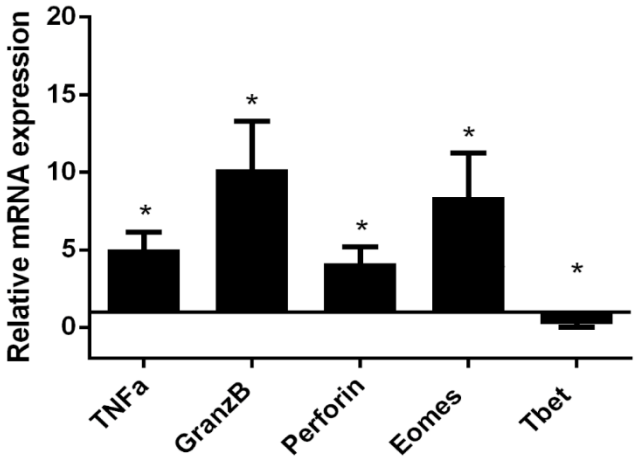


Figure V-4 **HLA-DR+ cytotoxic T cells express more cytotoxicity-related molecules**. mRNA level of *TNF-α*, *Granzyme B* (GranzB), *Perforin*, *Eomes*, and *Tbet* in cytotoxic T cells (CTLs) with HLA-DR (n=4, *p<0.05). The relative mRNA expression was performed with the expression of these genes in CTLs without HLA-DR (represented as 1).

4. HLA-DR+ cytotoxic T cells are active, proliferating and functional T cells and some present exhaustion markers

To deepen our knowledge regarding the profile of HLA-DR+ CTLs we have used a flow cytometry panel of distinct antibodies and compared the results with HLA-DR negative CTLs from breast cancer biopsies. The antibody panel comprised both IFN-γ and Granzyme B, and also CD69, Ki67, CD39, CD103, CD127, PD-1 and Tim3 (Figure V-6, 7, Saraiva *et al* manuscript in preparation).

We have chosen these cell surface markers to supplement the functional/developmental characterization derived from the above referred gene expression analysis. Although our first impression, due to the high production of cytotoxicity-related molecules, such as IFN-γ, Granzyme B and Perforin, would be that HLA-DR+ CTLs are effector T cells, the fact that they had a $Tbet^{low}/Eomes^{high}$ transcription profile suggested that this conclusion might be too simplistic.

As referred above, besides effector T cells, there are several other subsets of CTLs (Figure V-5). Naïve T cells are the subtype of CTLs that have never encountered an antigen. After T cells are presented with an antigen, a subset will acquire memory. Between memory T cells, there are several subtypes, with different expression profiles and functions. Stem cell memory T cells (T_{SCM}) are a subtype of memory T cells with stem cell-like properties, i.e. high self-renewal capacity. They have been described as an early developmental stage of memory T cells. Central

memory T cells (T_{CM}) have less self-renewal capacity than T_{SCM} and are mainly located in lymph nodes. Between T_{CM} and effector memory T cells (T_{EM}) there is a transitional subtype (T_{TM}) that has a phenotype close to T_{EM} with the loss of CCR7 expression but without effector capacity. T_{EM} has a high capacity to recirculate between tissues but is mainly recruited to inflamed tissues. This subtype has the capacity to produce IFN-γ and release cytotoxicity-related molecules. Tissue resident memory T cells (T_{RM}) also have effector functions but lost the capacity to recirculate. Terminally differentiated effector T cells (T_{TE}) are similar to T_{EM}, but instead of expressing CD45RO (a memory marker), express CD45RA (a naïve marker). Additionally, T_{TE} has a lower proliferative capacity, when compared to T_{EM}. Effector T cells (T_{eff}) is the most cytotoxic subtype of CTLs, have no memory to antigens but respond to them by releasing cytotoxic molecules. After T_{eff} cells have performed their cytotoxic function, the majority of them undergo apoptosis. Exhausted T cells (T_{Exh}) were initially effector T cells but due to their demanding cytotoxic function have become exhausted, and lose the capacity to proliferate and to respond to an insult. Interestingly, T_{CM}, T_{EM}, T_{RM} and T_{TE} have exhaustion markers.

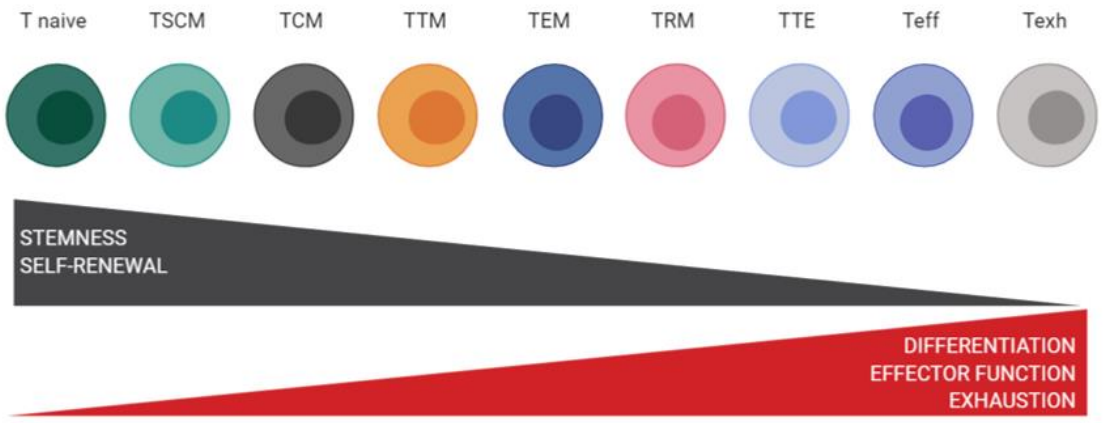


Figure V-5 **Subtypes of cytotoxic T cells (CTLs), from naïve to memory, effector (T_{eff}) and exhausted (T_{Exh}).** Between memory subtypes of CTLs, there are stem cell memory T cells (T_{SCM}), central memory T cells (T_{CM}), transitional memory T cells (T_{TM}), effector memory T cells (T_{EM}), tissue resident memory T cells (T_{RM}), and terminally differentiated effector T cells (T_{TE}). The scheme was adapted from (206) and produced with Biorender.

To assess if HLA-DR+ CTLs fitted to one of these subtypes, we performed a thorough characterization of cell surface markers expression and compared the results with HLA-DR negative CTLs (Figure V-6). Besides quantifying the percentage of these markers in HLA-DR+ and negative CTLs (Figure V-6), we also performed a visual automatic analysis (Figure V-7). For that, we conducted a t-SNE (t-distributed stochastic neighbor embedding) analysis to reduce the dimensionality of the samples and distinguish visually the different markers distribution (Figure V-7). HLA-DR+ CTLs, produced both IFN-γ and Granzyme B, as opposite to HLA-DR negative CTLs that show no expression of these molecules ($p<0.001$ and $p<0.0001$,

respectively, Figure V-6). Also, in the t-SNE analysis, the expression of these two markers co-localized with HLA-DR expression (Figure V-7A). Both IFN-γ and Granzyme B are produced by T_{EM}, T_{TE} and effector T cells (Table V-1).

CD69 is an early activation marker in T cells, as referred in the introduction (section 2.5 of Chapter I, (71–73)). This marker is more produced by HLA-DR+ CTLs when compared to HLA-DR negative CTLs where the presence of this marker is only observed in approximately 5% of the cells ($p<0.05$). Although HLA-DR is a late activation marker, approximately 40% of HLA-DR+ CTLs are also positive for CD69, revealing that this marker can remain in the cell surface when HLA-DR expression increases (Figure V-6). With the t-SNE visualization, it is possible to observe this marker in the majority of HLA-DR-expressing CTLs (Figure V-7A). Furthermore, this result also confirms that HLA-DR negative CTLs are not active T cells. CD69 expression is mainly related with the T_{EM} phenotype (Table V-1).

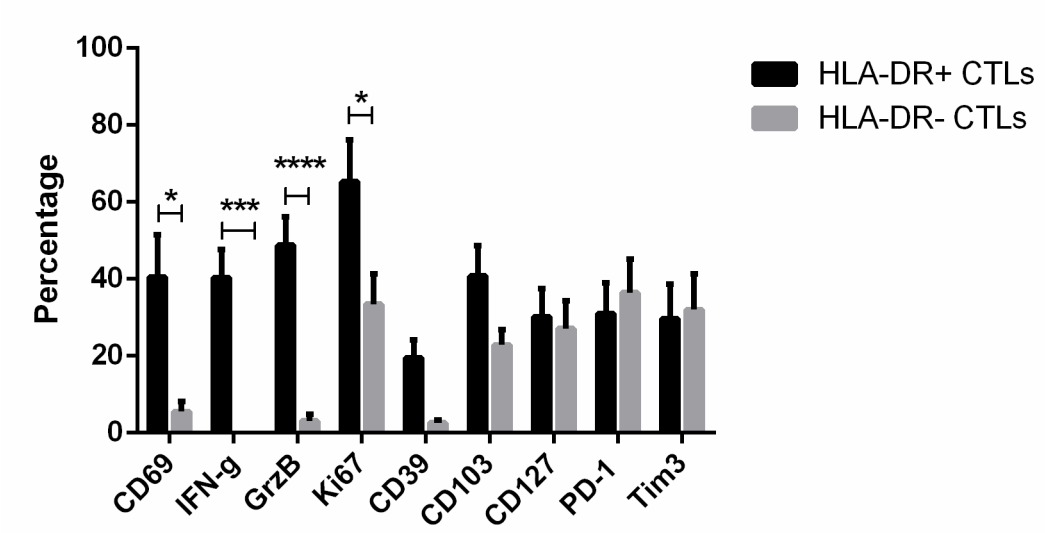


Figure V-6 **HLA-DR+ and HLA-DR negative cytotoxic T cells have differential surface markers expression**. Percentage of CD69, IFN-γ, Granzyme B (GrzB), Ki67, CD39, CD103, CD127, PD-1 and Tim3 inside the gate of HLA-DR+ cytotoxic T cells (black bars, n=16 biopsies) and HLA-DR negative cytotoxic T cells (grey bars, n=16 biopsies). The expression of these markers was analyzed by flow cytometry. Data represent mean ± SEM, *$p<0.05$, ***$p<0.001$, ****$p<0.0001$.

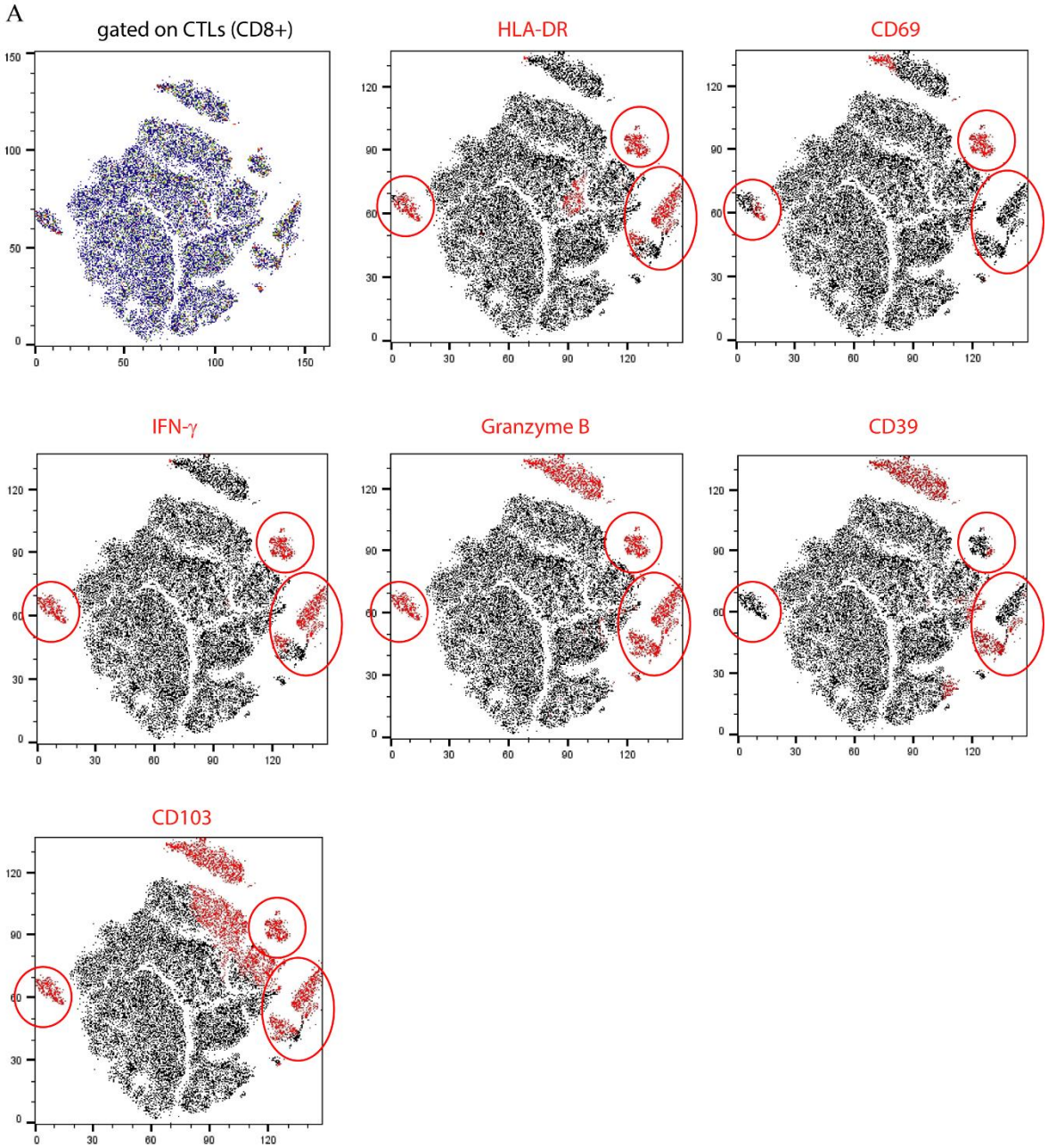
Normally, when CTLs become active and recognize specific antigens, a round of proliferation occurs to give rise to a higher number of antigen-specific activated CTLs. As such, Ki67 (a proliferation marker) is expressed in approximately 65% of HLA-DR+ CTLs ($p<0.05$), revealing that these cells are highly proliferative. Nonetheless, approximately 30% of HLA-DR negative CTLs also express Ki67, suggesting that these cells, although not active, are still proliferating (Figure V-6). By the t-SNE analysis, Ki67 is expressed in HLA-DR+ CTLs and also in a smaller representation in HLA-DR negative CTLs (Figure V-7B), confirming the results in Figure V-6. Additionally, Ki67 is normally present in T_{CM}, T_{TM}, T_{EM} and effector CTLs (Table V-1).

PD-1, Tim3 and CD127 are T cell exhaustion markers. The presence of these exhaustion markers is similar when comparing HLA-DR+ to HLA-DR negative CTLs. The three exhaustion markers are expressed by approximately 30% of HLA-DR+ CTLs (Figure V-6). By the t-SNE analysis, we observed that CD127 and PD-1 are also highly present in HLA-DR+ CTLs, although their expression is also observed in HLA-DR negative CTLs (Figure V-7B), corroborating the analysis of Figure V-6. This result suggests that T cell activation and exhaustion is a dynamic process and although HLA-DR+ CTLs are activated with the capacity to produce cytotoxicity-related molecules, the permanent contact with tumor antigens can leave them exhausted. Furthermore, this result points out that HLA-DR+ CTLs are not simply effector T cells, as suspected given the $Tbet^{low}$/$Eomes^{high}$ transcriptional profile. Exhaustion markers, besides being present in exhausted T cells, are also produced by different subsets of memory T cells, such as T_{EM} (Table V-1).

We also wondered if HLA-DR+ CTLs with cytotoxic capacities against tumor cells are tumor infiltrating or are tissue resident CTLs. To assess this question, we analyzed the expression of CD103. CD103 is an integrin, also known as integrin αE, essential for the retention of CTLs in the tissue (207). T_{RM} cells have an advantage when compared to tumor infiltrating T cells because they are adapted to the tumor microenvironment. This microenvironment can have extreme conditions that will inhibit the function of infiltrating T cells. These extreme conditions include high levels of hypoxia and decreased pH due to the presence of lactate derived from the preferential energy metabolism based on aerobic glycolysis of tumor cells. T_{RM} cells, even in the presence of such surroundings will be adapted and capable of exerting their functions. As such, CD103+ T_{RM} CTLs have been reported to have an important role in tumor cell elimination and indeed have been categorized as a good prognostic factor in different types of cancer (208–211). Nevertheless, CD103 can also be upregulated in T_{EM}, since they have the capacity to recirculate but are retained in inflamed tissues (206). Interestingly, CD103 expression is observed in both HLA-DR+ and HLA-DR negative CTLs, suggesting that resident CTLs, be them T_{RM} or T_{EM} have the capacity to become activated and respond to tumor-specific antigens. CD103 is present in approximately 40% of HLA-DR+ CTLs, revealing that this subset comprises both tissue resident and tumor-infiltrating CTLs (Figure V-6). There is a co-localization of CD103 and HLA-DR+ CTLs (t-SNE analysis), although its expression is also observed in HLA-DR negative CTLs (Figure V-7A), again corroborating the analysis of Figure V-6.

CD39 is an ectoenzyme capable of converting extracellular ATP and ADP to adenosine (212). Extracellular ATP can induce CTLs apoptosis; hence, CTLs might increase the expression of CD39 at their surface to escape this inhibitory mechanism (213). On the other hand, the presence of adenosine in the tumor microenvironment, the product of ATP hydrolysis, is known to be immunosuppressive, inhibiting CTLs activation and function, inducing suppressive immune cells such as Tregs and potentiating tumor cells proliferation and invasiveness capacity (reviewed in (213)). Additionally, CD39 was demonstrated to be increased in activated CTLs and is present in T_{TE}, T_{CM} and T_{EM} (213). Although present in

activated CTLs, some authors claim that CD39 mark exhausted CTLs (214). CD39 is normally encountered in tumor infiltrating T lymphocytes (215–217) and CD39+ CTLs were shown to have a Tbet^{low}/Eomes^{high} transcriptional profile (214,217). Co-expression of CD39 and CD103 can also represent T_{RM} (215). Here we observed that approximately 20% of HLA-DR+ CTLs are positive for CD39, whereas only 3% of HLA-DR negative CTLs produce this enzyme (Figure V-6). By the t-SNE analysis, there is a representation of CD39 in HLA-DR+ CTLs (Figure V-7). Again, this result confirms that HLA-DR+ CTLs are a heterogeneous population with distinct levels of activation/exhaustion. Indeed, a proportion of these cells acquired CD39 cell surface marker, which is not only present in activated T cells but also in exhausted T cells.



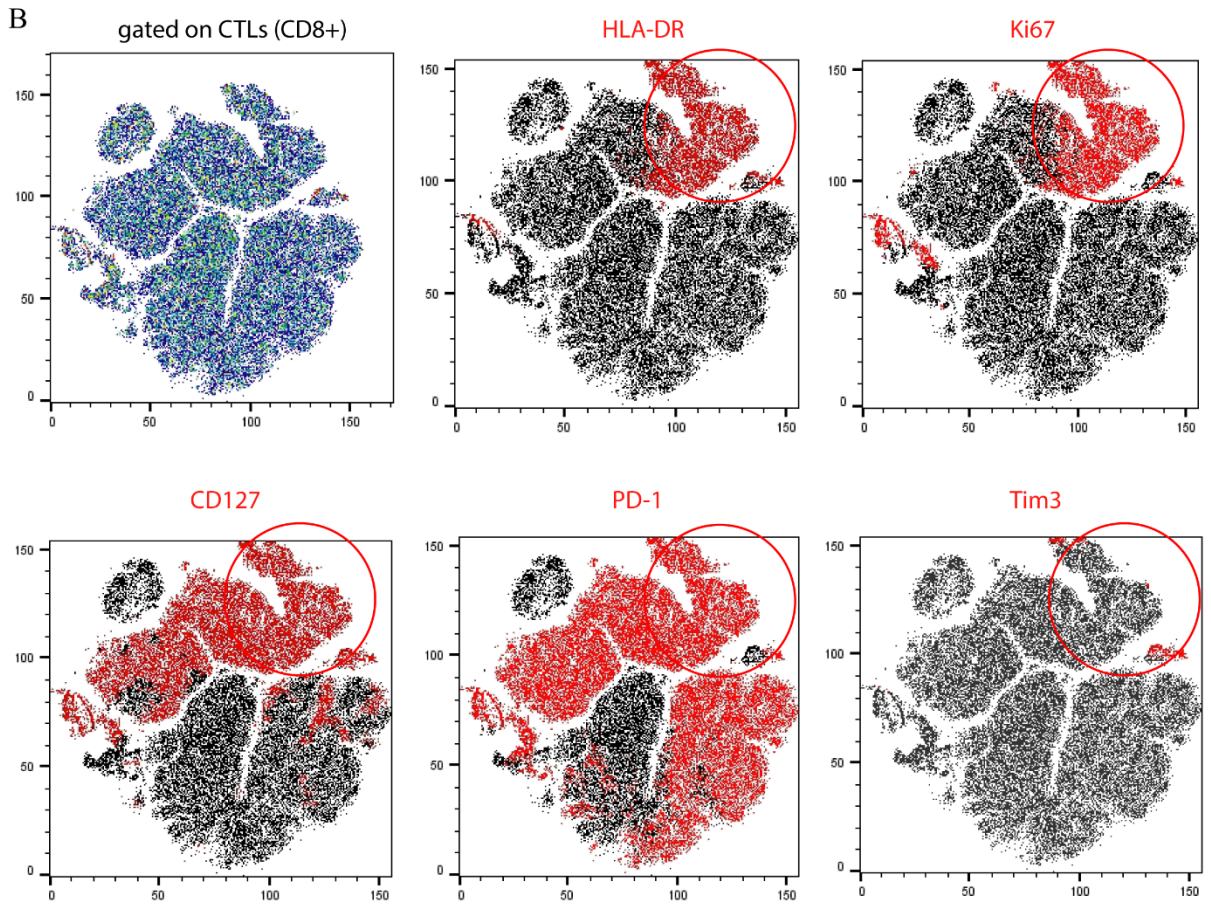


Figure V-7 **t-SNE analysis of distinct markers gated on cytotoxic T cells (CTLs).** A dimensionality reduction analysis (t-SNE) was performed in FlowJo software for CTLs analysed by flow cytometry in a concatenated file comprising the results from the different patients' biopsies. Inside the CTLs gate, several surface markers were analysed. Namely, **(A)** HLA-DR, CD69, IFN-γ, Granzyme B, CD39, CD103; and **(B)** Ki67, CD127, PD-1 and Tim3. Positive regions for each marker are in red. HLA-DR+ CTLs region is marked with a red circle and it is represented in the other surface markers analysed.

In general, the most noteworthy differences between HLA-DR+ and negative CTLs were the production of IFN-γ and Granzyme B and the expression of CD69 and Ki67, highlighting the higher level of CTLs activation and the capacity to produce cytotoxicity-related molecules (Figure V-6).

Combining the results from this section with the results from the previous one, where the gene expression differences between HLA-DR+ and negative CTLs were compared, we can conclude that the majority of HLA-DR+ cells have an IFN-γ ^{high}, Granzyme B ^{high}, Perforin ^{high}, TNF-α ^{high}, Eomes ^{high}, Tbet ^{low} phenotype, and some express CD69, Ki67, CD39, CD103, CD127, PD-1 and Tim3. This phenotype suggests that HLA-DR+ CTLs are functionally active, cytotoxic T cells, although, at least some of the cells amongst the population exhibit an intermediate level of exhaustion.

As referred throughout these results, there are several subtypes of CTLs. Namely, exhausted CTLs, naïve T cells, stem cell memory T cells (T_{SCM}), central memory T cells (T_{CM}), transitional memory T cells (T_{TM}), effector memory T cells (T_{EM}), tissue resident memory T cells (T_{RM}) and terminally differentiated effector T cells (T_{TE}). We have combined the results from this subsection with the gene expression analysis and, based on the literature (206,213,218,219), have constructed a summary of the results (Table V-1).

Table V-1 **Expression of different markers in HLA-DR+ CTLs and the correlation with the different subsets of memory T cells, effector and exhausted T cells.**

Marker	HLA-DR+ CTLs	T naïve	T_{SCM}	T_{CM}	T_{TM}	T_{EM}	T_{RM}	T_{TE}	T_{eff}	T_{Exh}
$Tbet^{lo}/Eomes^{hi}$	+			+		+		+		+
TNF-α	+					+	+		+	
Granzyme B	+				+	+	+	+	+	
IFN-γ	+					+	+	+	+	
Perforin	+				+	+	+	+	+	
CD69	+					+	+			
Ki67	+			+	+	+	+		+	
CD39	+/-			+		+	+		+	+
CD103	+					+	+			
CD127	+	+	+	+	+	+	+			+
PD-1	+			+		+/-	+	+/-		+
Tim-3	+					+/-	+			+
HLA-DR	+					+	+		+	

T_{SCM} - stem cell memory T cells; T_{CM} - central memory T cells; T_{TM} - transitional memory T cells; T_{EM} - effector memory T cells; T_{RM} – tissue resident memory T cells; T_{TE} - terminally differentiated effector T cells; T_{eff} – effector T cells; T_{Exh} – exhausted T cells.

By the analysis of the table above, we can suggest that HLA-DR+ CTLs have a predominant effector memory T cell phenotype. T_{EM} cells have the capacity to home to tissues and also to recirculate, they have the ability to rapidly release effector molecules and differentiate in effector CTLs. This seem consistent with the fact that in breast cancer patients their presence is required for the anti-tumor response that might contribute to NACT. Other markers characteristic of this specific subset should be further analyzed to corroborate this suggestion.

Chapter VI

3D system of breast cancer cells and patient-derived immune cells to clarify the role of HLA-DR+ cytotoxic T cells in NACT response and optimize alternative therapeutic strategies

Chapter VI - 3D system of breast cancer cells and patient-derived immune cells to clarify the role of HLA-DR+ cytotoxic T cells in NACT response and optimize alternative therapeutic strategies

We have observed that HLA-DR-expressing CTLs are highly represented in biopsies of breast cancer with response to neoadjuvant chemotherapy (NACT, section 3 of Chapter IV, (191)). Additionally, we have concluded that HLA-DR+ CTLs produce more cytotoxicity-related molecules, namely IFN-γ, Granzyme B and Perforin, when compared to the HLA-DR negative counterpart (section 3 of Chapter V, (191)).

To better understand the importance of HLA-DR+ CTLs in the response to the treatment, we developed an *in vitro* platform of 3D co-cultures of breast cancer cell lines and immune cells (peripheral blood mononuclear cells - PBMCs) isolated from the patients' blood. Moreover, with this allogenic platform, we can explore new therapeutic strategies to have an alternative treatment for patients without response to NACT.

1. Establishment of 3D co-culture of breast cancer cell line and patient-derived immune cells

As referred in Chapter II, a growing body of evidence suggested that 3D cell culture systems, in contrast to the 2D cultures, represent more accurately the actual microenvironment where cells reside in tissues. Thus, the behavior of 3D cultured cells is more reflective of *in vivo* cellular responses.

Therefore, in order to clarify the role of HLA-DR+ CTLs in the success of NACT and to validate this immune feature as a biomarker of patients' response to treatment *in vitro*, we have established a platform of 3D cultures for two breast cancer cell lines - MCF-7 and MDA-MB-231 (Figure VI-1). Then, we optimized a co-culture with the cell lines and patients' PBMCs, from both NACT-responders and non-responders (Figure VI-3).

These 3D systems were based in tumor spheroids, since they recapitulate essential features present in a tumor microenvironment, namely 3D cellular aggregates of cancer cells, immune infiltration, nutrients and oxygen gradients from normal to hypoxic in the center of the structure (220). To form the 3D spheroids, we have performed a liquid overlay technique, which consists in coating the plate with a non-adhesive component, such as agarose, so that cell-cell interaction is enhanced, allowing the spontaneous formation of the 3D structure.

Similar to what has been described for MCF-7 spheroid formation (188), spheroids of this cell line were completely formed after 3 days of culture, as at this point there is no visible blank spaces within the multicellular aggregate (Figure VI-1). MDA-MB-231 spheroids needed further optimization, but we were able to observe a fully formed 3D structure on day 6 (Figure VI-1, Saraiva *et al*, submitted).

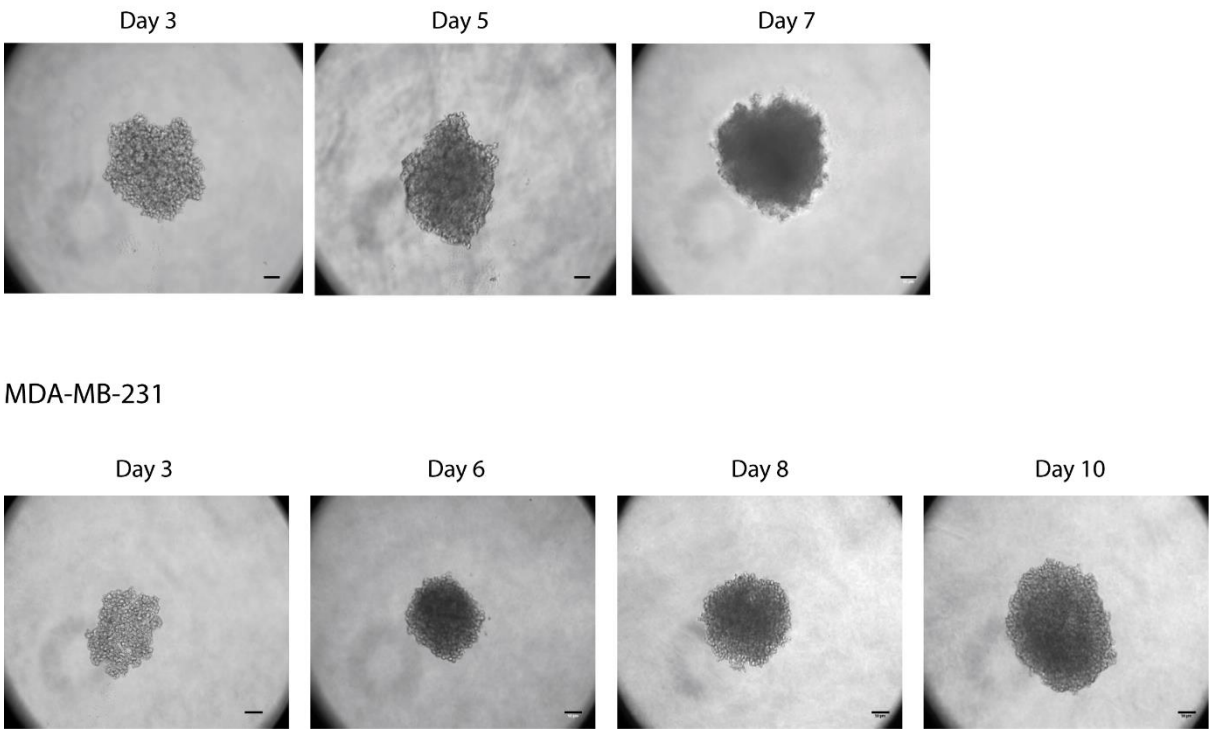


Figure VI-1 **Establishment of 3D spheroids with MCF-7 and MDA-MB-231 breast cancer cell lines**. Bright field images of MCF-7 spheroids at days 3, 5 and 7 of culture and of MDA-MB-231 spheroids at days 3, 6, 8 and 10. Scale bars – 50 μm.

Since our experiments with MCF-7 and MDA-MB-231 spheroids were performed during a period of 7 and 10 days, respectively, we analyzed the percentage of live cells at these time points (Figure VI-2A). We verified that both cell lines were more than 80% viable at day 7 and 10 for MCF-7 and MDA-MB-231 cell lines, respectively. Moreover, we also quantified the spheroid area throughout the days of culture for both cell lines (Figure VI-2B-C). The spheroid area of MCF-7 increases from day 3 to day 5 ($p<0.0001$) and to day 7 ($p<0.0001$, Figure VI-2B); while the spheroid area of MDA-MB-231 increases from day 3 to day 6 ($p=0.01$), day 8 ($p=0.0003$) and day 10 ($p=0.0009$, Figure VI-2C, Saraiva *et al*, submitted).

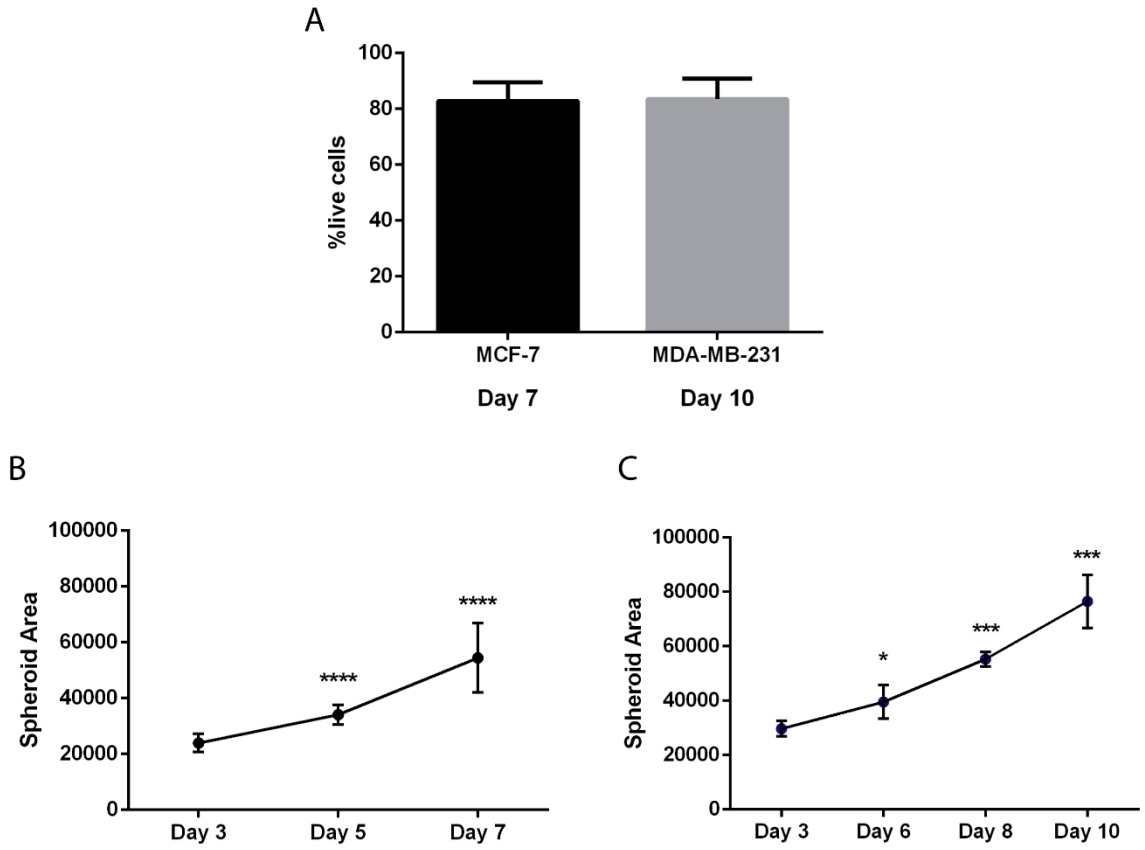


Figure VI-2 **3D spheroids maintain breast cancer cell lines viable, increasing the spheroid area throughout the culture**. **(A)** Percentage of live MCF-7 cells on day 7 of the 3D spheroid culture (n=10) and percentage of live MDA-MB-231 cells at day 10 of the 3D spheroid culture (n=11). **(B)** Spheroid area of MCF-7 spheroids at day 3 (n=9), day 5 (n=8) and day 7 (n=18). **(C)** Spheroid area of MDA-MB-231 spheroids at day 3 (n=4), day 6 (n=7), day 8 (n=4) and day 10 (n=10). *p<0.05, ***p<0.001, ****p<0.0001.

For the co-culture to be as physiological as possible, we added the patient-derived PBMCs after spheroid formation, as *in vivo* the immune cells are recruited to the already formed tumor structure. Additionally, although it is often reported in the literature co-cultures of 10:1 ratio (PBMCs to cancer cells, (185,221)), we optimized our co-culture to have a ratio of 1:1 (PBMCs to cancer cells). This should accurately reflect the tumor microenvironment, since we observed in section 1 of Chapter IV that rarely breast tumors have more than 50% of their mass composed by immune cells.

Interestingly, we observed that either PBMCs from NACT-responders or from non-responders were able to invade both types of spheroids 24h after (Figure VI-3).

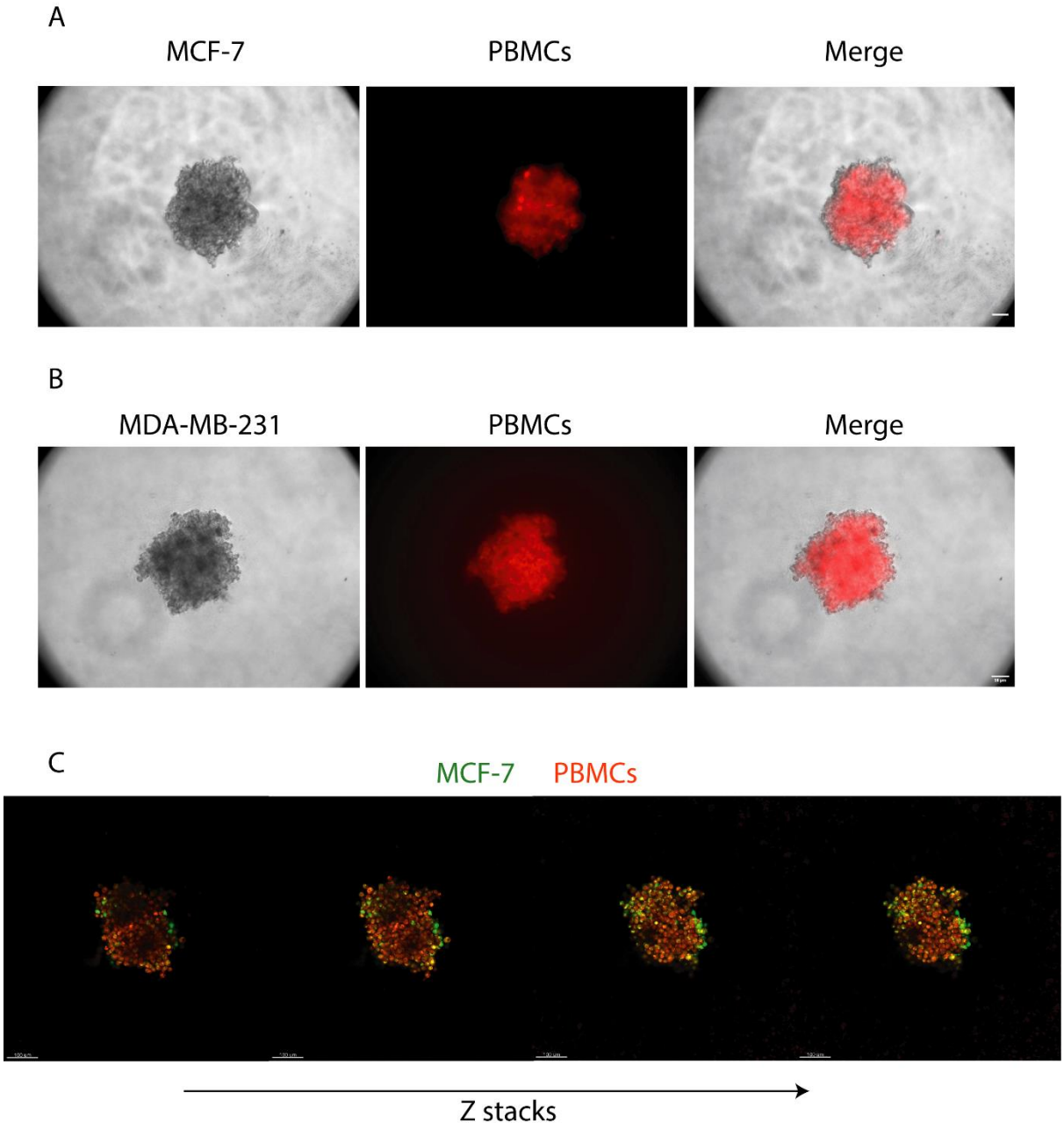


Figure VI-3 **Patient-derived immune cells are able to infiltrate 3D spheroids of breast cancer cell lines**. **(A)** Bright field of MCF-7 spheroid (left panel), patient-derived PBMCs stained in red with a cell tracer dye (middle panel) and the two photos merged (right panel), at day 4 of the culture (24h after the addition of the PBMCs); scale bar 50 μm. **(B)** Bright field of MDA-MB-231 spheroid (left panel), patient-derived PBMCs stained in red with a cell tracer dye (middle panel) and the two photos merged (right panel), at day 7 of the culture (24h after the addition of the PBMCs); scale bar 50 μm. **(C)** Confocal images of 3D spheroids of MCF-7 cell line (stained in green with CFSE) and patient-derived PBMCs (stained in red with a cell tracer dye), at day 4 of culture (24h after the addition of the PBMCs). Stills were acquired for different Z focal planes, to demonstrate the immune infiltration in a 3D structure; scale bar 100 μm.

2. PBMCs from NACT-responders exhibited anti-tumor activity, contrarily to PBMCs from NACT non-responders

Patient-derived PBMCs were added at day 3 for MCF-7 spheroids and at day 6 for MDA-MB-231 spheroids since at these time points the cell line spheroids were completely formed. The co-culture was maintained for 4 more days. To assess the effect of the addition of the patient-derived PBMCs to the 3D spheroids we analyzed the changes in spheroid area, by imaging and analyzing in Fiji software the spheroid area (determined by applying an automatic threshold to the image, Figure VI-4).

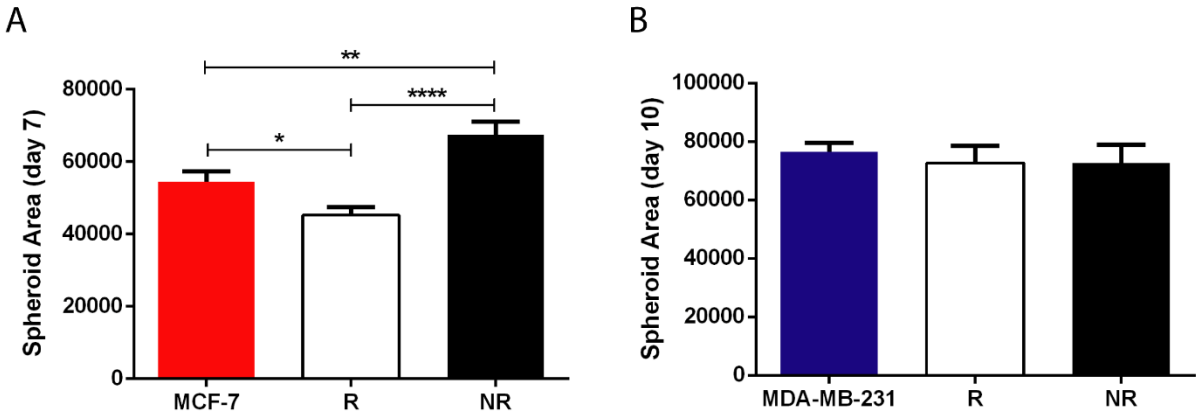


Figure VI-4 **MCF-7 spheroid area decreases with the addition of NACT-responders' PBMCS. (A)** Spheroid area of MCF-7 cells in monoculture (red bar, n=18), in co-culture with NACT-responders PBMCs (R, white bar, n=18) and in co-culture with NACT non-responders PBMCs (NR, black bar, n=18) at day 7 of the culture. **(B)** Spheroid area of MDA-MB-231 cells in monoculture (blue bar, n=12), in co-culture with NACT-responders PBMCs (R, white bar, n=12) and in co-culture with NACT non-responders PBMCs (NR, black bar, n=12) at day 10 of the culture. Data is represented as mean ± SD. *p<0.05, **p<0.01, ****p<0.0001.

Interestingly, we observed that the addition of patient-derived PBMCs from NACT-responders decreased the area of the MCF-7 spheroids (p=0.04), suggesting a potential effect of the patients' immune cells on the viability and/or proliferation of this breast cancer cell line (Figure VI-4A). On the other hand, the addition of patient-derived PBMCs from NACT non-responders increased the area of the MCF-7 spheroids (p=0.003, Figure VI-4A). This observation could be explained by the fact that NACT non-responders' immune cells are unable, by themselves, to deploy an effect on the breast cancer cell line, and the cell line is capable to continue proliferating. In the case of MDA-MB-231 spheroids, no effect on the area of these structures was observed, either in the presence of NACT-responders or non-responders' PBMCs (Figure VI-4B).

To assess if there is an effect of the PBMCs addition on the viability of the cell lines, we next quantified the viability of MCF-7 and MDA-MB-231 at the end of the culture, using a live/dead

dye and analyzing by flow cytometry (Figure VI-5). As expected based on the area analysis, in the co-culture of MCF-7 with NACT-responders' PBMCs, the viability of the cell line is decreased (p=0.03), highlighting the cytotoxic activity of the immune cells present in the blood of breast cancer patients with response to NACT (Figure VI-5A). Contrarily, the addition of NACT non-responders' PBMCs has no effect on the viability of the MCF-7 cell line, demonstrating that the immune cells from the blood of breast cancer patients with no response to NACT have less cytotoxic capacities and potentially more tumor-protective abilities (Figure VI-5A).

Although no effect on the MDA-MB-231 spheroid area was observed when NACT-responders' PBMCs were added, there is a significant reduction on the viability of this cell line (p=0.03, Figure VI-5B), similarly to MCF-7, confirming that immune cells from the blood of NACT-responders indeed possess cytotoxic activity, independently of the breast cancer cells present in the co-culture. Moreover, the addition of NACT non-responders' PBMCs had no effect on the viability of MDA-MB-231 cells, emphasizing that these PBMCs should have a more tumor-protective phenotype.

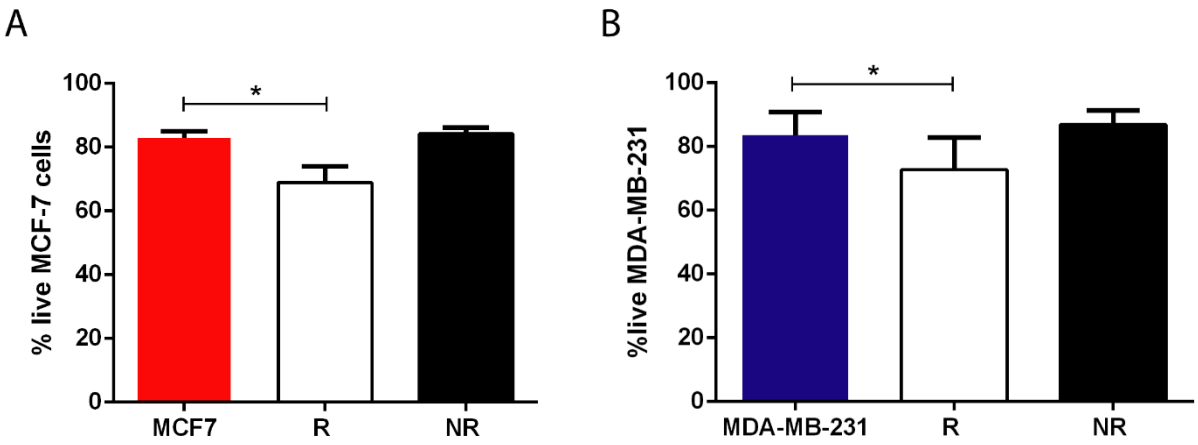


Figure VI-5 **NACT-responders' PBMCs are able to reduce the viability of the breast cancer cell lines in a 3D conformation**. **(A)** Percentage of live MCF-7 cells in monoculture (red bar, n=10), in co-culture with NACT-responders' PBMCs (R, white bar, n=6) and in co-culture with NACT non-responders' PBMCs (NR, black bar, n=5), at day 7 of the culture. **(B)** Percentage of live MDA-MB-231 cells in monoculture (blue bar, n=11), in co-culture with NACT-responders' PBMCs (R, white bar, n=6) and in co-culture with NACT non-responders' PBMCs (NR, black bar, n=6) at the day 10 of the culture. Data are represented as mean ± SD. *p<0.05.

Moreover, here we demonstrate that the established allogenic co-cultures of tumor cell spheroids and patients' immune cells are a powerful tool to study *ex vivo* anti-tumor immune responses. Indeed, we were able to generate a precise and reproducible co-culture setting and a reliable readout based on assessing the spheroid area and tumor cells' viability at the end of the co-culture (Saraiva *et al*, manuscript in preparation).

3. HLA-DR+ cytotoxic T cells have the capacity to reduce the viability of tumor cells

To this point, the experiments with the 3D system were performed with the total PBMCs isolated from the patients' blood. Since we have observed that HLA-DR+ CTLs, mainly present in NACT-responders (section 3 of Chapter IV), have a cytotoxic profile, due to the production of cytotoxic-related molecules, namely Granzyme B, IFN-γ and Perforin (section 3 of Chapter V), we hypothesized that HLA-DR+ CTLs were the cell type responsible for the above observed reduction of cancer cells' viability. So, to further tackle this question, we developed an experiment to specifically evaluate the cytotoxic ability of HLA-DR+ CTLs, compared with HLA-DR negative CTLs, taking advantage of the established 3D co-culture protocol of breast cancer cell lines and allogenic PBMCs.

Particularly in this experiment, we opted for PBMCs isolated from buffy coats of healthy donors instead of using patient-derived PBMCs, due to the limitation of the number of cells in these samples. Therefore, we isolated PBMCs from the buffy coats of healthy donors, stimulated them *in vitro* to obtain activated CTLs and then sorted (by fluorescence activated cell sorting) HLA-DR+ CTLs, HLA-DR negative CTLs and the other PBMCs (except CTLs). This last population comprises antigen presenting cells (monocytes and B cells) and CD4+ T cells and will be labeled herein as CTLs negative fraction. With these sorted populations we could test specifically the effect of HLA-DR+ (relatively to HLA-DR negative CTLs) in the viability of the MCF-7 cells, and confirm their cytotoxic capacity. Moreover, with the addition of the CTLs negative fraction, which includes antigen presenting cells, we could analyze if antigen presentation of tumor antigens is necessary to deploy a cytotoxic response or if HLA-DR+ CTLs were sufficiently activated and capable by themselves to eliminate the allogenic tumor cells.

In summary, we performed the co-culture of MCF-7 cells in the following conditions:

MCF-7 with i) HLA-DR+ CTLs;

ii) HLA-DR negative CTLs;

iii) CTLs negative fraction;

iv) HLA-DR+ CTLs and the CTLs negative fraction;

v) HLA-DR negative CTLs and the CTLs negative fraction.

After the addition of these populations at day 3, the co-culture was maintained for another 4 days, after which the spheroids were collected and their viability analyzed by flow cytometry (Figure VI-6A). Interestingly, the addition of HLA-DR+ CTLs to the MCF-7 spheroid (condition i) reduced significantly their viability ($p=0.01$), contrarily to the addition of HLA-DR negative CTLs (condition ii), which did not affect the MCF-7 viability, suggesting that HLA-DR+ CTLs indeed possess cytotoxic abilities. The addition of the CTLs negative fraction (condition iii) also did not affect the viability of the cell line. However, when the CTLs negative fraction was added in combination with HLA-DR+ CTLs (condition iv) or with HLA-DR negative CTLs (condition v), the

viability of the tumor cells reduced significantly (p=0.03 and p=0.01, respectively). Thus, although HLA-DR negative CTLs by themselves have no cytotoxic capacity, it seems that the presence of antigen presenting cells and CD4+ T cells in the co-culture, allow their activation, leading to higher cytotoxic profile and the capacity to reduce the viability of the tumor cells.

In conclusion, HLA-DR+ CTLs are already reactive and have cytotoxic capacity without the contribution of antigen presenting cells and CD4+ T cells. HLA-DR negative CTLs do not have this cytotoxic capacity, but this property can be reverted if HLA-DR negative CTLs are assisted by other immune cells (Saraiva *et al*, manuscript in preparation).

We also analyzed the viability of the PBMCs in this co-culture assay, since there is a report claiming that HLA-DR+ CTLs have a suppressive role and that are cytotoxic against autologous immune cells (222). In our experiment, even when HLA-DR+ CTLs are in co-culture with the CTLs negative fraction, there is no loss on the viability of these cells (Figure VI-6B), supporting the fact that these cells are reactive T cells with cytotoxicity against tumor cells and not against autologous immune cells.

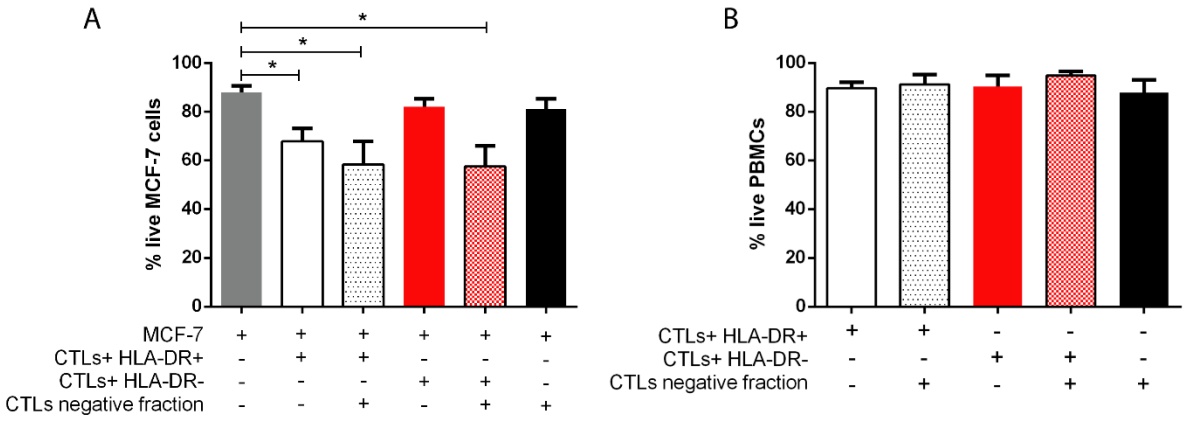


Figure VI-6 **HLA-DR+ CTLs are responsible for the reduction of breast cancer cell lines viability**. **(A)** Viability of MCF-7 spheroids either in monoculture (grey bar, n=5) or in co-culture with sorted immune populations – HLA-DR+ CTLs (white bar, n=8), HLA-DR+ CTLs with the CTLs negative fraction (white dotted bar, n=7), HLA-DR negative CTLs (red bar, n=8), HLA-DR negative CTLs with the CTLs negative fraction (red dotted bar, n=7) and only the CTLs negative fraction (black bar, n=7). **(B)** Viability of the PBMCs in the same culture conditions as in (A). Data are represented as mean ± SEM, *p<0.05.

This experiment corroborates the idea that PBMCs from NACT-responders exhibit cytotoxic properties against tumor cells due to the higher expression of HLA-DR in CTLs. Moreover, in the light of these results, we hypothesized that in NACT non-responders, the tumor microenvironment is immunosuppressive and the tumor cells escape the immune system by releasing immunosuppressive cytokines or by expressing immune checkpoints, for instance. So, in this case, the antigen presenting cells adopt a more tolerogenic profile, impairing the

activation of the CD4+ T cells, and consequently of CTLs (therefore not allowing the increase of their HLA-DR level).

PMBCs from healthy donors, on the other hand, were not in an immunosuppressive environment. Thus, we could obtain, *in vitro*, cytotoxic abilities of sorted HLA-DR negative CTLs in the presence of autologous antigen presenting cells and CD4+ T cells.

4. PBMCs from NACT-responders act synergistically with doxorubicin against tumor cells

After verifying that PBMCs from NACT-responders are able *per se* to decrease the viability of breast cancer cell lines (section 2 of this Chapter), we performed a drug assay on the cancer spheroids with doxorubicin (one of the chemotherapeutic agents used in NACT regimens), in the presence of patients' PBMCs. Our objective with this experiment was to analyze the influence of patients' PBMCs on drug-tumor elimination. Specifically, we intended to determine if the immune cells from NACT-responders' blood had a synergistic effect with the drug and, oppositely, if the immune cells from NACT non-responders' blood were unable to reduce the viability of the breast cancer cells even in the presence of doxorubicin. This would replicate the clinical observations.

Thus, briefly, doxorubicin was added 2 days after the establishment of the co-culture (day 5 for MCF-7 spheroids and day 8 for MDA-MB-231 spheroids) and the co-cultures were maintained for 2 more days (day 7 for MCF-7 spheroids and day 10 for MDA-MB-231 spheroids, Figure VI-7). It is known that the elimination of tumor cells by chemotherapeutic drugs can release tumor antigens to the medium and activate the immune response, in a process called immunogenic cell death. So the doxorubicin concentration used in the 3D culture (0.5 μg/mL for MCF-7 spheroids and 0.1 μg/mL for MDA-MB-231 spheroids) was chosen after several optimization steps, so that we could observe an effect of the drug on the viability of the breast cancer cell lines without compromising considerably the viability of the patient-derived PBMCs.

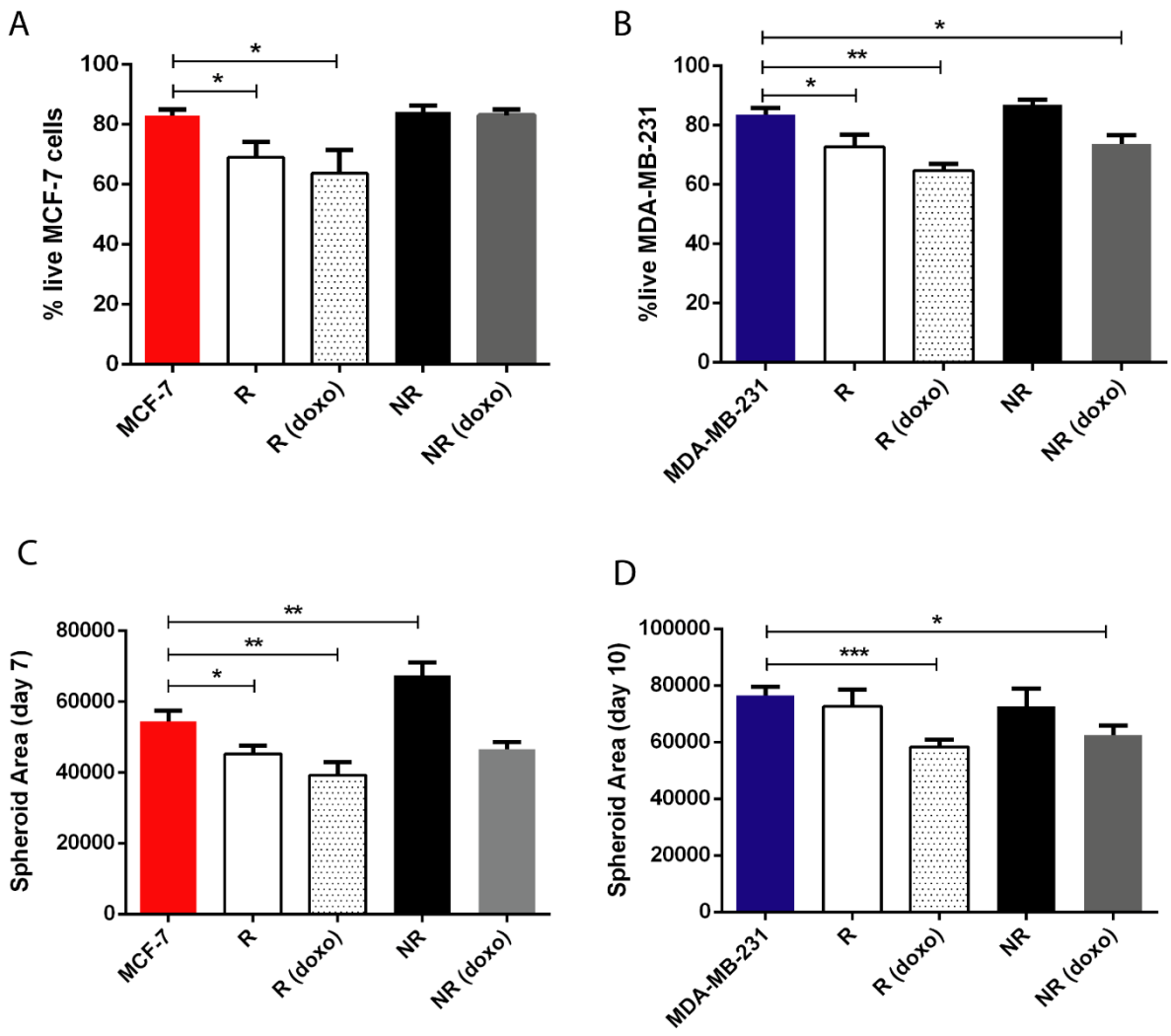


Figure VI-7 **NACT-responders' PBMCs reduce the viability of the breast cancer cell lines and the addition of doxorubicin potentiates this effect**. **(A)** Percentage of live MCF-7 cells in monoculture (MCF-7, red bar, n=10), in co-culture with NACT-responders' PBMCs without the addition of doxorubicin (R, white bar, n=6), and with the addition of doxorubicin (R(doxo), white dotted bar, n=5), in co-culture with NACT non-responders' PBMCs (NR, black bar, n=5) and with the addition of doxorubicin (NR(doxo), grey bar, n=5), at day 7 of culture. **(B)** Percentage of live MDA-MB-231 cells in the same conditions as in (A) (n=11 for MDA-MB-231, n=6 for R, n=4 for R(doxo), n=6 for NR and n=4 for NR(doxo)) at day 10 of culture. **(C)** Spheroid area of the different conditions for MCF-7 spheroids (n=18 per condition) at day 7 of culture. **(D)** Spheroid area of the different conditions for MDA-MB-231 spheroids (n=12 per condition), at day 10 of culture. Data is represented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

As previously observed, the addition of NACT-responders' PBMCs to either MCF-7 or MDA-MB-231 spheroids has an impact on the viability of the breast cancer cells (p=0.03, Figure VI-7A-B). NACT-responders' PBMCs in the presence of doxorubicin also decreased the viability of both cell lines, having an obvious synergistic effect, more pronounced for MDA-MB-231 spheroids (p=0.03, p=0.002, respectively, Figure VI-7A-B). For MCF-7 spheroids, the addition of NACT non-responders' PBMCs even in the presence of doxorubicin has no effect on the

viability of the breast cancer cells (Figure VI-7A), highlighting the fact that immune cells isolated from non-responder patients are immunosuppressed. The viability of MDA-MB-231 spheroids suffered a reduction when NACT non-responders' PBMCs were added in the presence of doxorubicin (p=0.03); although no obvious reduction was observed without doxorubicin (Figure VI-7B). So, in this case, the loss of viability of the cancer cells is related with the effect of the doxorubicin and not with the cytotoxic activity of the immune cells, probably because MDA-MB-231 cell line is less resistant to chemotherapeutic agents when compared to MCF-7, especially in a 3D conformation (223). The addition of doxorubicin to the cell lines (without the co-culture with patient-derived PBMCs) showed a small decrease in their viability, although not significantly (data not shown).

The spheroid area was also quantified for the different culture conditions (Figure VI-7C-D). For MCF-7 spheroids, both the addition of NACT-responders' PBMCs with or without doxorubicin decrease the spheroid area (p=0.004 and p=0.04, respectively, Figure VI-7C). On the other hand, the addition of NACT non-responders' PBMCs increased the spheroid area (p=0.003), while the addition of doxorubicin to this co-culture had no effect on the spheroid area when compared to the monoculture condition (Figure VI-7C). For MDA-MB-231 spheroids, only the presence of doxorubicin decreased the spheroid area for both co-culture with NACT-responders' PBMCs (p=0.0002) and with NACT non-responders' PBMCs (p=0.02, Figure VI-7D), confirming the viability results.

Overall, we were able to perform a 3D co-culture assay with spheroids of breast cancer cell lines and patient-derived PBMCs. With this platform, we confirmed with an *in vitro* approach that PBMCs isolated from the blood of NACT-responders are cytotoxic and can eliminate tumor cells. This effect is potentiated with the addition of the chemotherapeutic agent. On the other hand, PBMCs isolated from NACT non-responders maintain their immunosuppressed phenotype and are unable to execute cytotoxic functions against tumor cells even in the presence of doxorubicin.

Taking in consideration that PBMCs from NACT-responders have CTLs with higher levels of HLA-DR than PBMCs from NACT non-responders, these *in vitro* assays helped to understand the role of these cells in the success of NACT treatment. Additionally, as the results here presented mirrored the clinical observations (HLA-DR+ CTLs at the biopsy are essential for the chemotherapeutic success), these experiments also validate the potential use of this immune feature as a biomarker of patients response to chemotherapy (Saraiva *et al*, manuscript in preparation).

5. PBMCs from NACT non-responders are able to act against tumor cells if previously stimulated

Since the difference observed between patients with response to NACT and without response to this treatment relies on the presence of activated CTLs (higher levels of HLA-DR, section 3 of Chapter IV), we asked if the CTLs of NACT non-responders' PBMCs could be activated *ex vivo* (as previously demonstrated in section 1 of Chapter V), therefore increasing their HLA-DR expression, and potentially reverting their immunosuppressed phenotype.

To achieve this aim we have stimulated patients' PBMCs with the canonical stimulus of PMA and ionomycin. PMA is able to diffuse through the cell membrane and activate protein kinase C; whereas ionomycin is a calcium ionophore and triggers the intracellular release of calcium. The stimulation with these compounds is receptor-independent and leads to the activation of several signaling pathways, likely resulting in T cell activation and production of several inflammatory cytokines.

However, the stimulation with PMA/ionomycin is non-specific and can boost a general immune response. Thus, to have a targeted T cell stimulation, similar to a specific antigen stimulation, we used a T cell receptor (TCR) stimulus. Namely, we used anti-CD3 (5 μg/mL) and anti-CD28 (1 μg/mL) antibodies with a crosslinking agent (anti-mouse IgG1, 5 μg/mL) that allow the stimulation of the T cells alone. This specific stimulation could then be used in future experiments envisioning a potential clinical use.

So, PBMCs from NACT-responders and non-responders, previously stimulated as above referred, were added to the 3D spheroids, similarly to the protocol mentioned in section 2 of this Chapter.

First, we quantified the percentage of HLA-DR+ CTLs that we could obtain in patients' PBMCs after stimulation. We observed that with both methods, the percentage of HLA-DR+ CTLs increased, i.e. CTLs became activated not only in NACT-responders but also in non-responders' PBMCs. However, TCR engagement led to a slightly higher percentage of HLA-DR+ CTLs when compared to PMA/ionomycin stimulation (Figure VI-8A).

In MCF-7 spheroids, the addition of NACT-responders' PBMCs previously stimulated with PMA/ionomycin, significantly decreases the viability of the tumor cells ($p=0.004$). Additionally, when doxorubicin was also used, the percentage of live MCF-7 cells decreased even more ($p<0.0001$), corroborating the synergistic effect of NACT-responders' PBMCs with the chemotherapeutic agent (Figure VI-8B).

Remarkably, NACT non-responders' PBMCs were able to transform their previously suppressed phenotype in an activated one, when stimulated with PMA/ionomycin, therefore becoming cytotoxic and able to decrease the viability of MCF-7 cancer cells ($p<0.0001$ when compared to the condition without stimulation or doxorubicin). When both stimulation and

doxorubicin were employed, the viability of MCF-7 cells reduced even further ($p<0.0001$), similar to the effect of NACT-responders' PBMCs (Figure VI-8B).

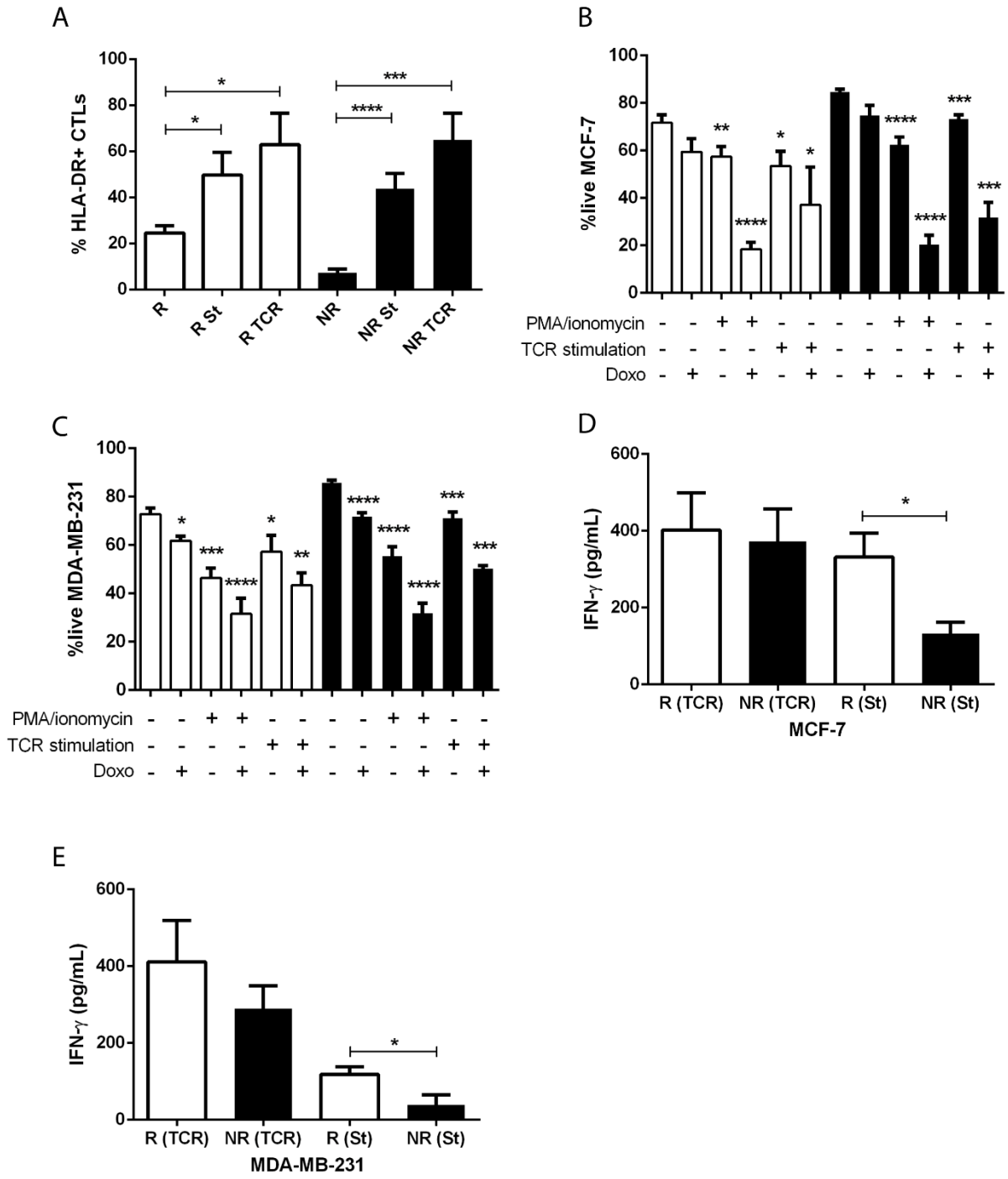


Figure VI-8 **NACT non-responders' PBMCs previously stimulated with PMA/ionomyccin or by TCR engagement are capable of reducing the breast cancer cell lines' viability. (A)** Percentage of HLA-DR+ cytotoxic T cells (CTLs) in PBMCs of NACT-responders (R, white bar, n=12), NACT-responders with a canonical stimulation of PMA/ionomycin (R St, white bar, n=10), NACT-responders with TCR stimulation (R TCR, white bar, n=4), NACT non-responders (NR, black bar, n=11), NACT non-responders stimulated with PMA/ionomycin (NR St, black bar, n=12) and NACT non-responders with TCR stimulation (NR TCR, black bar, n=6). **(B)** Viability of MCF-7 cells in co-culture with NACT-responders' PBMCs (R, white bars, n=4-11) or with NACT non-responders' PBMCs (NR, black bars, n=6-11) in the

presence/absence of doxorubicin (Doxo), with or without previous canonical stimulation (PMA/ionomycin) and with or without previous TCR stimulation. **(C)** Viability of MDA-MB-231 cells in co-culture with NACT-responders' PBMCs (R, white bars, n=4-10) or with NACT non-responders' PBMCs (NR, black bars, n=6-12) in the presence/absence of doxorubicin (Doxo), with or without previous canonical stimulation (PMA/ionomycin) and with or without previous TCR stimulation. **(D)** IFN-γ production of stimulated NACT-responders' PBMCs with PMA/ionomycin (R (St), white bar, n=11) or with TCR stimulation (R (TCR), white bar, n=4); and stimulated NACT non-responders' PBMCs with PMA/ionomycin (NR (St), black bar, n=12) or with TCR stimulation (NR (TCR), black bar, n=6) after 4 days of co-culture with MCF-7 cells. **(E)** IFN-γ production of stimulated NACT-responders' PBMCs with PMA/ionomycin (R (St), white bar, n=6) or with TCR stimulation (R (TCR), white bar, n=4); and stimulated NACT non-responders' PBMCs with PMA/ionomycin (NR (St), black bar, n=9) or with TCR stimulation (NR (TCR), black bar, n=6) after 4 days of co-culture with MDA-MB-231 cells. Data is represented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

When PBMCs either from NACT-responders or non-responders were TCR-stimulated, results were similar to the ones abovementioned. Namely, NACT-responders and non-responders' PBMCs reduced the viability of MCF-7 cells (p=0.01 and p=0.0003, respectively, Figure VI-8B). The addition of doxorubicin had a synergistic effect with the TCR stimulation for NACT-responders' PBMCs and especially for non-responders' PBMCs (p=0.049 and p=0.0002, respectively, Figure VI-8B).

Regarding 3D co-culture systems with MDA-MB-231 cell line, similar results were obtained (Figure VI-8C). Namely, PMA/ionomycin stimulated PBMCs, either from NACT-responders or non-responders, decreased significantly the percentage of viable cancer cells, compared to unstimulated/no doxorubicin condition (p=0.0002 and p<0.0001, respectively, Figure VI-8C). As previously reported, the addition of the chemotherapeutic agent (doxorubicin) to the stimulated PBMCs further improved the anti-tumor effect of these cells (p<0.0001 for co-culture with NACT-responders and non-responders, Figure VI-8C), corroborating, also in this cell line, the synergistic effect between activated immune cells and doxorubicin.

With the TCR stimulation we could obtain the same results. Actually, co-culture with NACT-responders or non-responders' PBMCs led to a significant reduction of the breast cancer cell line viability (p=0.049, p=0.0001, respectively, Figure VI-8C). Again, the addition of doxorubicin proved the synergistic effect with stimulated PBMCs from both groups of patients, in reducing the viability of cancer cells (p=0.002 for co-culture with NACT-responders' PBMCs and p=0.0001 for co-culture with NACT non-responders' PBMCs, Figure VI-8C).

An additional readout of the stimulation of the PBMCs and, consequently increased activated CTLs (higher percentage of HLA-DR+ CTLs), is their ability to release IFN-γ. Hence, we have quantified this molecule in the supernatant of the MCF-7 and MDA-MB-231 3D co-cultures (Figure VI-8D-E). As expected, stimulated PBMCs (with PMA/ionomycin or by TCR engagement) from either NACT-responders or non-responders produce IFN-γ, in both cell lines co-culture.

However, the PBMCs from NACT-responders stimulated with PMA/ionomycin are able to release higher levels of IFN-γ to the medium when compared to NACT non-responders' PBMCs with the same stimulation method (p=0.01 for MCF-7 spheroids and for MDA-MB-231 spheroids, Figure VI-8D-E), showcasing the hardwired capacity of PBMCs derived from NACT-responders' patients to become highly activated. The same was not observed for PBMCs stimulated by TCR engagement. Actually, there was no difference in the IFN-γ detected in the 3D culture with NACT-responders' PBMCs or non-responders' PBMCs with TCR stimulation for either co-culture with MCF-7 (Figure VI-8D) or MDA-MB-231 cell lines (Figure VI-8E). This result further illustrates that TCR engagement is sufficient to activate equivalently the PBMCs from both NACT-responders and non-responders.

When comparing both stimulation methods, we concluded that not only the TCR engagement is able to increase the percentage of HLA-DR+ CTLs in PBMCs derived either from NACT-responders and non-responders' blood (Figure VI-8A), but also leads to a higher production of IFN-γ when PBMCs from both groups of patients are in co-culture with breast cancer cell lines (Figure VI-8D-E). Nevertheless, stimulation by TCR engagement of NACT non-responders' PBMCs resulted in slightly less reduction of the tumor cells' viability, when compared to PMA/ionomycin stimulated PBMCs

Taken together, the results obtained in this section highlight the fact that it is possible to effectively stimulate CTLs and boost their anti-tumor activity, even in CTLs isolated from the blood of NACT non-responders, which present a higher immunosuppressed phenotype. This suggests that the stimulation of the immune cells could be a potential therapeutic strategy to be given in combination with NACT agents, particularly for non-responder breast cancer patients and ameliorate the treatment of conventional chemotherapy (Saraiva *et al*, manuscript in preparation).

6. Immune checkpoint blockade combined with doxorubicin is not sufficient to revert the immunosuppressed phenotype of NACT non-responders' PBMCs

As previously observed, we were able to increase the immune response/tumor cell elimination of NACT non-responders' PBMCs with the PMA/ionomycin stimulation and by TCR engagement. Nevertheless, in a search for a therapeutic strategy already utilized in a clinical setting, we used immune checkpoint inhibitors, such as anti-PD-1 and anti-PD-L1. Although this approach is still not used in early breast cancer (it is only accepted for metastatic TNBC), it is widely administered in lung cancer and melanoma patients, for instance. It is known that tumor cells are able to escape the immune system and can employ the immune checkpoints to dampen CTLs cytotoxic function. Briefly, tumor cells can express PD-L1 which binds to PD-1 present in the surface of CTLs. Considering that CTLs from NACT non-responder patients are

likely to be more affected by the inhibitory immune checkpoints, our rationale was to use antibodies against either PD-1 or PD-L1 to impede the suppression on their CTLs, potentially provoked by the tumor cells, and consequently increasing the activation level of these cells (Figure VI-9).

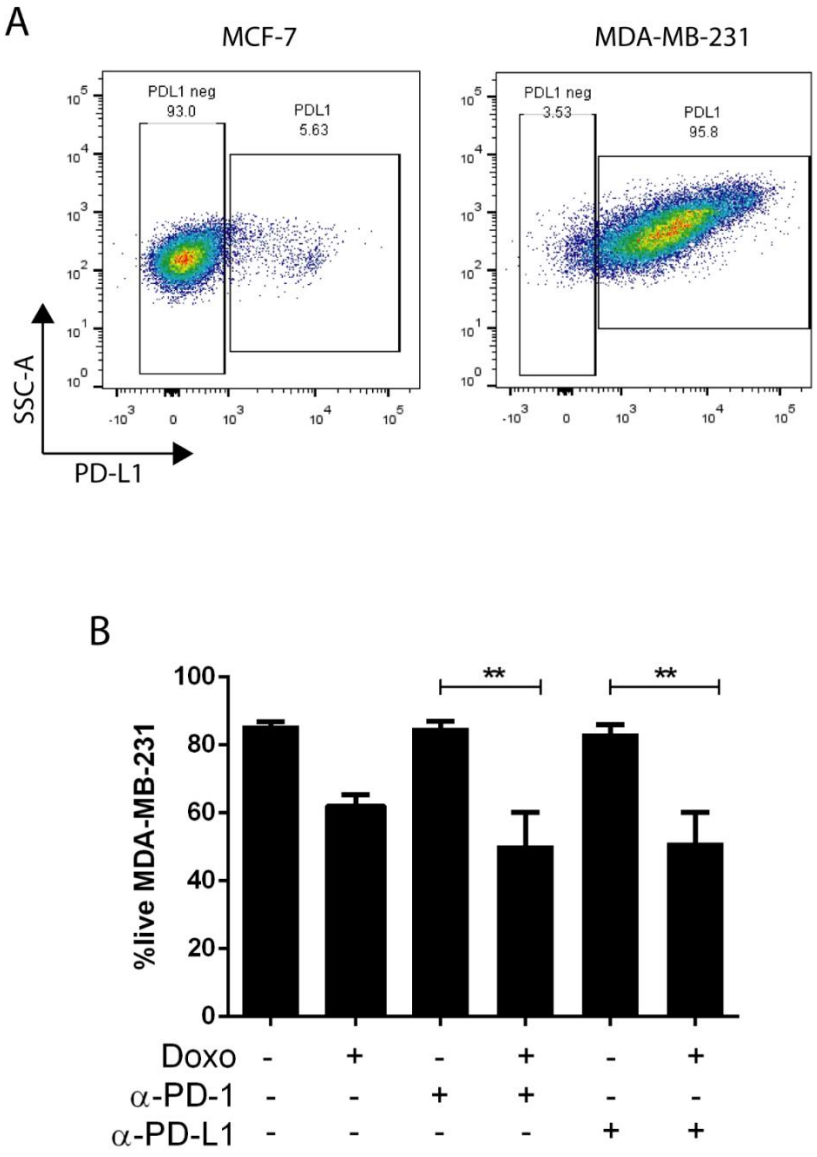


Figure VI-9 **Immune checkpoint blockade with anti-PD-1 and anti-PD-L1 is not sufficient to release the immunosuppressive phenotype of NACT non-responders' PBMCs to be able to reduce the viability of breast cancer cells**. **(A)** Representation of flow cytometry analysis of PD-L1 expression on both breast cancer cell lines – MCF-7 and MDA-MB-231. **(B)** Viability of MDA-MB-231 cells in co-culture with NACT non-responders' PBMCs (NR, black bars, n=6) in the presence of different combinations between doxorubicin, anti-PD-1 and anti-PD-L1. Data is represented as mean ± SEM, **p<0.01.

First of all, we analyzed the expression of PD-L1 in the breast cancer cell lines used previously – MCF-7 and MDA-MB-231 (Figure VI-9A). Although both were positive for PD-L1, only

approximately 5% of MCF-7 cells were positive for this surface marker, whereas more than 95% of MDA-MB-231 were PD-L1 positive (Figure VI-9A). Thus, we decided to use immune checkpoint blockade only in this cell line. Moreover, due to the need to search for alternative strategies especially for NACT non-responder patients, for this experiment we decided to focus only in the PBMCs isolated from these patients to perform the 3D co-culture. We used both antibodies (anti-PD-1 and –PD-L1) alone or in combination with doxorubicin in the same platform as previously explained.

In MDA-MB-231 spheroids (Figure VI-9B), the percentage of viable cells did not reduce when the blocking antibodies alone were added to the co-culture. With the addition of doxorubicin in combination with anti-PD-1 or PD-L1, NACT non-responders' PBMCs were capable to decrease the tumor cells' viability (Figure VI-9B). Nevertheless, although the cell line used was PD-L1 positive, the potential blockade of PD-1/PD-L1 axis *per se* did not impact the viability of the cancer cells.

Moreover, as an alternative readout of the possible CTLs' activity, due to the release of the immunosuppressive brakes possibly employed by immune checkpoints, we also analyzed IFN-γ levels in the supernatant of the 3D co-culture in this experiment. However, no visible IFN-γ secretion was detected in the co-culture of MDA-MB-231 cells with NACT non-responders' PBMCs in the presence of immune checkpoint inhibitors, corroborating that the blockade of PD-1/PD-L1 axis did not contribute to improve of CTLs' function.

The obtained results could be explained by the fact that all the CTLs isolated from the NACT non-responders' PBMCs used in these assays had a low percentage of PD-1 at their surface. Actually, approximately 5% of the CTLs were PD-1 positive, which could not be sufficient to be influenced by the immune checkpoints' blockade here tested (Figure VI-10).

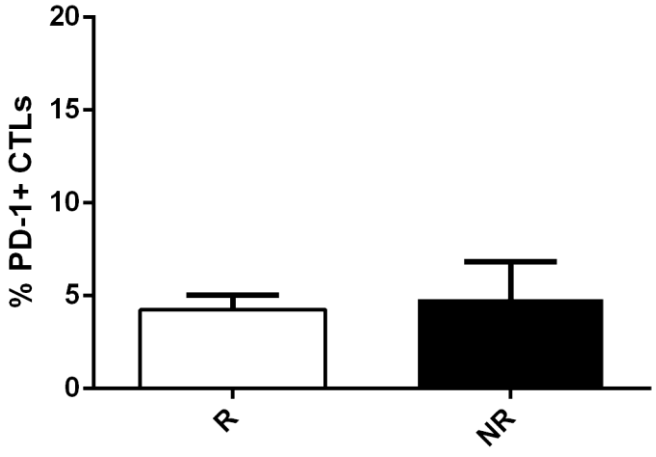


Figure VI-10 **PD-1 positive cytotoxic T cells (CTLs) are scarce in immune cells isolated from patients' blood**. Percentage of PD-1 positive CTLs in PBMCs from NACT-responders (R, white bar, n=4) and from NACT non-responders' PBMCs (NR, black bar, n=4). Data is represented as mean ± SEM.

Nevertheless, we have to take into account that at least some breast cancer patients could have increased PD-1 expression in their tumor infiltrating CTLs (even if not in circulating CTLs). Therefore, we cannot exclude that immune checkpoint blockade, alone or in combination with chemotherapeutic agents, could be advantageous in particular cases.

In addition, we demonstrated that the 3D model here establish is also a remarkable tool to test several immunomodulatory drugs or antibody combinations, in a throughput manner. In fact, breast cancer patients, with immunosuppressed CTLs, where the suppressing PD-1/PD-L1 axis is absent, might have alternative suppressive mechanisms increased, such as high expression of CTLA4 or Tim3 in T cells. Therefore, we can use this 3D platform to assess the response to other therapeutic strategies, namely, the combination of chemotherapy and other blocking antibodies, such as the anti-CTLA4, -Tim3 or – Galectin 9 (Tim3 ligand in tumor cells), to name a few.

Altogether, we have demonstrated in this chapter that we are able to recapitulate clinical observations, such as the response to neoadjuvant chemotherapy, with an *in vitro* approach. This platform developed in a 3D structure composed by breast cancer cell lines and patient-derived immune cells (PBMCs) revealed that PBMCs isolated from NACT-responders are cytotoxic against tumor cells; while PBMCs isolated from NACT non-responders did not show this effect. The immunosuppressive effect of NACT non-responders' PBMCs could be reverted by the immune stimulation with PMA/ionomycin and/or by TCR engagement. This platform can be further used for new therapeutic screening assays.

Chapter VII

General Discussion

Chapter VII – General Discussion

Breast cancer is the most common cancer type in women worldwide and accounts for up to 15% of cancer-related deaths in women around the globe (224). Currently, breast cancer patients have approximately an 85 to 99% overall survival (depending on the breast cancer subtype) when the disease is in an early stage (141). When the disease is locally advanced, i.e. tumors with a size of >2 cm and/or with disease extended to the axillary lymph node, the treatment relies on neoadjuvant chemotherapy (NACT), independently on the breast cancer subtype. Although NACT is effective in reducing the size of the primary tumor to allow for more conservative surgery instead of mastectomy, disease down-staging and pathological complete response (pCR) is achieved by less than 50% of the patients (142,149). For instance, from the total of 52 breast cancer patients that performed NACT whose samples we have included in the biomarker study, only 20 patients indeed responded to this treatment, meaning that 62% of the patients received this aggressive treatment for 6 months without any benefit.

As such, there is an urgent clinical need to find new biomarkers that could predict response to NACT and, additionally, to find new and alternative therapies for NACT non-responders, since the alternative first line treatment nowadays is surgery which is not possible in all cases.

Recently, there has been an international effort to find new predictive markers for response to NACT. These markers have been mainly related to immune cell infiltration. Namely, tumor infiltrating lymphocytes (TILs) has been pointed as predictive biomarkers of response in a neoadjuvant setting (152,154–156). However, while some studies use this biomarker for the three subtypes of breast cancer, others only found a positive correlation with response for TNBC patients. Additionally, combining all the lymphocytes for prediction seems counterintuitive since there are effector lymphocytes with cytotoxicity capacity against tumor cells (such as the cytotoxic T cells – CTLs), but also immunosuppressive and tumor-promoting lymphocytes, namely regulatory T cells (Tregs). Another issue is the fact that TILs are assessed by immunohistochemistry and several antibodies can be used to detect TILs. Although a TILs working group was created to standardize TILs assessment (151), there is no translation to a clinical setting and TILs as a predictive factor in breast cancer response to NACT is not yet used.

Another predictive marker that has been studied is the neutrophil to lymphocyte ratio (NLR), analyzed in circulation. Similarly to the TILs, some studies use NLR for the prediction of NACT response for the three subtypes of breast cancer, while others use only for TNBC patients or only ER+ breast cancer patients. Moreover, the cut-off point or the threshold for NLR is controversial and, again, no translation for a clinical setting was yet established (163–169).

Hence, in this thesis, we aimed to uncover new biomarkers that could be used to predict breast cancer patients' response to NACT. To tackle this question, we also focused on the infiltration of the immune cells to the tumor bed, but instead of analyzing all lymphocytes

together, have divided them into different populations and analyzed different activation and/or suppression molecules. Our main hypothesis was that lymphocytes, and especially the anti-tumor CTLs *per se*, could not have a predictive function since their anti-tumor capacity could be suppressed by an impeditive tumor microenvironment. Thus, more than the presence of CTLs, their activation status could be important.

In this thesis we also establish a new platform, using 3D cellular structures, to clarify *in vitro* the clinical results and develop alternative therapeutic strategies for NACT non-responders. To achieve both aims we have used fresh patients' material, from biopsies, surgical specimens and blood.

1. HLA-DR expressing cytotoxic T cells as a biomarker of response to neoadjuvant chemotherapy in breast cancer patients

Considering that HLA-DR-expressing T cells could distinguish breast cancer aggressiveness (see Figure IV-1) but were not different when patients' age, body mass index, and tumor histological grade, dimension and breast cancer subtype were stratified (see Figures IV-3-7), we wanted to investigate whether this marker could also distinguish breast cancer patients' response to NACT. For this, we have evaluated a cohort of 30 patients and analyzed their biopsies before NACT implementation. After NACT we divided the patients into responders and non-responders. Our criteria for this separation was different from the RECIST (Response Evaluation Criteria in Solid Tumors) criteria, which is used in some clinical studies. In RECIST, the response is divided into complete response, partial response, progressive disease and stable disease (225). Complete response is evaluated as no tumor and non-pathological lymph nodes present; partial response comprises tumors with at least 30% reduction in diameter; the progressive disease is used when the tumor has a diameter increase of at least 20% or when new lesions occur; stable disease refers to when no tumor diameter change occurred. Although these criteria were published to standardize how clinicians evaluated response, there are still few clinical trials or biomarker studies that use these criteria, as most of them prefer to use pCR and no-pCR.

In the case of the cohorts evaluated in these thesis, no progressive disease nor stable disease was found. As such, we decided to use a dichotomy of responder and non-responder, instead of dividing in complete or partial response. Actually, since pCR following NACT is difficult to occur (142,149), we decided to join patients who achieved pCR and patients bearing no lymph node metastasis and with a tumor mass less than 10% of the initial mass, which is clinically a significant response to treatment.

As it occurred for the aggressiveness status, the only difference found was for the expression level of HLA-DR in T cells, especially in CTLs. which was higher in NACT-responders when compared to NACT non-responders (see Figure IV-8). Interestingly, we observed that this biomarker segregated NACT-responders from non-responders independently of the breast

cancer subtype, which is an advantage when comparing to other predictive biomarker studies, only focused on TNBC patients. This attention can be explained by the fact that TNBC has no targeted therapy and, as such, there is an effort to find new biomarkers of response and alternative treatments especially for breast cancer patients with this subtype. Nevertheless, we have to take into account that TNBC represents around 15% of all breast cancer cases and that ER+ and HER2+ breast cancer patients are also selected for NACT, frequently without any advantage. Actually, in our first cohort of patients, approximately 60% of NACT non-responders were not TNBC patients.

In order to find a threshold for HLA-DR-expressing CTLs, we performed a ROC curve (Table IV-1). With this analysis, we obtained a threshold of 8.943, value beneath which patients would be NACT non-responders, with high sensitivity (94.12%) and specificity (100%). Of the 30 samples evaluated, only one did not fit the model.

We also analyzed a few post-NACT surgical specimens of non-matched patients. We observed that even after the chemotherapy treatment, patients that responded to the treatment had a higher expression level of HLA-DR in CTLs, contrarily to non-responders. As referred in the introduction, chemotherapeutic agents can modulate the immune response, either by inducing the immunogenic cell death of tumor cells, which will consequently increase anti-tumor specific responses, or by recruiting CTLs and pro-inflammatory myeloid cells and eliminating Tregs. Altogether, chemotherapy should induce activation of CTLs, therefore increasing their HLA-DR level and effector function, contributing to the elimination of tumor cells. Interestingly, here it seems that in NACT non-responders the chemotherapy is not able to boost an anti-tumor immune response, probably due to the enrichment of suppressive mechanisms, evidencing the importance of an immune infiltrate with particular features, i.e high frequency of activated CTLs/poor immunosuppressive mechanisms, to assist NACT treatment. Nevertheless, to truly observe the chemotherapeutic effect on the HLA-DR expression level in CTLs, both biopsies and matched post-NACT surgical specimens (from the same patient) should have been analyzed. However, we could not performed this analysis due to limitations in obtaining these samples.

A biomarker study should follow the REMARK (Reporting recommendations for tumor marker prognostic studies) criteria, since it was an international effort to standardize how biomarker studies should be reported to have less non-translatable biomarkers (196). For that, validation in a second independent cohort and specific statistical evaluation should be performed. A validation, so far with 22 patients, corroborate that HLA-DR-expressing CTLs are able to differentiate between responders and non-responders (see Figure IV-12). Using the threshold value calculated in the ROC curve of the first cohort, only 4 patients did not fit in the parameters, by a short margin. As such, we calculated a new ROC curve to assess the differences in this model, and the threshold was different by only 3 decimal points (8.943 and 8.63, see Table IV-2).

Moreover, by grouping all the pre-NACT analysis (30 from the initial cohort and 22 from the validation cohort), we assure that HLA-DR-expressing CTLs is a strong factor for response prediction, since it is the only statistical significant marker by univariable analysis (see Table IV-3) and it is an independent predictive factor by multivariable analysis (see Table IV-4 and Figure IV-13). This means that even when taking into account patients' age, breast cancer subtype and other tumor immune-related markers, HLA-DR-expressing CTLs will be a clinically relevant predictive biomarker. Therefore, even with a small sample population size we were able to create a predictive statistical model that allow us to infer *a priori* if a patient will benefit or not from NACT (Figure IV-14).

Although HLA-DR expression level in CTLs is a validated and independent predictive factor for the response to NACT, this treatment is administered only to approximately 20-25% of the breast cancer patients in the hospitals with whom we collaborate. Hence, a prognostic marker that correlates with survival, should also be considered to embrace all breast cancer patients, independently on the treatment implemented. As such, we did an analysis with patients that undergone NACT and patients that first undergone surgery followed by adjuvant chemotherapy and performed a 3 years follow-up (see Figures IV-15 and 16). Intriguingly, patients with an HLA-DR expression level above the threshold calculated in the ROC curve of the first cohort show a higher progression-free survival. Nonetheless, for significant results, more patients should be followed-up. Additionally, no differences were found for overall survival. This last result can be explained by the fact that overall survival in early breast cancer patients is very high, accounting for approximately 95% of patients in the first years following diagnosis (141,224). For a more conclusive result, patients should be followed for 5-10 years. In conclusion, HLA-DR expression level in CTLs besides being predictive for NACT response, it also showed a tendency to be prognostic (correlated with progression-free survival) for breast cancer patients where NACT or surgery followed by adjuvant chemotherapy was administered.

HLA-DR is an antigen presenting molecule expressed at high levels on professional antigen presenting cells, but its expression on effector T lymphocytes upon their activation has also been intensively described in some diseases, such as auto-immune diseases and viral infections (176,177). The increase of HLA-DR at CTLs' surface, upon stimulation, could also be required to boost the anti-tumor immune response. Indeed, HLA-DR+ CTLs were found to have the machinery needed for antigen processing and loading on HLA-DR molecules and, additionally, could express CD86 and CD80, which are the co-stimulatory molecules of antigen presenting cells that are necessary for the proper T cell effector function (37). Moreover, it was described that T cell-T cell synapsis occurs to allow T cells to secrete IFN-γ towards each other, compelling the differentiation of more protective T cells (76). These T cell-T cell interactions and mutual antigen presentation can be essential for mounting a suitable anti-tumor response.

HLA-DR molecule, by itself, assessed in tumor cells, has been reported to serve as a favorable prognostic marker for other types of cancers, such as colorectal carcinoma (226) or glioma

(227). It was even shown that HLA-DR+ melanoma cells predict the response to anti-PD-1/PD-L1 immunotherapy (228). However, to our knowledge, the expression of HLA-DR in CTLs, besides its extensive application in the study of viral infections and chronic inflammatory diseases, was never recommended as a biomarker in cancer.

The fact that not all tumor infiltrating CTLs acquire HLA-DR in a process of T cell activation, despite being in the presence of tumor cells, can be multifactorial. As previously stated, tumors have the capacity to escape the immune system. This escape can be performed, for instance, by the release of anti-inflammatory cytokines (such as IL-10 or TGF-β), or by the activation of immune checkpoints, such as the PD-1/PD-L1 axis, both will prevent the cytotoxicity function of CTLs (Figure VII-1). When these escape mechanisms are not yet engaged, CTLs can become activated when they recognize an antigen, and produce effector molecules such as Granzyme B, leading to tumor cell elimination (Figure VII-1).

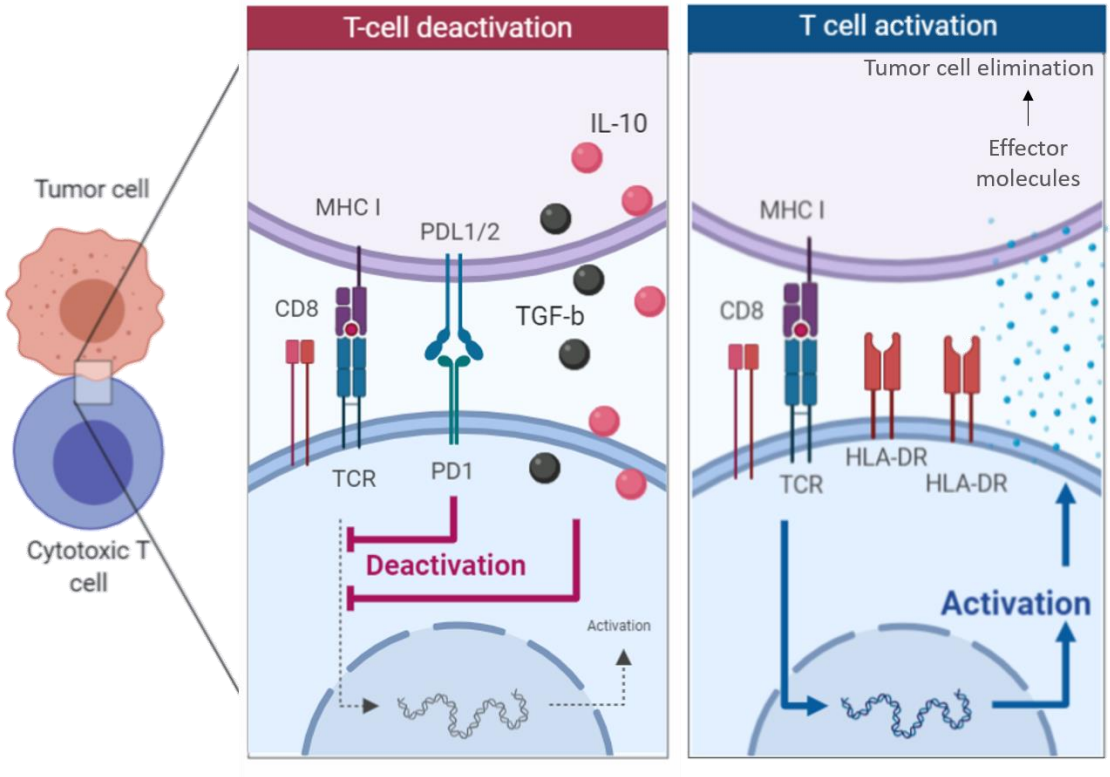


Figure VII-1 **Mechanisms of CTLs' activation and deactivation when encountering a tumor cell.** Deactivation or suppression can be induced by escape mechanisms of tumor cells, such as the production of anti-inflammatory cytokines (IL-10 and TGF-β) or the activation of immune checkpoints, such as PD-L1. T cell activation occurs when T cells recognize an antigen and these inhibitory mechanisms are not released. Activation leads to an increase in HLA-DR production and the release of effector cytotoxicity molecules that will eliminate tumor cells. Figure performed in Biorender.

Actually, we observed that HLA-DR expression level in CTLs negatively correlated with immunosuppressive and pro-tumor molecules released by tumor cells, such as TGF-β, PD-L1, IL-6, IL-8, IL-1β, and IL-23/IL-12 (see Figure IV-17). As stated in the introduction, tumor cells

have the capacity to produce all these cytokines to help in tumor-promoting actions, such as the production of metalloproteinases (which are enzymes important for extracellular matrix degradation and consequent tumor invasion), angiogenesis (to induce tumor growth with the arrival of more nutrients) and activation of immunosuppressive cells, such as M2 macrophages or Tregs that will prevent CTLs cytotoxicity function.

As such, we may say that the HLA-DR expression in CTLs is a reflection and a consequence of the general tumor immune status. Indeed, an immunosuppressed environment will be unable to stimulate an appropriate immune response and should not give rise to high levels of HLA-DR in CTLs. As cell-to-cell contact and tumor-specific antigen presentation by antigen presenting molecules are required to elicit a specific cytotoxic T cell response against the tumor cells, we may infer that, *in vivo*, the antigens presented by tumor cells, alongside with the soluble factors, contribute to CTLs' effector function.

Altogether, the results from Chapter IV respond to our main objective – to find a new biomarker that could predict breast cancer patients' response to NACT. To highlight the importance of this immune trace – HLA-DR-expressing CTLs - and how it can differentiate between responders and non-responders, we elaborated the scheme of Figure VII-2.

When comparing HLA-DR-expressing CTLs to other biomarkers, such as TILs or the NLR it has several advantages. The drawbacks of TILs and NLR are (152,154–156), (163–169):

- their effectiveness is mainly on one subtype of breast cancer (TNBC);
- the fact that they are represented as a single population of cells, when in fact they encompass distinct immune cell populations with different functions, for instance, pro and anti-tumor T cells;
- analysis based on immunohistochemistry (TILs) or blood sampling (NLR);
- non-reproducible technical issues to assess the biomarkers – different antibodies for TILs or different cut-off points for NLR;
- the fact that some studies claim that they are predictive, while others claim they are only prognostic.

HLA-DR+ CTLs goes further as a predictive biomarker, firstly because it is a population of T cells with cytotoxic properties (CTLs) against tumor cells, which are activated (expressing high levels of HLA-DR). These activated CTLs reflect the overall immune status present in the tumor microenvironment, contrarily to TILs or the NLR. In fact, even if the tumor is enriched with effector T cells, they can be inhibited by molecules expressed by the tumor cells or by regulatory immune cells. As such, only the activated (HLA-DR+) CTLs will lead to anti-tumor immune responses.

Secondly, this biomarker is analyzed by flow cytometry, and although we cannot distinguish intratumor CTLs from stromal CTLs, as it is possible with immunohistochemistry (IHC), it is more representative of the whole biopsy than IHC, where only a slice of the biopsy mass, and not the whole 3D conformation, is used. Actually, several reports claim that stromal TILs are

more important than the tumor infiltrating to predict response to NACT (reviewed in (229)), while others report the opposite (230). With the flow cytometry, we can quantify both together, reducing the heterogeneity between studies.

Thirdly, it is a powerful predictive factor, which is possible to observe in two different cohorts of patients, by the ROC curve parameters, the univariable and the multivariable analysis. Moreover, it is an immune trait that also show promising results as a long-term prognostic biomarker. Nevertheless, to achieve that, a higher follow-up with more patients and during 5-10 years needs to be performed.

Lastly, it is a predictive factor independently of the breast cancer subtype and other clinical parameters, such as patients' age, reflecting the capacity to be used in all breast cancer patients submitted to NACT.

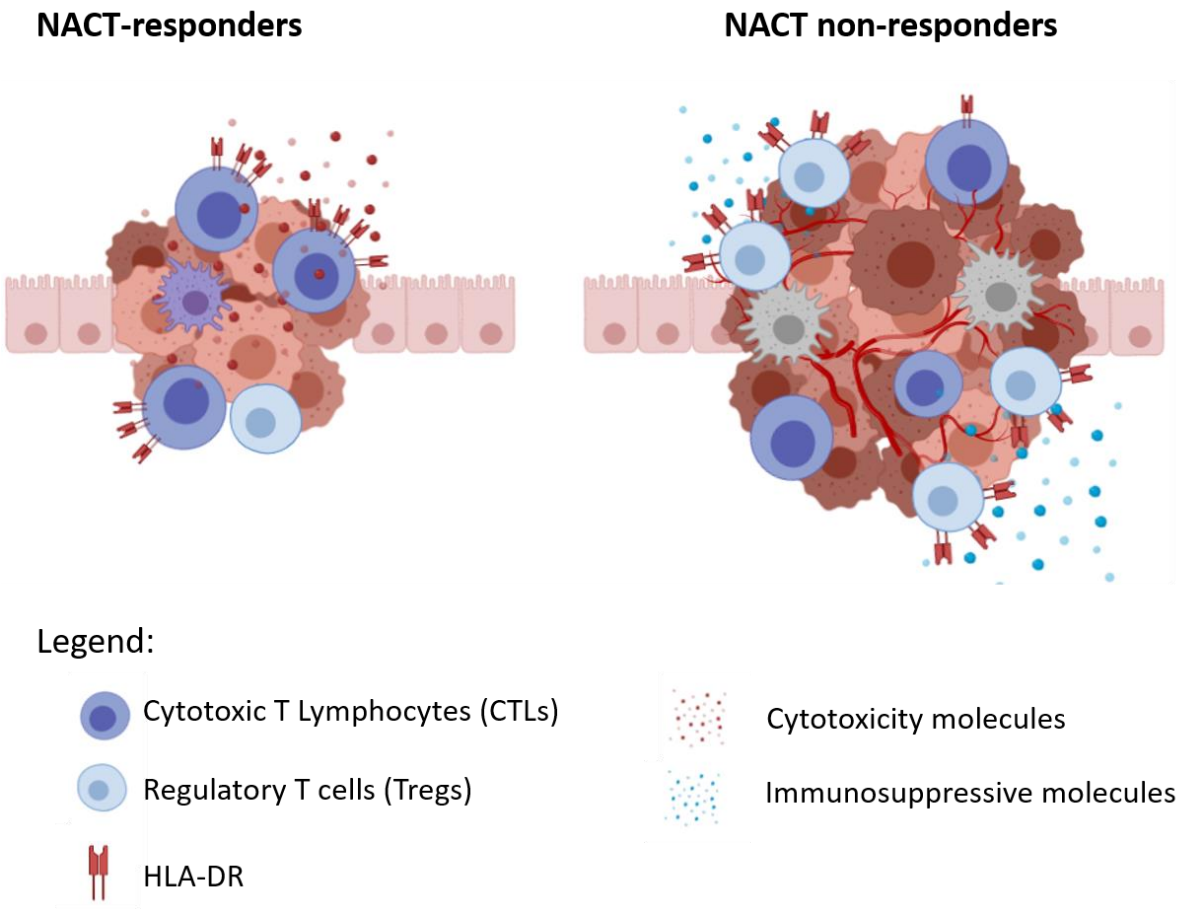


Figure VII-2 **Model of HLA-DR-expressing CTLs as a predictive biomarker for NACT response.** In patients with response to NACT, the tumor is infiltrated with several types of immune cells, including cytotoxic T cells (CTLs) that are active and express high levels of HLA-DR. Activated CTLs release cytotoxicity-related molecules, such as Granzyme B and Perforin, inducing tumor cell death. In NACT non-responders, the tumor can still be infiltrated with different immune cells, but due to the release of immunosuppressive molecules by the tumor cells, CTLs are inhibited and the level of HLA-DR is reduced. Figure performed in Biorender.

The main limitation of this study is the low sample size when comparing to biomarker studies that are conducted in parallel with clinical trials. However, we have to take into consideration that the patients who performed NACT are approximately 20% of the breast cancer patients enrolled in the hospitals with whom we collaborate to obtain the biopsy samples (HCD and HVFX). Additionally, we have used fresh biopsies and for that the radiologists have to perform an extra biopsy shot, besides the biopsy for Pathological diagnostic. After the biopsy is collected, we have to wait at least 6 months (the duration of NACT) to obtain the response to the treatment. Nevertheless, even with a small sample size, we have obtained remarkable results with great statistical power that allow the elaboration of a robust model, emphasizing that HLA-DR-expressing CTLs, assessed by flow cytometry, have a great potential to be used in clinical practice as a biomarker to predict NACT response.

Nevertheless, the future implementation of this biomarker in a clinical setting would be challenging. Although analyzing HLA-DR-expressing CTLs by flow cytometry is an advantage to obtain statistically relevant results, more representative from the whole biopsy (much more cells are analyzed per sample), this method is not routinely used in Hospitals' Pathology departments, as IHC is, for instance. Nevertheless, due to the significant results emphasized, we believe that an implementation in a clinical setting would be truly valuable to breast cancer patients. Our idea for the pipeline used to assess HLA-DR-expressing CTLs prior to treatment implementation is described in Figure VII-3. If patients have a low HLA-DR expression level in CTLs, different therapeutic strategies could be implemented, such as, a combination of chemotherapy with immune checkpoint inhibitors (as the PD-1/PD-L1 axis) or other immune modulators, even personalized vaccines or adoptive T cell transfer with activated autologous CTLs. Of course, none of these alternative treatments are yet optimized for breast cancer patients (although some new strategies are in clinical trials), but the implementation of this analysis in a near future at least could help clinicians to promptly direct patients with operable tumors to surgery, instead of recommend a 6 month-regime of an aggressive chemotherapy without any benefit for the patient.

To further corroborate our claims, we are still enrolling patients for the validation cohort to have a stronger representation of breast cancer patients. Additionally, we have optimized a platform to demonstrate *in vitro* that HLA-DR+ CTLs are required for NACT success and also analyze new therapeutic strategies that could help NACT non-responder patients (Chapter VI).

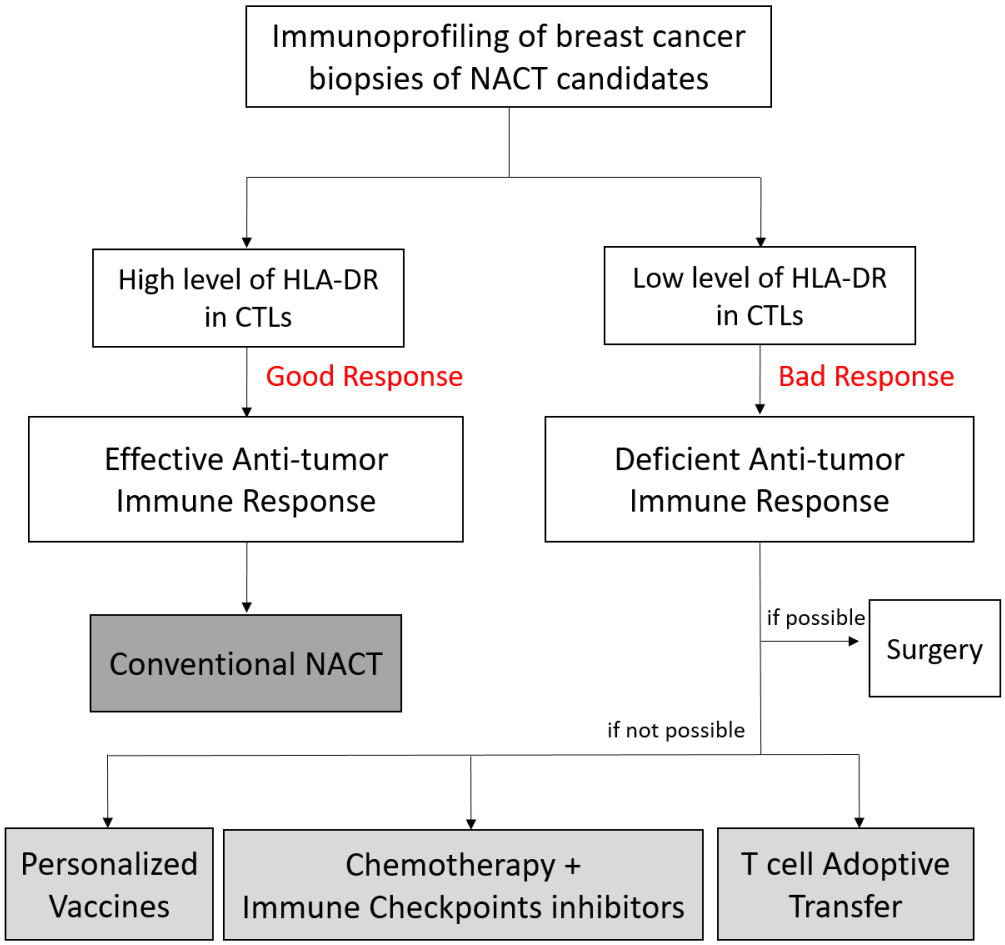


Figure VII-3 **Flowchart of clinical decisions that could be implemented with the use of HLA-DR-expressing CTLs as a predictive biomarker to assess breast cancer patients' response to NACT.** In biopsies of breast cancer patients (candidates to perform NACT) immune profiling is executed by flow cytometry. The patients are then divided into bearing tumors with high expression level of HLA-DR in CTLs and low expression level of HLA-DR in CTLs. The first group of patients has a prediction for a good response to NACT since they present an effective anti-tumor immune response. As such, conventional NACT can be administered to these patients. Patients with low levels of HLA-DR in CTLs have a deficient anti-tumor immune response and, consequently, a prediction for a poor response to NACT. These patients should be directed to surgery, if possible. If not possible, alternative therapeutic strategies should be implemented, such as adoptive T cell transfer, the use of standard chemotherapy combined with immune checkpoint inhibitors or personalized vaccines.

2. HLA-DR+ cytotoxic T cells produce cytotoxic molecules and exhibit effector memory T cell markers

Recent reports highlighted the importance of an analysis of the variations between a T cell subset profile/function (memory, effector, exhaustion) for the development of individual T cell-based immunotherapies. Thus, in order to better understand the profile of HLA-DR+ CTLs, envisaging their potential therapeutic value, we performed a thorough characterization of these cells, comparing to HLA-DR negative CTLs. First of all, we used isolated cells from the peripheral blood of patients and healthy donors to conduct these experiments, since the

material provided by biopsies or surgical specimens was limited. We observed that a culture of *ex vivo* peripheral blood mononuclear cells (PBMCs) in contact with a stimulus could increase the percentage of HLA-DR+ CTLs in NACT-responder patients but surprisingly, also in non-responders (see Figure V-1). Additionally, the stimulated PBMCs could produce higher levels of IFN-γ, which is the main cytokine produced by activated CTLs (see Figure V-1).

We also observed that this increase in HLA-DR+ CTLs was possible not only with a canonical stimulation, but also when PBMCs are in the presence of tumor cells (in this case a breast cancer cell line – Hs 578T) or even only in the presence of the factors released by the tumor cells to the extracellular medium (see Figure V-2). This result suggest that, in the tumor microenvironment, CTLs respond not only to the cellular contact derived from the connection between the T cell receptor (TCR) in CTLs and MHC I bearing antigens in tumor cells, but also to the soluble molecules released by the tumor cells. These molecules could be, for instance, cytokines, which mirrors our clinical result (see Figure IV-17) showing that the presence of immunosuppressive cytokines produced by the tumor cells was negatively correlated with the expression level of HLA-DR in CTLs.

The production of IFN-γ above described was induced in PBMCs by stimulation *in vitro*. We also assessed the levels of IFN-γ and Granzyme B directly in both intratumor and circulating HLA-DR+ CTLs (see Figure V-3). Both molecules were highly produced by HLA-DR+ CTLs when compared to HLA-DR negative CTLs, highlighting that HLA-DR is indeed a marker of T cell activation. Moreover, TNF-α, Perforin and Eomes were up-regulated in HLA-DR+ CTLs, whereas T-bet was down-regulated in these cells (see Figure V-4). Effector T cells are defined as T-bet^{high}/Eomes^{high} (206), which is not representative of these cells. With the profile T-bet^{low}/Eomes^{high}, HLA-DR+ CTLs could be either exhausted T cells, central memory (T_{CM}), effector memory T cells (T_{EM}) or terminally differentiated effector T cells (T_{TE}) (206). Additional flow cytometry analysis (see Figure V-6, 7) revealed that the majority of HLA-DR+ CTLs are proliferative cells (high levels of Ki67) and some present exhaustion markers, such as PD-1. This result points out that HLA-DR+ CTLs are in distinct levels of activation, and at least part of these cells could become exhausted due to the constant contact with tumor antigens. Indeed, in the past years, T cell exhaustion has been a topic of debate. While in the past exhausted CTLs were characterized as CTLs that had lost the capacity to produce cytokines (namely IFN-γ), had reduced proliferation and cytotoxicity function; now, these cells were shown to still have the capacity to release cytotoxic molecules and kill target cells (215,231). However, the reduced proliferative capacity of exhausted CTLs is still consensual in the field. As we observed that most HLA-DR+ CTLs are positive for Ki67, these cells cannot be typical exhausted T cells. T_{CM} cells were shown to have high levels of CD127, low levels of HLA-DR and no Granzyme B production (218). As such, HLA-DR+ CTLs are also not representative of T_{CM} subtype. The differences between T_{EM} cells and T_{TE} CTLs are based on CD127 (low expression in T_{EM} and no expression in T_{TE}), Ki67 (only present in T_{EM}), CD103 (only present in T_{EM}) and HLA-DR (only present in T_{EM}) (218). Moreover, while both T_{TE} and T_{EM} produce IFN-γ, only T_{EM} is able to produce TNF-α (218). Hence, HLA-DR+ CTLs appear to be closer to a T_{EM} phenotype.

Nevertheless, additional markers should be used to classified HLA-DR+ CTLs as effector memory CTLs. For instance, CD45RO that marks memory immune cells should be assessed. CCR7 and CD62L, which are upregulated in T_{CM}, are not expressed in T_{EM} and could also improve the segregation of these subsets of memory T cells. CD28 is highly expressed in T_{EM} and T_{CM} but not in T_{TE}. Besides continuing the characterization of the surface markers, a more detailed transcriptional profile should also be pursued. Actually, the transcription factor Hobit is mainly expressed in T_{RM}; TCF-1 is mainly present in T_{CM}; KLF7 is upregulated in T_{EM}, and KLF2 is downregulated in T_{RM} and upregulated in T_{EM} (232,233). Increasing the complexity of the markers and transcription factors analyzed it will be possible to better discriminate if HLA-DR+ CTLs are indeed T_{EM}.

Besides bearing differences in the surface markers and gene expression, the different CTLs subtypes have distinct metabolic states. Naïve T cells that have never encountered an antigen have low metabolic demand and perform mainly oxidative phosphorylation to obtain energy (234). On the opposite, after activation by antigen presentation, effector CTLs have a high energy demand and rewire their metabolism to rely mainly on aerobic glycolysis with lactate production (235). Memory CTLs also have different metabolic demands. Although they are in a steady-state, T_{EM} cells have a high mitochondrial mass to rapidly produce energy after antigen encounter (235,236). Similarly to effector CTLs, T_{EM} cells rely on aerobic glycolysis and also oxidative phosphorylation (235,236). The other memory subtypes of T cells, such as T_{CM} and T_{RM} cells, on the other hand, have a metabolic adaptation based on fatty acid oxidation (235). Hence, the analysis of the gene expression of different enzymes involved in the distinct metabolic pathways can be another way to better identify to which category HLA-DR+ CTLs belong to.

Although effector CTLs have a high cytotoxic capacity and are the ultimate anti-tumor immune cell population, they undergo fast apoptosis after performing their function. Additionally, the constant contact with antigen stimulation will turn the effector CTLs in exhausted with a progressive loss of their effector function. Besides effector and exhausted cells, a percentage of CTLs are memory T cells.

Tumor-immune microenvironment reports in the literature have been reiterating that from the different memory subsets, tissue resident memory T cells are the ones with the greatest capacity to act against tumor cells (207,208), despite being unable to recirculate. Although these cells are better adapted to the tumor microenvironment and can release cytotoxicity-related molecules, they are also able to produce IL-17 (237), which has been shown to induce the production of metalloproteinases, essential for the extracellular matrix degradation and tumor invasion (99). Additionally, IL-17 is known to stimulate Tregs, which would inhibit CTLs cytotoxicity function (94).

Effector memory T cells are the memory subtype with higher cytotoxic phenotype, proliferative capacity and can efficiently eliminate target cells (206). The phenotype of

effector memory CTLs is an advantage in the process of tumor cells elimination, since they are long-lived, retain in inflamed tumors and are capable of performing cytotoxic functions (206).

3. Clarification of the role of HLA-DR+ cytotoxic T cells in NACT response and optimization of alternative therapeutic strategies

In order to better understand the role of HLA-DR+ CTLs in the response to NACT and to develop a platform for screening new alternative therapies for NACT non-responders, we have constructed a 3D allogenic co-culture model of breast cancer cell lines and patient-derived immune cells.

We have used 3D models since they better replicate the complexity of a tumor microenvironment than classical 2D cultures. For instance, 3D cultures allow for different cell types to be cultured together and replicate essential tumor features, such as a hypoxia gradient towards the center of the structure and increased cell-cell contacts (182,183,220). With the aim of developing a platform for assessing therapeutic strategies, our 3D systems have to be simple at the same time, to allow a throughput screening. As such, we have used a scaffold-free technique, by reducing the contact of the cells to the culture plastic. Additionally, since we intended to use the same platform to assess different patients' response to alternative treatments, we have developed a co-culture of breast cancer cell lines with allogenic patient-derived immune cells, which have been shown by others to still possess cytotoxicity functions against target cells (184,185).

We have used two breast cancer cell lines – MCF-7 and MDA-MB-231 because they were isolated from patients with distinct breast cancer subtypes, ER+ and TNBC, respectively. Additionally, both can be cultured in 3D and they may have different chemo-sensitivities (188,223). Patient-derived immune cells were isolated from the blood (peripheral blood mononuclear cells – PBMCs) and both NACT-responders and non-responders patients' PBMCs were used for the co-culture. We have observed that both responders and non-responders derived PBMCs were able to infiltrate the tumor spheroids (see Figure VI-3).

Our first aim with this 3D platform was to clarify the role of HLA-DR+ CTLs in the tumor microenvironment and their influence in NACT efficacy. We already knew from Chapter V that HLA-DR+ CTLs had higher production of cytotoxicity-related molecules, such as Granzyme B, but lacked the proof that they had the capacity to eliminate tumor cells. To better clarify their cytotoxic abilities, we added only sorted HLA-DR+ CTLs to a MCF-7 spheroid, and observed a significant decrease in the viability of the cell line (see Figure VI-6). Moreover, no effect was observed with the addition of sorted HLA-DR negative CTLs alone or sorted CTLs negative fraction, highlighting that indeed HLA-DR+ CTLs are the immune subset with the capacity to eliminate tumor cells. Additionally, we observed that the cytotoxic function of HLA-DR+ CTLs was only directed to tumor cells and not autologous immune cells since the co-culture of HLA-

DR+ CTLs and the CTLs negative fraction revealed that the majority of the total PBMCs remain viable (see Figure VI-6B).

This result was not in agreement with two published reports from the same group claiming that HLA-DR+ CTLs are cytotoxic against autologous PBMCs (222,238), and thus have a suppressive role. In these reports, the authors show that HLA-DR+ CTLs are similar to typical Tregs, are positive for PD-1, TIGIT and CTLA-4, and can inhibit the proliferation of responding PBMCs and eliminate them when in co-culture. For this elimination, cell-to-cell contact must occur either via CTLA4 (238) or PD-1 (222). Moreover, the authors also claim that HLA-DR marks activated and proliferating CTLs and that they express IFN-γ and TNF-α, but not Granzyme B. However, our results prove that, in our developed platform, HLA-DR+ CTLs are cytotoxic against tumor cells and not autologous cells. The suppressive role of HLA-DR+ CTLs against autologous PBMCs was only demonstrated *in vitro* (with isolated cells from human peripheral blood) and may not be representative of an *in vivo* phenotype. Actually, to our knowledge, this group is the only one claiming that HLA-DR+ CTLs are a suppressive cell type. There are other reports describing a suppressive role of CD8+ T cells, but never associated with HLA-DR expression (239,240).

Interestingly, when the CTLs negative fraction, that comprised antigen presenting cells and helper T cells, was added to the spheroids together with HLA-DR negative CTLs, these non-activated CTLs became able to eliminate tumor cells, similarly to HLA-DR+ CTLs alone. Our hypothesis here is that antigen presenting cells are able to uptake tumor antigens, process and present them to helper T cells. Then, helper T cells will become activated and produce IFN-γ, that in turn will stimulate the CTLs, contributing to their activation (acquiring high expression levels of HLA-DR) and gain of cytotoxic function against tumor cells (Figure VII-4).

To test this hypothesis, represented in Figure VII-4, different co-culture experiments could be performed. For example, instead of sorting the CTLs negative fraction, the CD4+ T cells (Th) and the APCs (for instance, CD14+ cells) could be sorted. Then, with the MCF-7 3D spheroids, a co-culture with HLA-DR negative CTLs and Th cells could be performed and, in another experiment, HLA-DR negative with only the APCs could be added to the MCF-7 spheroids. The viability of the MCF-7 cells would then be analyzed. With this assay, we could assess if both cell types are necessary to activate HLA-DR negative CTLs (turning them into HLA-DR+) and confirm our hypothesis. Additionally, to assess the specific role of HLA-DR, namely if the expression of this molecule in CTLs leads to antigen presentation to Th cells, thus boosting IFN-γ production and cytotoxic capacity of CTLs, in a positive feedback loop, a blocking antibody against HLA-DR in sorted T cells (containing both Th and CTLs) in co-culture with spheroids could be added. IFN-γ released to the extracellular environment could then be quantified as well as the viability of the breast cancer cell line.

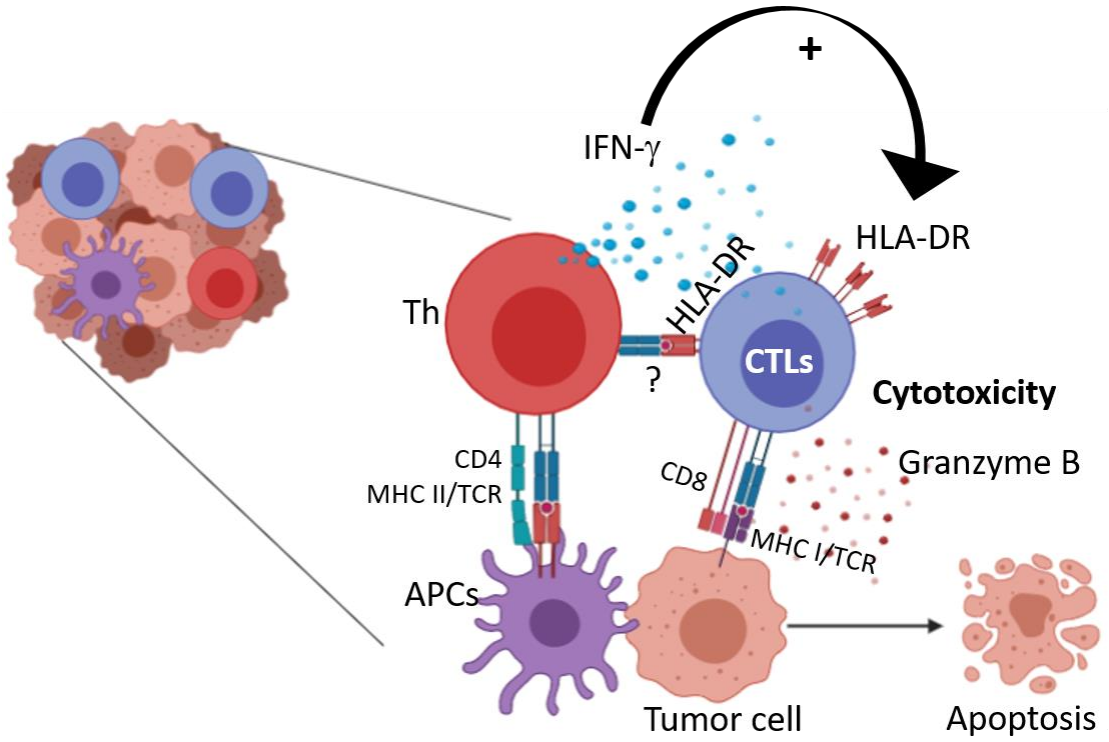


Figure VII-4 **Activation of CTLs by IFN-γ released by helper T cells that were stimulated by antigen presenting cells**. In a tumor microenvironment, antigen presenting cells (APCs) can recognize tumor antigens and present them by class II MHC to helper T cells (Th). This presentation will stimulate Th response, which comprises the release of IFN-γ. This cytokine is a known inducer of cytotoxic T cells (CTLs) and will activate them, increasing their HLA-DR expression level. Activated CTLs will be able to perform their effector cytotoxic function and release Granzyme B and Perforin. These cytotoxicity-related molecules will induce tumor cell apoptosis. It is also known that besides being an activation marker, HLA-DR presence in CTLs could act as a T cell-T cell synapsis, maybe even by the antigen presentation to Th cells, increasing the secretion of IFN-γ to the environment. Figure performed in Biorender.

So, why does this cycle (Figure VII-4) don't occur always in a tumor microenvironment? We have seen that in a tumor microenvironment, tumor cells are capable of releasing several tumor-protective mechanisms, such as immunosuppressive cytokines and inhibitor molecules (e.g. PD-L1). Hence, even the antigen presenting cells and/or the helper T cells could be hampered in this impeditive microenvironment and be unable to stimulate CTLs. We could hypothesize that treatment with personalized vaccines, such as dendritic vaccines, would help to present specific tumor antigens to helper T cells, which would stimulate CTLs, increasing their HLA-DR which will contribute to improve their own activation/function. Nevertheless, for this treatment to be effective, a combination with immune checkpoint inhibitors, for instance, has to be considered so that the inhibitory microenvironment is refrained.

Having assessed the cytotoxic function of HLA-DR+ CTLs, we analyzed what was the effect of adding the whole PBMCs to the 3D spheroids of MCF-7 and MDA-MB-231 cell lines. Interestingly, when the addition of the NACT-responders' PBMCs was performed, we

observed a significant decrease in the breast cancer cells' viability, without the need for chemotherapeutic agents (see Figure VI-5). This effect was not observed for NACT non-responders' PBMCs, emphasizing the immunosuppressed phenotype of the immune cells isolated from patients who did not respond to chemotherapy (known to possess low levels of HLA-DR in CTLs). Interestingly, the NACT-responders' PBMCs acted in synergy with doxorubicin (a chemotherapeutic agent normally used on NACT, see Figure VI-7) leading to an even greater effect on the viability of the breast cancer cells. This effect was not observed for the co-culture of the cell lines with NACT non-responders' PBMCs and doxorubicin. This result proves that we are able to replicate clinical observations *in vitro*.

Thus, we can conclude that we have constructed a reliable 3D platform to further test new therapeutic strategies, especially for the NACT non-responder patients.

We have seen in Chapter IV that the main difference between NACT-responders and non-responders was the expression level of HLA-DR in CTLs. Thus, we hypothesized that increasing this HLA-DR level in CTLs of NACT non-responders would revert their immunosuppressed phenotype, therefore increasing the anti-tumor function of these cells.

To achieve this we have used two different strategies – stimulation with PMA/ionomycin or TCR engagement. PMA/ionomycin is a canonical, receptor-independent stimulation that will activate protein kinase C and increase calcium influx (241). This method will lead to overall immune cells activation (including CTLs), proliferation, and release of inflammatory cytokines (241). T cell receptor stimulation with co-stimulatory signaling, on the other hand, is a more specific stimulation method that reproduces the natural activation of T cells. The TCR signaling cascade is a complex pathway that ultimately leads to T cell activation, proliferation, increased function and release of cytokines (242). As the TCR engagement is a specific T cell stimulation it can explain the fact that HLA-DR levels in CTLs and IFN-γ production are increased with this method, when compared to PMA/ionomycin (see Figure VI-8-A, D, E). Nevertheless, with both methods we were capable to increase the percentage of HLA-DR+ CTLs.

Interestingly, NACT non-responders' PBMCs previously stimulated by either method are able to eliminate tumor cells and this effect is synergistic with the addition of doxorubicin (see Figure VI-8-B, C), similarly to NACT-responders' PBMCs.

Moreover, we tried to revert the immunosuppression phenotype of NACT non-responders' PBMCs by a more translatable approach. To achieve this, we used immune checkpoint inhibitors, to remove the tumor impeditive action on the function of T cells. These inhibitors are already used in the treatment of several cancer types and are FDA approved in breast cancer only for patients with metastatic TNBC (118).

To perform this, we used antibodies against PD-1 and PD-L1 alone or in combination with doxorubicin. However, we were not able to revert the immunosuppressed phenotype of NACT non-responders' PBMCs (see Figure VI-9). The lack of immune checkpoint blockade specific effect can be explained by the low percentage of PD-1 at the surface of CTLs. We have to take

into account, however, that breast cancer patients are very heterogeneous, and in some cases, PD-1 expression can be increased in CTLs, especially in intratumor CTLs, and immune checkpoint blockade could be a strategy for these patients.

Altogether, the main output from these results is the developed 3D platform, that could be used as a tool to test different therapeutic strategies. Actually, there are other immunomodulatory drugs besides the PD-1/PD-L1 axis blockade. Antibodies against CTLA4 or Tim3 are other possible strategies, to release the immunosuppressive action on CTLs. Indeed, CTLA4 is already used to treat other types of cancer (Ipilimumab is FDA approved, (118)). Inhibiting indoleamine 2,3-dioxygenase (IDO), an enzyme that is related to immunosuppression of APCs (243), could also potentiate the circuit represented in Figure VII-4. Small drugs with immunomodulatory properties, could also be tested, as it is the case of the agonist for Ox40, that it might augment CTLs' cytotoxic activity (244).

Moreover, it would be interesting to perform these assays with CTLs isolated from TILs. Conceivably, CTLs from the tumor are already hardwired to fight specific tumor cells, due to the presence of specific tumor antigens, but are being suppressed by the tumor microenvironment, therefore bearing higher expression of PD-1 and other immune checkpoints, than peripheral CTLs. Hence, the immune checkpoint blockade therapy could be more effective in this case. Additionally, 3D co-culture experiments with tumor cells from the patient, instead of breast cancer cell lines, and autologous immune cells would give a more representative view of the interactions between tumor and immune cells. Here, a combination of stimulated CTLs with immune checkpoint blockade could be tackled, for instance.

Nevertheless, with the results shown so far in this thesis, we could envisage that stimulation of PBMCs (including CTLs with high levels of HLA-DR), should be considered as an alternative strategy to increase HLA-DR expression in CTLs in NACT non-responder patients.

Ideally, CTLs from the blood of the patients – where a high number of cells can be isolated, could be stimulated *ex vivo* by TCR engagement, increasing the levels of HLA-DR and the consequent production of cytotoxicity molecules. These cells could then be reinfused into the patients and have a more targeted approach against tumor cells. Nevertheless, the use of these cells as a therapeutic strategy is still far from being clinically tested. Several experiments should be performed to test the safety and efficacy of stimulated CTLs. Namely, stimulated CTLs should be further tested to clarify their expression profiles and their half-life. Moreover, these cells should be cultured with other cell types, including autologous immune cells, to determine if their cytotoxicity is only towards tumor cells or if they could have any side effects.

Actually, adoptive T cell transfer was already used in a metastatic ER+ breast cancer patient, as depicted in section 6.3.2 of Chapter I (173). Although this treatment was effective for this breast cancer patient it is a very personalized medicine approach, were tumor antigens of each patient have to be detected and reactive TILs against them screened. Moreover, it is necessary to take into account that this treatment has high costs due to the specific

techniques to find tumor antigens, such as whole exome sequencing and RNAseq, and that it is a very demanding and time-consuming to perform these techniques and to expand tumor-specific T cells in culture. As such, all efforts to discover new, effective, straight forward, less expansive alternatives should be considered to improve breast cancer treatment and patients' quality of life.

4. Concluding remarks

Overall, with this thesis, we discovered a new biomarker to predict breast cancer patients' response to NACT that can be more precise than the classical TILs and NLR. This biomarker – HLA-DR-expression level in CTLs, mainly assessed at the biopsy level, but with a systemic reflection, can accurately distinguish *a priori* patients that will respond to the treatment from the ones who will not respond. HLA-DR+ CTLs were shown to be active, with production of IFN-γ, Perforin and Granzyme B, and with cytotoxicity against tumor cells and not autologous immune cells. Precise biomarkers to predict response to NACT are a medical need, due to the high percentage of non-responder patients, but the biomarker studies should run in parallel with the finding of new treatments, due to the lack of alternative therapeutic strategies. The alternative treatment available nowadays would be surgery followed by radiotherapy and/or adjuvant chemotherapy. However, patients selected for NACT include patients with inflammatory and inoperable tumors, and for those surgery without prior tumor down-staging is impossible.

This thesis also aimed to develop a new platform to perform a screening of new therapeutic strategies, especially for patients without response to NACT. First of all, an *in vitro* validation of the need of HLA-DR+ CTLs for NACT success was performed. Additionally, we observed that increasing the HLA-DR levels in CTLs derived from the blood of non-responder patients could improve their anti-tumor response and showed a synergistic effect with doxorubicin – a NACT agent.

Nevertheless, further studies need to be performed, namely there is a need to increase the sample size for the biomarker study and to perform 3D cultures with patient-derived tumors to have a more physiological platform to test several therapeutic strategies.

Chapter VIII

Bibliography

Chapter VIII – Bibliography

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57–70.
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar;144(5):646–74.
3. Warburg O. On the origin of cancer cells. Science. 1956;123(3191):309–14.
4. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer Metastasis Rev. 2010;29(2):309–16.
5. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140(6):883–99.
6. Pages F, Galon J, Dieu-Nosjean M-C, Tartour E, Sautes-Fridman C, Fridman W-H. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene. 2010;29(8):1093–102.
7. Thomas L, Lawrence HS. Cellular and humoral aspects of the hypersensitive states. New York Hoeber-Harper. 1959;529–32.
8. Burnet M. Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. Br Med J. 1957;1(5023):841–7.
9. Kaplan HS. Role of immunologic disturbance in human oncogenesis: some facts and fancies. Br J Cancer. 1971;25(4):620.
10. Stutman O. Immunodepression and malignancy. In: Advances in cancer research. Elsevier; 1976. p. 261–422.
11. Boon T, van der Bruggen P. Human tumor antigens recognized by T lymphocytes. J Exp Med. 1996;183(3):725–9.
12. Schulz TF. Cancer and viral infections in immunocompromised individuals. Int J cancer. 2009;125(8):1755–63.
13. Chen M-R. Epstein-barr virus, the immune system, and associated diseases. Front Microbiol. 2011;2:5.
14. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. Lancet. 2007;370(9581):59–67.
15. Gallagher MP, Kelly PJ, Jardine M, Perkovic V, Cass A, Craig JC, et al. Long-term cancer risk of immunosuppressive regimens after kidney transplantation. J Am Soc Nephrol. 2010;21(5):852–8.
16. Pawelec G, Derhovanessian E, Larbi A. Immunosenescence and cancer. Crit Rev Oncol Hematol. 2010;75(2):165–72.
17. Kiniwa Y, Miyahara Y, Wang HY, Peng W, Peng G, Wheeler TM, et al. CD8+ Foxp3+

regulatory T cells mediate immunosuppression in prostate cancer. Clin Cancer Res. 2007;13(23):6947–58.

18. Liakou CI, Narayanan S, Ng Tang D, Logothetis CJ, Sharma P. Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human bladder cancer. Cancer Immun. 2007;7:10.

19. Santoiemma PP, Powell Jr. DJ. Tumor infiltrating lymphocytes in ovarian cancer. Cancer Biol Ther. 2015;16(6):807–20.

20. Teng F, Mu D, Meng X, Kong L, Zhu H, Liu S, et al. Tumor infiltrating lymphocytes (TILs) before and after neoadjuvant chemoradiotherapy and its clinical utility for rectal cancer. Am J Cancer Res. 2015;5(6):2064–74.

21. Hornychova H, Melichar B, Tomsova M, Mergancova J, Urminska H, Ryska A. Tumor-infiltrating lymphocytes predict response to neoadjuvant chemotherapy in patients with breast carcinoma. Cancer Invest. 2008;26(10):1024–31.

22. Clemente CG, Mihm MCJ, Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. Cancer. 1996;77(7):1303–10.

23. Liudahl SM, Coussens LM. Chapter 8 - To Help or To Harm: Dynamic Roles of CD4+ T Helper Cells in Solid Tumor Microenvironments. In: Hayat MABT-I, editor. Academic Press; 2018. p. 97–116.

24. Bevan MJ. Helping the CD8(+) T-cell response. Nat Rev Immunol. 2004;4(8):595–602.

25. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med. 2010;207(3):637–50.

26. Hennequin A, Derangère V, Boidot R, Apetoh L, Vincent J, Orry D, et al. Tumor infiltration by Tbet+ effector T cells and CD20+ B cells is associated with survival in gastric cancer patients. Oncoimmunology. 2016;5(2):e1054598.

27. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, Th2, Treg, Th17) in patients with colorectal cancer. Cancer Res. 2011;71(4):1263–71.

28. Gao Q, Wang X-Y, Qiu S-J, Zhou J, Shi Y-H, Zhang B-H, et al. Tumor stroma reaction-related gene signature predicts clinical outcome in human hepatocellular carcinoma. Cancer Sci. 2011;102(8):1522–31.

29. Kusuda T, Shigemasa K, Arihiro K, Fujii T, Nagai N, Ohama K. Relative expression levels of Th1 and Th2 cytokine mRNA are independent prognostic factors in patients with ovarian cancer. Oncol Rep. 2005;13(6):1153–8.

30. Togashi Y, Shitara K, Nishikawa H. Regulatory T cells in cancer immunosuppression - implications for anticancer therapy. Nat Rev Clin Oncol. 2019;16(6):356–71.

31. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008;322(5899):271–5.

32. Jarnicki AG, Lysaght J, Todryk S, Mills KHG. Suppression of antitumor immunity by IL-10 and TGF-beta-producing T cells infiltrating the growing tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. J Immunol. 2006;177(2):896–904.

33. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10(9):942–9.

34. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol. 2006;24(34):5373–80.

35. Correale P, Rotundo MS, Del Vecchio MT, Remondo C, Migali C, Ginanneschi C, et al. Regulatory (FoxP3+) T-cell tumor infiltration is a favorable prognostic factor in advanced colon cancer patients undergoing chemo or chemoimmunotherapy. J Immunother. 2010;33(4):435–41.

36. Yeong J, Thike AA, Lim JCT, Lee B, Li H, Wong S-C, et al. Higher densities of Foxp3(+) regulatory T cells are associated with better prognosis in triple-negative breast cancer. Breast Cancer Res Treat. 2017;163(1):21–35.

37. Farhood B, Najafi M, Mortezaee K. CD8(+) cytotoxic T lymphocytes in cancer immunotherapy: A review. J Cell Physiol. 2019;234(6):8509–21.

38. Trapani JA, Smyth MJ. Killing by cytotoxic T cells and natural killer cells: multiple granule serine proteases as initiators of DNA fragmentation. Immunol Cell Biol. 1993;71 (Pt 3):201–8.

39. Nagata S. Apoptosis by death factor. Cell. 1997;88(3):355–65.

40. Bertrand F, Montfort A, Marcheteau E, Imbert C, Gilhodes J, Filleron T, et al. TNFalpha blockade overcomes resistance to anti-PD-1 in experimental melanoma. Nat Commun. 2017;8(1):2256.

41. Al-Saleh K, Abd El-Aziz N, Ali A, Abozeed W, Abd El-Warith A, Ibraheem A, et al. Predictive and prognostic significance of CD8(+) tumor-infiltrating lymphocytes in patients with luminal B/HER 2 negative breast cancer treated with neoadjuvant chemotherapy. Oncol Lett. 2017;14(1):337–44.

42. Zhao Y, Ge X, He J, Cheng Y, Wang Z, Wang J, et al. The prognostic value of tumor-infiltrating lymphocytes in colorectal cancer differs by anatomical subsite: a systematic review and meta-analysis. World J Surg Oncol. 2019;17(1):85.

43. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010;11(10):889–96.

44. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14–20.

45. Poh AR, Ernst M. Targeting Macrophages in Cancer: From Bench to Bedside . Vol. 8, Frontiers in Oncology . 2018. p. 49.

46. Di Caro G, Cortese N, Castino GF, Grizzi F, Gavazzi F, Ridolfi C, et al. Dual prognostic significance of tumour-associated macrophages in human pancreatic adenocarcinoma treated or untreated with chemotherapy. Gut. 2016;65(10):1710–20.

47. Campbell MJ, Tonlaar NY, Garwood ER, Huo D, Moore DH, Khramtsov AI, et al. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. Breast Cancer Res Treat. 2011;128(3):703–11.

48. Hanada T, Nakagawa M, Emoto A, Nomura T, Nasu N, Nomura Y. Prognostic value of tumor-associated macrophage count in human bladder cancer. Int J Urol. 2000;7(7):263–9.

49. Forssell J, Öberg Å, Henriksson ML, Stenling R, Jung A, Palmqvist R. High Macrophage Infiltration along the Tumor Front Correlates with Improved Survival in Colon Cancer. Clin Cancer Res. 2007;13(5):1472 LP-1479.

50. Veglia F, Gabrilovich DI. Dendritic cells in cancer: the role revisited. Curr Opin Immunol. 2017;45:43–51.

51. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CMT, Pryer N, et al. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. Cancer Cell. 2014;26(5):623–37.

52. Kruger P, Saffarzadeh M, Weber ANR, Rieber N, Radsak M, von Bernuth H, et al. Neutrophils: Between host defence, immune modulation, and tissue injury. PLoS Pathog. 2015;11(3):e1004651.

53. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: “N1” versus “N2” TAN. Cancer Cell. 2009;16(3):183–94.

54. Parekh A, Das S, Parida S, Das CK, Dutta D, Mallick SK, et al. Multi-nucleated cells use ROS to induce breast cancer chemo-resistance in vitro and in vivo. Oncogene. 2018;37(33):4546–61.

55. Jablonska E, Puzewska W, Grabowska Z, Jablonski J, Talarek L. VEGF, IL-18 and NO production by neutrophils and their serum levels in patients with oral cavity cancer. Cytokine. 2005;30(3):93–9.

56. Pang Y, Gara SK, Achyut BR, Li Z, Yan HH, Day C-P, et al. TGF-beta signaling in myeloid cells is required for tumor metastasis. Cancer Discov. 2013;3(8):936–51.

57. Gonda K, Shibata M, Sato Y, Washio M, Takeshita H, Shigeta H, et al. Elevated neutrophil-to-lymphocyte ratio is associated with nutritional impairment, immune suppression, resistance to S-1 plus cisplatin, and poor prognosis in patients with stage IV gastric cancer. Mol Clin Oncol. 2017;7(6):1073–8.

58. Graziano V, Grassadonia A, Iezzi L, Vici P, Pizzuti L, Barba M, et al. Combination of peripheral neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio is predictive of pathological complete response after neoadjuvant chemotherapy in breast cancer patients. Breast. 2019;44:33–8.

59. Doi H, Nakamatsu K, Anami S, Fukuda K, Inada M, Tatebe H, et al. Neutrophil-to-

Lymphocyte Ratio Predicts Survival After Whole-brain Radiotherapy in Non-small Cell Lung Cancer. In Vivo. 2019;33(1):195–201.

60. Gabrilovich DI. Myeloid-Derived Suppressor Cells. Cancer Immunol Res. 2017;5(1):3–8.

61. Weber R, Fleming V, Hu X, Nagibin V, Groth C, Altevogt P, et al. Myeloid-Derived Suppressor Cells Hinder the Anti-Cancer Activity of Immune Checkpoint Inhibitors . Vol. 9, Frontiers in Immunology . 2018. p. 1310.

62. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1–10.

63. Blank CU, Haanen JB, Ribas A, Schumacher TN. Cancer Immunology. The “cancer immunogram”. Science (80-). 2016;352(6286):658–60.

64. Motz GT, Coukos G. Deciphering and reversing tumor immune suppression. Immunity. 2013;39(1):61–73.

65. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol. 2006;6(10):715–27.

66. Whiteside TL. Tumor-induced death of immune cells: its mechanisms and consequences. Semin Cancer Biol. 2002;12(1):43–50.

67. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Adv Immunol. 2000;74:181–273.

68. Palazon A, Aragones J, Morales-Kastresana A, de Landazuri MO, Melero I. Molecular pathways: hypoxia response in immune cells fighting or promoting cancer. Clin Cancer Res. 2012;18(5):1207–13.

69. Mendler AN, Hu B, Prinz PU, Kreutz M, Gottfried E, Noessner E. Tumor lactic acidosis suppresses CTL function by inhibition of p38 and JNK/c-Jun activation. Int J cancer. 2012;131(3):633–40.

70. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3(11):991–8.

71. Santis AG, Campanero MR, Alonso JL, Tugores A, Alonso MA, Yague E, et al. Tumor necrosis factor-alpha production induced in T lymphocytes through the AIM/CD69 activation pathway. Eur J Immunol. 1992;22(5):1253–9.

72. D’Ambrosio D, Trotta R, Vacca A, Frati L, Santoni A, Gulino A, et al. Transcriptional regulation of interleukin-2 gene expression by CD69-generated signals. Eur J Immunol. 1993;23(11):2993–7.

73. Reddy M, Eirikis E, Davis C, Davis HM, Prabhakar U. Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function. J Immunol Methods. 2004;293(1–2):127–42.

74. Theze J, Alzari PM, Bertoglio J. Interleukin 2 and its receptors: recent advances and new immunological functions. Immunol Today. 1996;17(10):481–6.

75. Rea IM, McNerlan SE, Alexander HD. CD69, CD25, and HLA-DR activation antigen expression on CD3+ lymphocytes and relationship to serum TNF-alpha, IFN-gamma, and sIL-2R levels in aging. Exp Gerontol. 1999;34(1):79–93.

76. Gerard A, Khan O, Beemiller P, Oswald E, Hu J, Matloubian M, et al. Secondary T cell-T cell synaptic interactions drive the differentiation of protective CD8+ T cells. Nat Immunol. 2013;14(4):356–63.

77. Elaraj DM, Weinreich DM, Varghese S, Puhlmann M, Hewitt SM, Carroll NM, et al. The role of interleukin 1 in growth and metastasis of human cancer xenografts. Clin Cancer Res. 2006;12(4):1088–96.

78. Gemma A, Takenaka K, Hosoya Y, Matuda K, Seike M, Kurimoto F, et al. Altered expression of several genes in highly metastatic subpopulations of a human pulmonary adenocarcinoma cell line. Eur J Cancer. 2001;37(12):1554–61.

79. Barille S, Akhoundi C, Collette M, Mellerin MP, Rapp MJ, Harousseau JL, et al. Metalloproteinases in multiple myeloma: production of matrix metalloproteinase-9 (MMP-9), activation of proMMP-2, and induction of MMP-1 by myeloma cells. Blood. 1997;90(4):1649–55.

80. Konishi N, Miki C, Yoshida T, Tanaka K, Toiyama Y, Kusunoki M. Interleukin-1 receptor antagonist inhibits the expression of vascular endothelial growth factor in colorectal carcinoma. Oncology. 2005;68(2–3):138–45.

81. Lewis AM, Varghese S, Xu H, Alexander HR. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. J Transl Med. 2006;4:48.

82. Fisher DT, Appenheimer MM, Evans SS. The two faces of IL-6 in the tumor microenvironment. Semin Immunol. 2014;26(1):38–47.

83. Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, et al. Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. Clin Cancer Res. 2006;12(1):11–9.

84. Xie T-X, Wei D, Liu M, Gao AC, Ali-Osman F, Sawaya R, et al. Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. Oncogene. 2004;23(20):3550–60.

85. Wei L-H, Kuo M-L, Chen C-A, Chou C-H, Lai K-B, Lee C-N, et al. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. Oncogene. 2003;22(10):1517–27.

86. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol. 2003;170(6):3369–76.

87. Yao C, Lin Y, Chua M-S, Ye C-S, Bi J, Li W, et al. Interleukin-8 modulates growth and invasiveness of estrogen receptor-negative breast cancer cells. Int J cancer. 2007;121(9):1949–57.

88. Araki S, Omori Y, Lyn D, Singh RK, Meinbach DM, Sandman Y, et al. Interleukin-8 is a

molecular determinant of androgen independence and progression in prostate cancer. Cancer Res. 2007;67(14):6854–62.

89. De Larco JE, Wuertz BRK, Furcht LT. The potential role of neutrophils in promoting the metastatic phenotype of tumors releasing interleukin-8. Clin Cancer Res. 2004;10(15):4895–900.

90. Zeng L, O'Connor C, Zhang J, Kaplan AM, Cohen DA. IL-10 promotes resistance to apoptosis and metastatic potential in lung tumor cell lines. Cytokine. 2010;49(3):294–302.

91. Sheikhpour E, Noorbakhsh P, Foroughi E, Farahnak S, Nasiri R, Neamatzadeh H. A Survey on the Role of Interleukin-10 in Breast Cancer: A Narrative. Reports Biochem Mol Biol. 2018;7(1):30–7.

92. García-Hernández ML, Hernández-Pando R, Gariglio P, Berumen J. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. Immunology. 2002;105(2):231–43.

93. Llanes-Fernandez L, Alvarez-Goyanes RI, Arango-Prado MDC, Alcocer-Gonzalez JM, Mojarrieta JC, Perez XE, et al. Relationship between IL-10 and tumor markers in breast cancer patients. Breast. 2006;15(4):482–9.

94. Benevides L, da Fonseca DM, Donate PB, Tiezzi DG, De Carvalho DD, de Andrade JM, et al. IL17 Promotes Mammary Tumor Progression by Changing the Behavior of Tumor Cells and Eliciting Tumorigenic Neutrophils Recruitment. Cancer Res. 2015;75(18):3788–99.

95. Steiner GE, Newman ME, Paikl D, Stix U, Memaran-Dagda N, Lee C, et al. Expression and function of pro-inflammatory interleukin IL-17 and IL-17 receptor in normal, benign hyperplastic, and malignant prostate. Prostate. 2003;56(3):171–82.

96. Zhang B, Rong G, Wei H, Zhang M, Bi J, Ma L, et al. The prevalence of Th17 cells in patients with gastric cancer. Biochem Biophys Res Commun. 2008;374(3):533–7.

97. Reppert S, Boross I, Koslowski M, Tureci O, Koch S, Lehr HA, et al. A role for T-bet-mediated tumour immune surveillance in anti-IL-17A treatment of lung cancer. Nat Commun. 2011;2:600.

98. Wu S, Rhee K-J, Albesiano E, Rabizadeh S, Wu X, Yen H-R, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat Med. 2009;15(9):1016–22.

99. Benevides L, Cardoso CRB, Tiezzi DG, Marana HRC, Andrade JM, Silva JS. Enrichment of regulatory T cells in invasive breast tumor correlates with the upregulation of IL-17A expression and invasiveness of the tumor. Eur J Immunol. 2013;43(6):1518–28.

100. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6(11):1123–32.

101. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, et al. IL-23 promotes tumour incidence and growth. Nature. 2006;442(7101):461–5.

102. Deckers M, van Dinther M, Buijs J, Que I, Lowik C, van der Pluijm G, et al. The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res. 2006;66(4):2202–9.

103. Derynck R, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet. 2001;29(2):117–29.

104. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limon P. The polarization of immune cells in the tumour environment by TGFbeta. Nat Rev Immunol. 2010;10(8):554–67.

105. Gong D, Shi W, Yi S, Chen H, Groffen J, Heisterkamp N. TGFbeta signaling plays a critical role in promoting alternative macrophage activation. BMC Immunol. 2012;13:31.

106. Fuchs Y, Steller H. Live to die another way: modes of programmed cell death and the signals emanating from dying cells. Nat Rev Mol Cell Biol. 2015;16(6):329–44.

107. van Kempen TS, Wenink MH, Leijten EFA, Radstake TRDJ, Boes M. Perception of self: distinguishing autoimmunity from autoinflammation. Nat Rev Rheumatol. 2015;11(8):483–92.

108. Galluzzi L, Buque A, Kepp O, Zitvogel L, Kroemer G. Immunogenic cell death in cancer and infectious disease. Nat Rev Immunol. 2017;17(2):97–111.

109. Galluzzi L, Buque A, Kepp O, Zitvogel L, Kroemer G. Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents. Cancer Cell. 2015;28(6):690–714.

110. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. J Exp Med. 2005;202(12):1691 LP-1701.

111. Ding Z-C, Lu X, Yu M, Lemos H, Huang L, Chandler P, et al. Immunosuppressive myeloid cells induced by chemotherapy attenuate antitumor CD4+ T-cell responses through the PD-1-PD-L1 axis. Cancer Res. 2014;74(13):3441–53.

112. Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, et al. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother. 2007;56(5):641–8.

113. Wu J, Waxman DJ. Metronomic cyclophosphamide eradicates large implanted GL261 gliomas by activating antitumor Cd8(+) T-cell responses and immune memory. Oncoimmunology. 2015;4(4):e1005521.

114. Schiavoni G, Sistigu A, Valentini M, Mattei F, Sestili P, Spadaro F, et al. Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. Cancer Res. 2011;71(3):768–78.

115. Demaria S, Volm MD, Shapiro RL, Yee HT, Oratz R, Formenti SC, et al. Development of tumor-infiltrating lymphocytes in breast cancer after neoadjuvant paclitaxel chemotherapy. Clin Cancer Res. 2001;7(10):3025–30.

116. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov. 2011;1(1):54–67.

117. Pfannenstiel LW, Lam SSK, Emens LA, Jaffee EM, Armstrong TD. Paclitaxel enhances early dendritic cell maturation and function through TLR4 signaling in mice. Cell Immunol. 2010;263(1):79–87.

118. Cancer Research Insitute. Immunotherapy - Timeline of Progress [Internet]. 2019. Available from: https://www.cancerresearch.org/immunotherapy/timeline-of-progress

119. Koury J, Lucero M, Cato C, Chang L, Geiger J, Henry D, et al. Immunotherapies: Exploiting the Immune System for Cancer Treatment. J Immunol Res. 2018;2018:9585614.

120. Morales A, Eidinger D, Bruce AW. Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. J Urol. 1976;116(2):180–3.

121. Lotze MT, Custer MC, Sharrow SO, Rubin LA, Nelson DL, Rosenberg SA. In vivo administration of purified human interleukin-2 to patients with cancer: development of interleukin-2 receptor positive cells and circulating soluble interleukin-2 receptors following interleukin-2 administration. Cancer Res. 1987;47(8):2188–95.

122. Chen L, Han X. Anti–PD-1/PD-L1 therapy of human cancer: past, present, and future. J Clin Invest. 2015;125(9):3384–91.

123. Perkins D, Wang Z, Donovan C, He H, Mark D, Guan G, et al. Regulation of CTLA-4 expression during T cell activation. J Immunol. 1996;156(11):4154 LP-4159.

124. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med. 2010;207(10):2175–86.

125. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature. 2002;415(6871):536–41.

126. Kageshita T, Kashio Y, Yamauchi A, Seki M, Abedin MJ, Nishi N, et al. Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. Int J cancer. 2002;99(6):809–16.

127. Banerjee H, Kane LP. Immune regulation by Tim-3. F1000Research. 2018;7:316.

128. Yan J, Zhang Y, Zhang J-P, Liang J, Li L, Zheng L. Tim-3 expression defines regulatory T cells in human tumors. PLoS One. 2013;8(3):e58006.

129. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, et al. Nivolumab and Ipilimumab versus Ipilimumab in Untreated Melanoma. N Engl J Med. 2015;372(21):2006–17.

130. Motzer RJ, Tannir NM, McDermott DF, Arén Frontera O, Melichar B, Choueiri TK, et al. Nivolumab plus Ipilimumab versus Sunitinib in Advanced Renal-Cell Carcinoma. N Engl J Med. 2018 Mar 21;378(14):1277–90.

131. Overman MJ, Lonardi S, Wong KYM, Lenz H-J, Gelsomino F, Aglietta M, et al. Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair-Deficient/Microsatellite Instability-High Metastatic Colorectal Cancer. J Clin Oncol. 2018;36(8):773–9.

132. Gandhi L, Rodriguez-Abreu D, Gadgeel S, Esteban E, Felip E, De Angelis F, et al. Pembrolizumab plus Chemotherapy in Metastatic Non-Small-Cell Lung Cancer. N Engl J Med. 2018;378(22):2078–92.

133. Fares CM, Van Allen EM, Drake CG, Allison JP, Hu-Lieskovan S. Mechanisms of Resistance to Immune Checkpoint Blockade: Why Does Checkpoint Inhibitor Immunotherapy Not Work for All Patients? Am Soc Clin Oncol Educ B. 2019;(39):147–64.

134. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. Science. 2015;348(6230):62–8.

135. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, et al. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A. 2002;99(25):16168–73.

136. Miliotou AN, Papadopoulou LC. CAR T-cell Therapy: A New Era in Cancer Immunotherapy. Curr Pharm Biotechnol. 2018;19(1):5–18.

137. WorldHealthOrganization. The Global Cancer Observatory. 2018.

138. Cardoso F, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rubio IT, et al. Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol Off J Eur Soc Med Oncol. 2019;

139. Kamińska M, Ciszewski T, Łopacka-Szatan K, Miotła P, Starosławska E. Breast cancer risk factors. Menopause Rev. 2015;14(3):196–202.

140. Sun Y-S, Zhao Z, Yang Z-N, Xu F, Lu H-J, Zhu Z-Y, et al. Risk Factors and Preventions of Breast Cancer. Int J Biol Sci. 2017;13(11):1387–97.

141. Waks AG, Winer EP. Breast Cancer Treatment: A Review. JAMA. 2019;321(3):288–300.

142. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. Lancet. 2014;384(9938):164–72.

143. Jones SE, Savin MA, Holmes FA, O'Shaughnessy JA, Blum JL, Vukelja S, et al. Phase III trial comparing doxorubicin plus cyclophosphamide with docetaxel plus cyclophosphamide as adjuvant therapy for operable breast cancer. J Clin Oncol. 2006;24(34):5381–7.

144. Francis PA, Pagani O, Fleming GF, Walley BA, Colleoni M, Lang I, et al. Tailoring Adjuvant Endocrine Therapy for Premenopausal Breast Cancer. N Engl J Med. 2018;379(2):122–37.

145. Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of

continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet. 2013;381(9869):805–16.

146. Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Press M, et al. Adjuvant trastuzumab in HER2-positive breast cancer. N Engl J Med. 2011;365(14):1273–83.

147. Saraiva DP, Guadalupe Cabral M, Jacinto A, Braga S. How many diseases is triple negative breast cancer: the protagonism of the immune microenvironment. ESMO open. 2017;2(4):e000208.

148. Qin H, Liu L, Sun S, Zhang D, Sheng J, Li B, et al. The impact of PI3K inhibitors on breast cancer cell and its tumor microenvironment. PeerJ. 2018;6:e5092.

149. DeMichele A, Yee D, Esserman L. Mechanisms of Resistance to Neoadjuvant Chemotherapy in Breast Cancer. N Engl J Med. 2017;377(23):2287–9.

150. Denkert C. The immunogenicity of breast cancer--molecular subtypes matter. Vol. 25, Annals of oncology : official journal of the European Society for Medical Oncology. England; 2014. p. 1453–5.

151. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol Off J Eur Soc Med Oncol. 2015;26(2):259–71.

152. Denkert C, Loibl S, Noske A, Roller M, Müller BM, Komor M, et al. Tumor-Associated Lymphocytes As an Independent Predictor of Response to Neoadjuvant Chemotherapy in Breast Cancer. J Clin Oncol. 2009;28(1):105–13.

153. Denkert C, Loibl S, Salat C, Sinn B V, Schem C, Endris V, et al. Abstract S1-06: Increased tumor-associated lymphocytes predict benefit from addition of carboplatin to neoadjuvant therapy for triple-negative and HER2-positive early breast cancer in the GeparSixto trial (GBG 66). Cancer Res. 2013;73(24 Supplement):S1-6-S1-6.

154. Issa-Nummer Y, Darb-Esfahani S, Loibl S, Kunz G, Nekljudova V, Schrader I, et al. Prospective validation of immunological infiltrate for prediction of response to neoadjuvant chemotherapy in HER2-negative breast cancer--a substudy of the neoadjuvant GeparQuinto trial. PLoS One. 2013;8(12):e79775.

155. Denkert C, von Minckwitz G, Brase JC, Sinn B V, Gade S, Kronenwett R, et al. Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy With or Without Carboplatin in Human Epidermal Growth Factor Receptor 2–Positive and Triple-Negative Primary Breast Cancers. J Clin Oncol. 2014;33(9):983–91.

156. Loibl S, Untch M, Burchardi N, Huober J, Sinn B V, Blohmer J-U, et al. A randomised phase II study investigating durvalumab in addition to an anthracycline taxane-based neoadjuvant therapy in early triple negative breast cancer - clinical results and biomarker analysis of GeparNuevo study. Ann Oncol Off J Eur Soc Med Oncol. 2019;

157. Salgado R, Denkert C, Campbell C, Savas P, Nuciforo P, Aura C, et al. Tumor-Infiltrating Lymphocytes and Associations With Pathological Complete Response and Event-Free

Survival in HER2-Positive Early-Stage Breast Cancer Treated With Lapatinib and Trastuzumab: A Secondary Analysis of the NeoALTTO Trial. JAMA Oncol. 2015;1(4):448–54.

158. Ladoire S, Arnould L, Apetoh L, Coudert B, Martin F, Chauffert B, et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. Clin Cancer Res. 2008;14(8):2413–20.

159. Garcia-Martinez E, Gil GL, Benito AC, Gonzalez-Billalabeitia E, Conesa MAV, Garcia Garcia T, et al. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. Breast Cancer Res. 2014;16(6):488.

160. Miyashita M, Sasano H, Tamaki K, Chan M, Hirakawa H, Suzuki A, et al. Tumor-infiltrating CD8+ and FOXP3+ lymphocytes in triple-negative breast cancer: its correlation with pathological complete response to neoadjuvant chemotherapy. Breast Cancer Res Treat. 2014;148(3):525–34.

161. Asano Y, Kashiwagi S, Goto W, Kurata K, Noda S, Takashima T, et al. Tumour-infiltrating CD8 to FOXP3 lymphocyte ratio in predicting treatment responses to neoadjuvant chemotherapy of aggressive breast cancer. Br J Surg. 2016;103(7):845–54.

162. Oda N, Shimazu K, Naoi Y, Morimoto K, Shimomura A, Shimoda M, et al. Intratumoral regulatory T cells as an independent predictive factor for pathological complete response to neoadjuvant paclitaxel followed by 5-FU/epirubicin/cyclophosphamide in breast cancer patients. Breast Cancer Res Treat. 2012;136(1):107–16.

163. Asano Y, Kashiwagi S, Onoda N, Noda S, Kawajiri H, Takashima T, et al. Predictive Value of Neutrophil/Lymphocyte Ratio for Efficacy of Preoperative Chemotherapy in Triple-Negative Breast Cancer. Ann Surg Oncol. 2016;23(4):1104–10.

164. Enriquez D, De la Cruz Ku GA, Fernandez M, Eyzaguirre E, Luque R, Paitan D, et al. Predictive value of neutrophil-to-lymphocyte ratio on pathological complete response in triple negative breast cancer. J Clin Oncol. 2017;35(15_suppl):e12012–e12012.

165. Li X, Dai D, Chen B, Tang H, Xie X, Wei W. The value of neutrophil-to-lymphocyte ratio for response and prognostic effect of neoadjuvant chemotherapy in solid tumors: A systematic review and meta-analysis. J Cancer. 2018;9(5):861–71.

166. Koh YW, Lee HJ, Ahn J-H, Lee JW, Gong G. Prognostic significance of the ratio of absolute neutrophil to lymphocyte counts for breast cancer patients with ER/PR-positivity and HER2-negativity in neoadjuvant setting. Tumour Biol. 2014;35(10):9823–30.

167. Xu J, Ni C, Ma C, Zhang L, Jing X, Li C, et al. Association of neutrophil/lymphocyte ratio and platelet/lymphocyte ratio with ER and PR in breast cancer patients and their changes after neoadjuvant chemotherapy. Clin Transl Oncol. 2017;19(8):989–96.

168. Marin Hernandez C, Pinero Madrona A, Gil Vazquez PJ, Galindo Fernandez PJ, Ruiz Merino G, Alonso Romero JL, et al. Usefulness of lymphocyte-to-monocyte, neutrophil-to-monocyte and neutrophil-to-lymphocyte ratios as prognostic markers in breast cancer patients treated with neoadjuvant chemotherapy. Clin Transl Oncol.

2018;20(4):476–83.

169. Chen Y, Chen K, Xiao X, Nie Y, Qu S, Gong C, et al. Pretreatment neutrophil-to-lymphocyte ratio is correlated with response to neoadjuvant chemotherapy as an independent prognostic indicator in breast cancer patients: a retrospective study. BMC Cancer. 2016;16:320.

170. Vonderheide RH, Domchek SM, Clark AS. Immunotherapy for Breast Cancer: What Are We Missing? Clin Cancer Res. 2017;23(11):2640–6.

171. Planes-Laine G, Rochigneux P, Bertucci F, Chrétien A-S, Viens P, Sabatier R, et al. PD-1/PD-L1 Targeting in Breast Cancer: The First Clinical Evidences Are Emerging. A Literature Review. Cancers (Basel). 2019;11(7).

172. Cimino-Mathews A, Thompson E, Taube JM, Ye X, Lu Y, Meeker A, et al. PD-L1 (B7-H1) expression and the immune tumor microenvironment in primary and metastatic breast carcinomas. Hum Pathol. 2016;47(1):52–63.

173. Zacharakis N, Chinnasamy H, Black M, Xu H, Lu Y-C, Zheng Z, et al. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. Nat Med. 2018;24(6):724–30.

174. Ko HS, Fu SM, Winchester RJ, Yu DT, Kunkel HG. Ia determinants on stimulated human T lymphocytes. Occurrence on mitogen- and antigen-activated T cells. J Exp Med. 1979 Aug;150(2):246–55.

175. Bastidas S, Graw F, Smith MZ, Günthard HF, Oxenius A, Oxenius A. CD8 + T Cells Are Activated in an Antigen-Independent Manner in HIV-Infected Individuals. 2016;

176. Saez-Cirion A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, et al. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proc Natl Acad Sci U S A. 2007 Apr;104(16):6776–81.

177. Viallard JF, Blanco P, Andre M, Etienne G, Liferman F, Neau D, et al. CD8+HLA-DR+ T lymphocytes are increased in common variable immunodeficiency patients with impaired memory B-cell differentiation. Clin Immunol. 2006/01/18. 2006;119(1):51–8.

178. Viallard JF, Bloch-Michel C, Neau-Cransac M, Taupin JL, Garrigue S, Miossec V, et al. HLA-DR expression on lymphocyte subsets as a marker of disease activity in patients with systemic lupus erythematosus. Vol. 125, Clinical and Experimental Immunology. 2001. p. 485–91.

179. Pantaleo G, Koenig S, Baseler M, Lane HC, Fauci AS. Defective clonogenic potential of CD8+ T lymphocytes in patients with AIDS. Expansion in vivo of a nonclonogenic CD3+CD8+DR+CD25- T cell population. J Immunol. 1990 Mar;144(5):1696–704.

180. Pantaleo G, De Maria A, Koenig S, Butini L, Moss B, Baseler M, et al. CD8+ T lymphocytes of patients with AIDS maintain normal broad cytolytic function despite the loss of human immunodeficiency virus-specific cytotoxicity. Vol. 87, Proceedings of the National Academy of Sciences of the United States of America. 1990. p. 4818–22.

181. Holling TM, Schooten E, van Den Elsen PJ. Function and regulation of MHC class II

molecules in T-lymphocytes: of mice and men. Hum Immunol. 2004;65(4):282–90.

182. Charoen KM, Fallica B, Colson YL, Zaman MH, Grinstaff MW. Embedded multicellular spheroids as a biomimetic 3D cancer model for evaluating drug and drug-device combinations. Biomaterials. 2014 Feb;35(7):2264–71.

183. Weiswald L-B, Bellet D, Dangles-Marie V. Spherical cancer models in tumor biology. Neoplasia. 2015 Jan;17(1):1–15.

184. Herter S, Morra L, Schlenker R, Sulcova J, Fahrni L, Waldhauer I, et al. A novel three-dimensional heterotypic spheroid model for the assessment of the activity of cancer immunotherapy agents. Cancer Immunol Immunother. 2016/11/17. 2017 Jan;66(1):129–40.

185. Courau T, Bonnereau J, Chicoteau J, Bottois H, Remark R, Assante Miranda L, et al. Cocultures of human colorectal tumor spheroids with immune cells reveal the therapeutic potential of MICA/B and NKG2A targeting for cancer treatment. J Immunother Cancer. 2019;7(1):74.

186. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676–82.

187. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–8.

188. Froehlich K, Haeger J-D, Heger J, Pastuschek J, Photini SM, Yan Y, et al. Generation of Multicellular Breast Cancer Tumor Spheroids: Comparison of Different Protocols. J Mammary Gland Biol Neoplasia. 2016;21(3–4):89–98.

189. Mao Y, Qu Q, Zhang Y, Liu J, Chen X, Shen K. The value of tumor infiltrating lymphocytes (TILs) for predicting response to neoadjuvant chemotherapy in breast cancer: a systematic review and meta-analysis. PLoS One. 2014;9(12):e115103.

190. Cianfrocca M, Goldstein LJ. Prognostic and predictive factors in early-stage breast cancer. Oncologist. 2004;9(6):606–16.

191. Saraiva DP, Jacinto A, Borralho P, Braga S, Cabral MG. HLA-DR in Cytotoxic T Lymphocytes Predicts Breast Cancer Patients' Response to Neoadjuvant Chemotherapy. Front Immunol. 2018;9:2605.

192. Biselli R, Matricardi PM, D'Amelio R, Fattorossi A. Multiparametric flow cytometric analysis of the kinetics of surface molecule expression after polyclonal activation of human peripheral blood T lymphocytes. Scand J Immunol. 1992;35(4):439–47.

193. Cao S-S, Lu C-T. Recent perspectives of breast cancer prognosis and predictive factors. Oncol Lett. 2016;12(5):3674–8.

194. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. Science (80-). 2006;313(5795):1960 LP-1964.

195. Medler TR, Cotechini T, Coussens LM. Immune response to cancer therapy: mounting an effective antitumor response and mechanisms of resistance. Vol. 1, Trends in cancer.

2015. p. 66–75.

196. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer. 2005;93(4):387–91.

197. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;19(1):40–50.

198. Jakowlew SB. Transforming growth factor-beta in cancer and metastasis. Cancer Metastasis Rev. 2006;25(3):435–57.

199. Sun C, Mezzadra R, Schumacher TN. Regulation and Function of the PD-L1 Checkpoint. Immunity. 2018;48(3):434–52.

200. Waugh DJJ, Wilson C. The interleukin-8 pathway in cancer. Clin Cancer Res. 2008;14(21):6735–41.

201. Xiang T, Long H, He L, Han X, Lin K, Liang Z, et al. Interleukin-17 produced by tumor microenvironment promotes self-renewal of CD133+ cancer stem-like cells in ovarian cancer. Oncogene. 2015;34(2):165–76.

202. Intlekofer AM, Takemoto N, Wherry EJ, Longworth SA, Northrup JT, Palanivel VR, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nat Immunol. 2005;6(12):1236–44.

203. Pearce EL, Mullen AC, Martins GA, Krawczyk CM, Hutchins AS, Zediak VP, et al. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science. 2003;302(5647):1041–3.

204. Matsui M, Moriya O, Yoshimoto T, Akatsuka T. T-bet is required for protection against vaccinia virus infection. J Virol. 2005;79(20):12798–806.

205. Kallies A, Good-Jacobson KL. Transcription Factor T-bet Orchestrates Lineage Development and Function in the Immune System. Trends Immunol. 2017;38(4):287–97.

206. Reiser J, Banerjee A. Effector, Memory, and Dysfunctional CD8(+) T Cell Fates in the Antitumor Immune Response. J Immunol Res. 2016;2016:8941260.

207. Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. Nat Rev Immunol. 2016;16(2):79–89.

208. Djenidi F, Adam J, Goubar A, Durgeau A, Meurice G, de Montpréville V, et al. CD8+ CD103+ Tumor–Infiltrating Lymphocytes Are Tumor-Specific Tissue-Resident Memory T Cells and a Prognostic Factor for Survival in Lung Cancer Patients. J Immunol. 2015;194(7):3475 LP-3486.

209. Bo W, Shaoxu W, Hong Z, Zhuowei L, Wen D, Wang H, et al. CD103+ Tumor Infiltrating Lymphocytes Predict a Favorable Prognosis in Urothelial Cell Carcinoma of the Bladder. J Urol. 2015;194(2):556–62.

210. Webb JR, Milne K, Nelson BH. PD-1 and CD103 Are Widely Coexpressed on Prognostically Favorable Intraepithelial CD8 T Cells in Human Ovarian Cancer. Cancer Immunol Res. 2015;3(8):926 LP-935.

211. Workel HH, Komdeur FL, Wouters MCA, Plat A, Klip HG, Eggink FA, et al. CD103 defines intraepithelial CD8+ PD1+ tumour-infiltrating lymphocytes of prognostic significance in endometrial adenocarcinoma. Eur J Cancer. 2016;60:1–11.

212. Antonioli L, Pacher P, Vizi ES, Haskó G. CD39 and CD73 in immunity and inflammation. Trends Mol Med. 2013;19(6):355–67.

213. Bono MR, Fernández D, Flores-Santibáñez F, Rosemblatt M, Sauma D. CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. FEBS Lett. 2015;589(22):3454–60.

214. Gupta PK, Godec J, Wolski D, Adland E, Yates K, Pauken KE, et al. CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. PLOS Pathog. 2015;11(10):e1005177.

215. Duhen T, Duhen R, Montler R, Moses J, Moudgil T, de Miranda NF, et al. Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. Nat Commun. 2018;9(1):2724.

216. Canale FP, Ramello MC, Nunez N, Araujo Furlan CL, Bossio SN, Gorosito Serran M, et al. CD39 Expression Defines Cell Exhaustion in Tumor-Infiltrating CD8(+) T Cells. Cancer Res. 2018 Jan;78(1):115–28.

217. Lee Y, Park J, Park S-H, Shin E-C. CD39+ CD8+ T cells exhibit a distinct phenotype among tumor-infiltrating tumor-antigen specific CD8+ T cells. J Immunol. 2019;202(1 Supplement):195.2 LP-195.2.

218. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: Human memory T-cell subsets. Eur J Immunol. 2013;43(11):2797–809.

219. Sabins NC, Chornoguz O, Leander K, Kaplan F, Carter R, Kinder M, et al. TIM-3 Engagement Promotes Effector Memory T Cell Differentiation of Human Antigen-Specific CD8 T Cells by Activating mTORC1. J Immunol. 2017;199(12):4091 LP-4102.

220. Di Modugno F, Colosi C, Trono P, Antonacci G, Ruocco G, Nistico P. 3D models in the new era of immune oncology: focus on T cells, CAF and ECM. J Exp Clin Cancer Res. 2019;38(1):117.

221. Koeck S, Kern J, Zwierzina M, Gamerith G, Lorenz E, Sopper S, et al. The influence of stromal cells and tumor-microenvironment-derived cytokines and chemokines on CD3+CD8+ tumor infiltrating lymphocyte subpopulations. Oncoimmunology. 2017;6(6):e1323617.

222. Machicote A, Belén S, Baz P, Billordo LA, Fainboim L. Human CD8(+)HLA-DR(+) Regulatory T Cells, Similarly to Classical CD4(+)Foxp3(+) Cells, Suppress Immune Responses via PD-1/PD-L1 Axis. Front Immunol. 2018;9:2788.

223. Lovitt CJ, Shelper TB, Avery VM. Doxorubicin resistance in breast cancer cells is mediated by extracellular matrix proteins. BMC Cancer. 2018;18(1):41.

224. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov;68(6):394–424.

225. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009 Jan;45(2):228–47.

226. Sconocchia G, Eppenberger-Castori S, Zlobec I, Karamitopoulou E, Arriga R, Coppola A, et al. HLA class II antigen expression in colorectal carcinoma tumors as a favorable prognostic marker. Neoplasia. 2014 Jan;16(1):31–42.

227. Diao J, Xia T, Zhao H, Liu J, Li B, Zhang Z. Overexpression of HLA-DR is associated with prognosis of glioma patients. Int J Clin Exp Pathol. 2015;8(5):5485–90.

228. Johnson DB, Estrada M V, Salgado R, Sanchez V, Doxie DB, Opalenik SR, et al. Melanoma-specific MHC-II expression represents a tumour-autonomous phenotype and predicts response to anti-PD-1/PD-L1 therapy. Nat Commun. 2016 Jan;7:10582.

229. Pruneri G, Vingiani A, Denkert C. Tumor infiltrating lymphocytes in early breast cancer. Breast. 2018 Feb;37:207–14.

230. Khoury T, Nagrale V, Opyrchal M, Peng X, Wang D, Yao S. Prognostic Significance of Stromal Versus Intratumoral Infiltrating Lymphocytes in Different Subtypes of Breast Cancer Treated With Cytotoxic Neoadjuvant Chemotherapy. Appl Immunohistochem Mol Morphol AIMM. 2018 Sep;26(8):523–32.

231. Sakhdari A, Mujib S, Vali B, Yue FY, MacParland S, Clayton K, et al. Tim-3 negatively regulates cytotoxicity in exhausted CD8+ T cells in HIV infection. PLoS One. 2012;7(7):e40146.

232. Samji T, Khanna KM. Understanding memory CD8(+) T cells. Immunol Lett. 2017 May;185:32–9.

233. Braun J, Frentsch M, Thiel A. Hobit and human effector T-cell differentiation: The beginning of a long journey. Eur J Immunol. 2015 Oct 1;45(10):2762–5.

234. Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. Nat Rev Immunol. 2005 Nov;5(11):844–52.

235. Konjar Š, Veldhoen M. Dynamic Metabolic State of Tissue Resident CD8 T Cells. Front Immunol. 2019;10:1683.

236. van der Windt GJW, Everts B, Chang C-H, Curtis JD, Freitas TC, Amiel E, et al. Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. Immunity. 2012 Jan;36(1):68–78.

237. Liu Y, Ma C, Zhang N. Tissue-Specific Control of Tissue-Resident Memory T Cells. Crit Rev Immunol. 2018;38(2):79–103.

238. Arruvito L, Payaslian F, Baz P, Podhorzer A, Billordo A, Pandolfi J, et al. Identification and clinical relevance of naturally occurring human CD8+HLA-DR+ regulatory T cells. J Immunol. 2014 Nov;193(9):4469–76.

239. Xu Z, Ho S, Chang C-C, Zhang Q-Y, Vasilescu E-R, Vlad G, et al. Molecular and Cellular Characterization of Human CD8 T Suppressor Cells. Front Immunol. 2016;7:549.

240. Rifa'i M, Kawamoto Y, Nakashima I, Suzuki H. Essential roles of CD8+CD122+ regulatory T cells in the maintenance of T cell homeostasis. J Exp Med. 2004 Nov;200(9):1123–34.

241. Chatila T, Silverman L, Miller R, Geha R. Mechanisms of T cell activation by the calcium ionophore ionomycin. J Immunol. 1989 Aug;143(4):1283–9.

242. Huse M. The T-cell-receptor signaling network. J Cell Sci. 2009 May 1;122(9):1269 LP-1273.

243. Soliman H, Mediavilla-Varela M, Antonia S. Indoleamine 2,3-dioxygenase: is it an immune suppressor? Cancer J. 2010;16(4):354–9.

244. Linch SN, McNamara MJ, Redmond WL. OX40 Agonists and Combination Immunotherapy: Putting the Pedal to the Metal. Front Oncol. 2015/03/13. 2015;5:34.

Appendix

Published Paper

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ORIGINAL RESEARCH
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HLA-DR in Cytotoxic T Lymphocytes Predicts Breast Cancer Patients' Response to Neoadjuvant Chemotherapy

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Prediction of breast cancer response to Neoadjuvant Chemotherapy (NACT) is an urgent need to promptly direct non-responder patients to alternative therapies. Infiltrating T lymphocytes, namely cytotoxic T lymphocytes (CTLs) have been appointed as predictors of response. However, cancer cells have the ability to dampen CTLs' activity and thus, the prognostic value of the CTLs, *per se*, is debatable. Here, we disclose that more than the occurrence of CTLs, it is their activation state, revealed by HLA-DR expression, that can accurately predict response to NACT. Flow cytometry analysis of breast cancer biopsies showed that the frequency of CTLs and other lymphocytes were similar regardless disease stage and between NACT responders and non-responders. However, only breast cancer patients without axillary lymph node metastasis and NACT responders have HLA-DR^{hi} CTLs. Interestingly, HLA-DR levels in tumor CTLs is correlated with HLA-DR levels in systemic CTLs. These HLA-DR+ CTLs produce IFN-γ and Granzyme B, enlightening their effector and probable anti-tumor activity profile. Moreover, the level of HLA-DR in CTLs is negatively correlated with the level of HLA-DR in T regulatory lymphocytes and with immunosuppressive and pro-tumor molecules in the tumor microenvironment. Hence, HLA-DR levels in CTLs is a highly sensitive and specific potential predictive factor of NACT-response, which can be assessed in blood to guide therapeutic decisions.

Keywords: breast cancer, cytotoxic T lymphocytes, HLA-DR, prediction, neoadjuvant chemotherapy

INTRODUCTION

Breast cancer remains one of the main causes of cancer-related deaths in women worldwide (1). The disease can be divided in three different subtypes—hormone-positive (estrogen and/or progesterone—ER+ and/or PR+), human epidermal growth factor receptor 2 (HER2) positive and triple negative breast cancer (TNBC), which lacks the three mentioned markers. In the past years, advances in breast cancer treatment have been made, namely with the introduction of preoperative neoadjuvant chemotherapy (NACT) in selected cases of advanced tumors (with tumor size larger than 2 cm and/or disease extension to axillary lymph node) or inflammatory breast cancer. This treatment is effective in reducing the size of the primary tumor, allowing breast conservation in

selected cases, and some patients achieve a pathological complete response (pCR) (2). However, this only happens in less than 50% of the patients (3, 4) and residual disease after NACT is a strong predictor of relapse (2, 5). Hence, it is essential to find a good marker of response to NACT, in order to promptly direct patients to alternative therapies, therefore avoiding the misuse of resources and the potential toxicity associated with NACT.

Increasing evidence suggests that anti-cancer chemotherapy is influenced by the immune system (6). Indeed, tumors can be heavily infiltrated by immune cells, in particular, tumor infiltrating lymphocytes (TILs), which have been associated with good prognosis in various cancers, including breast cancer (6). Several reports have been advocating that TILs, especially cytotoxic T lymphocytes (CTLs), due to their anti-tumor cytotoxic activity, could serve as a robust marker for predicting pCR rate after NACT (7). However, even with an effort to standardize TILs evaluation (8), data are still conflicting, and other reports describe that TILs and their subsets could not show any predictive value, particularly in hormone-positive subtype (6), possibly because these patients are further treated with endocrine therapy for years. Furthermore, T regulatory cells (Tregs), by suppressing the effector immune response, are normally associated with poor prognosis. However, they are still described to have an ambiguous role (9).

Actually, tumor cells have mechanisms to escape the immune system, and the activity of CTLs can be hampered by them. For instance, tumors may express inhibitory molecules like programmed death-ligand 1 (PD-L1) which bind the inhibitory receptor programmed death 1 (PD-1) in CTLs, inducing negative regulatory pathways that limit the activity of these cells (10, 11). Other inhibitory immune checkpoints, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and the secretion of immunosuppressing molecules, such as IL-10, TGF-β or Indoleamine 2, 3-Dioxygenase (IDO), could also directly or indirectly (by recruiting and activating Tregs), negatively impact CTLs' activity (10, 11).

Considering the aforementioned, the presence of CTLs *per se* provides limited information regarding NACT outcome, and the activation level of these cells, as a result of the overall tumor immune status, should be taken into account in order to better predict response to NACT.

HLA-DR is recognized as a marker of T cell activation (12, 13) and has been shown to be increased in CTLs in autoimmune diseases (14) and in patients with HIV infection (15).

In this work, by flow cytometry analysis of fresh samples, we demonstrated that a subset of CTLs expressing HLA-DR is enriched in breast cancer without axillary lymph node metastasis comparing with breast cancer with axillary lymph node metastasis, although the average frequency of CTLs is similar between groups. Furthermore, biopsies from NACT responders also have HLA-DR^{hi} CTLs, while no differences were observed concerning the density of CTLs between NACT responders and non-responders. Interestingly, the profile of HLA-DR^{hi} CTLs was negatively correlated with high levels of pro-tumor and immunosuppressive molecules in the tumor immune microenvironment.

TABLE 1 | Characteristics of patients enrolled in this study (age, body mass index, and menopause).

Age	Median−58 (range: 35–87)
Body mass index (BMI)	Median−26.03 (range: 17.04–39.74)
Post-menopause	62.30%
ER+ (PR −/+)	68%
HER2+ including triple positive breast cancer	16%
TNBC	16%
Grade	G1–8.26%
	G2–58.68%
	G3–33.06%
Ki67	Median−21.75% (range: 2–98.4%)
Dimension (mm)	Median−25 (range: 7–90)
Axillary lymph node invasion status	Negative−51.09%
	Positive−48.91%
Response (if NACT)	Good response−43.33%
	Bad response−56.67%

Clinical data, such as subtype of breast cancer, grade, Ki67, tumor dimension, node status and response to treatment are also described.

Therefore, we propose that HLA-DR+ CTLs have the potential to be used in clinical settings to predict breast cancer patients' response to NACT, with the advantage of being easily accessed in blood.

MATERIALS AND METHODS

Patient Samples

Forty-eight fresh biopsies and ninety-six non-matched surgical samples of breast cancer (BC) patients were collected in saline solution. Matched peripheral blood from 31 BC patients was collected in Vaccutainer tubes with EDTA (BD Biosciences). Whole blood from 13 healthy donors was also collected for comparison studies. For *in vitro* studies, patients' peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll gradient (Merck Milipore) and PBMCs from buffy coats of 5 healthy donors, donated by Instituto Português do Sangue e da Transplantação, were used for control purposes. Formalin-fixed paraffin embedded (FFPE) tissue was collected from 12 matched patients divided according to the axillary lymph node invasion status and response to neoadjuvant chemotherapy (NACT). A summary of the patients characteristics are given in **Table 1**.

Fresh samples were handled within 1-day post collection. Fresh tumors and biopsies were mechanically dissociated with a BD Medicon (BD Bioscience), filtered and washed once with PBS 1X. PBMCs after isolation were cryopreserved in 90% fetal bovine serum (FBS, Biowest) and 10% dimethyl sulfoxide (DMSO, Sigma Aldrich) until further use.

Samples were gathered from Hospital CUF Descobertas, Hospital Prof. Doutor Fernando Fonseca and Hospital de Vila Franca de Xira. For each patient, written informed consent and approval by the Ethical Committee of the hospitals and of the NOVA Medical School were obtained. The study is in compliance with the Declaration of Helsinki.

Antibodies

For flow cytometry analysis, tumor and blood samples were stained with a cocktail of monoclonal mouse anti-human conjugated antibodies (mAbs): anti-CD45-PercP (clone HI30), anti-CD3-PercP (HIT3a), anti-CD3-APC (UCHT1), anti-CD19-PE (HIB19), anti-CD15-PE (HI98), anti-CD161-FITC (HP-3G10), anti-CD4-FITC (OKT4), anti-CD8-PE (HIT8a), anti-HLA-DR-APC (L243), anti-CD127-PE-Cy7 (A019D5), anti-CD1c-APC-Cy7 (L161), anti-CD163-PE (GHI/61), anti-CD206-APC-Cy7 (15–2), anti-PD-1-FITC (EH12.2H7), anti-PD-L1-APC (29E2A3), anti-CTLA4-PE (L3D10), anti-CD69-APC-Cy7 (FN50), anti-Tim3-APC (F38-2E2), anti-IL-8-APC (E8N1), anti-IFN-γ-PE (4S.B3), anti-IFN-γ-APC-Cy7 (4S.B3), anti-Granzyme B-FITC (QA16A02), anti-IL-1β-FITC (JK1B-1), anti-IL-2-PE-Cy7 (MQ1-17H12), anti-IL-6-APC (MQ2-13A5), anti-IL-17-FITC (BL168), anti-IL-23/IL-12-PE (C11.5), anti-TGF-β-APC (TW4-6H10), all from Biolegend; anti-IDO-PE (eyedio) and anti-IL-10-FITC (BT-10), both from eBioscience; anti-CD25-PE (MEM-181) and anti-CD11b-FITC (LT11) from ImmunoTools.

The antibodies used for immunofluorescence were: mouse monoclonal anti-human CD8 (32-M4) from Santa Cruz Biotechnology and rabbit polyclonal anti-human HLA-DRA from Sigma Aldrich.

Flow Cytometry

BC samples were stained with BD Horizon™ Fixable Viability Stain 450 (BD Biosciences) and with the cocktail of antibodies, fixed and permeabilized with Fix/Perm kit (eBiosciences) followed by intracellular staining. For whole blood, staining with antibodies was followed by a step of red blood cells lysis with RBC lysis buffer (Biolegend).

For immunophenotyping we classified cytotoxic T lymphocytes as CD45+/CD3+/CD8+; helper T lymphocytes as CD45+/CD3+/CD4+; regulatory T lymphocytes as CD45+/CD3+/CD4+/CD25^{hi}/CD127^{lo}; B lymphocytes as CD45+/CD19+; NK cells as CD45+/CD161+; M2 macrophages as CD45+/CD11b+/CD163+/CD206+; M1 macrophages as CD45+/CD11b+/CD163negative/CD206negative; dendritic cells as CD45+/CD1c+; and neutrophils as CD45+/CD15+.

Data was acquired in BD FACS Canto II cell analyzer with FACSDiva Software v8.0.1 (BD Biosciences) and the results were analyzed using FlowJo software v10. The data is presented as percentage of the populations in respect to the gate of single cells, following the gate strategy represented in **Figure S1**. To analyze the expression levels of HLA-DR in CTLs or Tregs, we considered the median fluorescent intensity of positive population and normalized it to the negative population, as previously described (16). The negative population was superimposed with the unstained control.

Ex vivo Stimulation Assay

Isolated PBMCs were cultured in 96 well-plates with U bottom (Sigma Aldrich) with RMPI-1640 (Gibco) supplemented with 10% FBS and 1% Penicillin/Streptomycin (GE Healthcare). Stimulation was performed during 4 h (or overnight for HLA-DR expression) with 35 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) and 1 μg/mL of ionomycin (Merck Milipore). Brefeldin A (Biolegend) was added for 4 h to stop the extracellular transport. Cells were collected and stained with mAbs and analyzed by flow cytometry.

Immunofluorescence

Immunofluorescence was performed in formalin-fixed paraffin embedded tissues (FFPE). Paraffin was removed in xylene (10 min) and passed through a gradient of alcohol (100, 96, and 70%) and dH_2O. Antigen retrieval was performed in 2 cycles of 15 mins each with 1 mM EDTA (Sigma Aldrich) with 0.05% Tween-20 (Sigma Aldrich) in the microwave (900W). The slides were permeabilized with PBS 1X + 0.3% Triton X-100 (ACROS Organics) and blocked with PBS 1X + 0.1% Triton X-100 + 1% bovine serum albumin (BSA, Sigma Aldrich) + 1.5% goat serum (Sigma Aldrich). Staining with primary antibody (1:100) was performed overnight at 4°C followed by the incubation with secondary antibody for 2 h in the dark at room temperature. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI; 0.001 mg/mL in PBS, Sigma-Aldrich) and the slides were mounted in Fluorescent Mounting Media (DAKO). Images were taken in a confocal microscope (LSM710, Zeiss) and analyzed in Fiji software (17).

ELISA

The patients' plasma was isolated from whole blood and frozen for further cytokine analysis. The quantity of secreted cytokines was measured using ELISA technique, namely human IL-10 (ImmunoTools) and IFN-γ (Biolegend) kits were used according to the manufacturer's instructions. Cytokine concentration was calculated using the specific standard curves.

Cell Sorting

PBMCs were stained with the viability dye followed by the mAbs anti-CD3-PercP, anti-CD8-PE and anti-HLA-DR-APC. Cells were sorted in CD3+/CD8+/HLA-DR+ and CD3+/CD8+/HLA-DRnegative in a FACS Aria III (BD Biosciences) with an efficiency of 98% (**Figure S2**).

qRT-PCR

Total RNA of sorted cells was extracted using RNeasy Micro Kit (Qiagen) and reverse transcribed with Transcriptor High Fidelity cDNA synthesis kit (Roche). Quantitative real-time PCR (qRT-PCR) was performed with several primers, described in **Table 2**, using Roche LightCycler 480 and FastStart Essential DNA Green Master Mix (Roche). Cyclic conditions were: 95°C for 10 min, followed by 45 amplification cycles, each consisting of 10 s at 95°C, 10 s at 56°C, and 20 s at 72°C, and finally a melting step of 10 s at 95°C, 60 s at 65°C, and 1 s at 97°C.

The relative mRNA levels were normalized against the values obtained for the housekeeping gene RPL13A and calculated by the formula $2^{-\Delta Ct} \times 1000$ that gives us the number of mRNA molecules of the gene of interest per 1000 molecules of the endogenous control (17, 18).

TABLE 2 | Genes assessed with qRT-PCR and the primers used.

Gene	Forward primer	Reverse primer
TNFα	AGATGATCTGACTGCCTGGG	CTGCTGCACTTTGGAGTGAT
Granzyme B	GGGGGACCCAGAGATTAAAA	CCATTGTTTGGTCCATAGGAG
Perforin	GCAATGTGCATGTGTCTGTG	GGGAGTGTGTACCACATGGA
Eomes	CAGCACCACCTCTACGAACA	CGCCACCAAACTGAGATGAT
RPL13A	TGCGTCTGAAGCCTACAAGA	TCCGTAGCCTCATGAGCTGTT

In vitro Co-culture Assay

HS 578T cell line was maintained for 4 days in DMEM (Biowest) with 10% FBS and 1% Penicillin/Streptomycin. After that period, the supernatant and cells were harvested for the co-culture. The supernatant was further centrifuged at 2000 rpm for 5 min to eliminate cellular debris and possible cellular antigens. PBMCs were plated on a 96 well plate U bottom in 4 conditions: monoculture, with the addition of the supernatant from HS 578T culture, with a canonical stimulus of PMA and ionomycin, and in co-culture on a ratio of 20:1 (PBMCs:HS 578T). PBMCs were collected 48 h after and analyzed by flow cytometry.

Statistical Analysis

Statistical analysis was performed in GraphPad Prism v6 and statistical significance was considered for $p < 0.05$. Comparison between samples was performed by a nonparametric Mann-Whitney test and correlations were calculated with Spearman *r*-test. ROC curves were performed to assign a threshold to divide NACT responders from non-responders. This analysis was executed for both HLA-DR expressing CTLs and Tregs. The determined area under the curve, sensitivity and specificity were taken into account. The cut-off point for the HLA-DR expression level in CTLs was a parameter also determined by ROC curve analysis, considering the expression value that corresponded to the maximum of sensitivity and specificity. Paired *t*-test was used for the analysis of the co-culture assay.

RESULTS

Clinical and Pathological Characteristics

For this study, 144 fresh samples (137 pre-treatment - 48 biopsies and 89 surgical specimens - and 7 post-NACT surgical specimens) were collected prospectively. Data from these patients are displayed in **Table 1**. Most patients have estrogen receptor (ER) positive breast cancer, have high body mass index and are post-menopausal.

These samples, except the post-NACT specimens, were used in the first phase of the study for a global analysis of tumor infiltrating immune populations. Then, aiming to find an adequate biomarker of response to NACT, from these samples, we used the first consecutive biopsies ($n = 30$) of patients that were selected for NACT and related the activation status of their lymphocytes with their response to treatment.

HLA-DR-expressing T Lymphocyte Populations Can Distinguish Breast Cancer Axillary Lymph Node Invasion Status

In the first phase of the study, and to get insight into the composition and the role of immune infiltrate in breast cancer (BC), we used a flow cytometry multipanel and analyzed several immune populations (gate strategy in **Figure S1**) in 137 BC patients prior to treatment implementation (48 biopsies and 89 surgical specimens). First, these patients were divided according to the axillary lymph node invasion status. Patients without axillary lymph node metastasis had a similar immunophenotype when compared with patients with axillary lymph node metastasis (**Figures 1A–C**). Indeed, although there was high heterogeneity in the degree of immune infiltration between patients, the average value of the frequency of T lymphocytes, B lymphocytes, NK cells (**Figure 1A**), neutrophils, dendritic cells, M1 and M2 macrophages (**Figure 1B**) infiltrating the tumor, were similar between the two groups of patients. Additionally, the average value of the frequency of T lymphocyte populations—helper (Th), cytotoxic (CTLs), and regulatory (Tregs)—were similar between these two groups (**Figure 1C**).

Then, we considered the activation state of T lymphocyte populations, by the analysis of established T cell activation markers, CD69 and HLA-DR. Interestingly, this analysis revealed significant differences between patients without axillary lymph node invasion and patients with axillary lymph node invasion, regarding the expression of HLA-DR. Namely, HLA-DR expression level was higher in CTLs ($p = 0.008$) and lower in Tregs ($p = 0.001$) in patients without axillary lymph node metastasis when compared to patients with axillary lymph node metastasis (**Figures 1D,F**). Furthermore, the expression of HLA-DR in CTLs was negatively correlated with the expression of this marker in Tregs ($r = -0.54$, $p < 0.0001$, **Figures 1E**, **6**).

No differences were observed between patients of the two groups regarding HLA-DR expression in Th cells, but we did not discriminate between Th subsets (e.g., Th1, Th2, Th17), that might influence cancer differently.

The analysis of CD69 expression in T lymphocyte populations did not lead to the same result obtained by the analysis of HLA-DR (data not shown). This may be due to the fact that CD69 is an early, transiently expressed, activation marker of T cells (18), while HLA-DR is increased later in the activation process of T cells (19), remaining in the cell surface.

Besides dividing the patients regarding the presence or absence of axillary lymph node invasion, we have also divided them regarding breast cancer subtype (ER+, HER2+, or TNBC), grade (G1, G2, or G3), age (<50 years old or >50 years old), tumor dimension (<20 or >20 mm) and body mass index (low, normal or overweight). HLA-DR expression in T cell populations was not significantly different in these other comparisons (results not shown).

Thus, independently of BC subtype, expression of HLA-DR in distinct T cell populations can differentiate patients with axillary lymph node metastasis (HLA-DR^{hi} Tregs) from patients without axillary lymph node metastasis (HLA-DR^{hi} CTLs). These results suggested that more than simply the presence

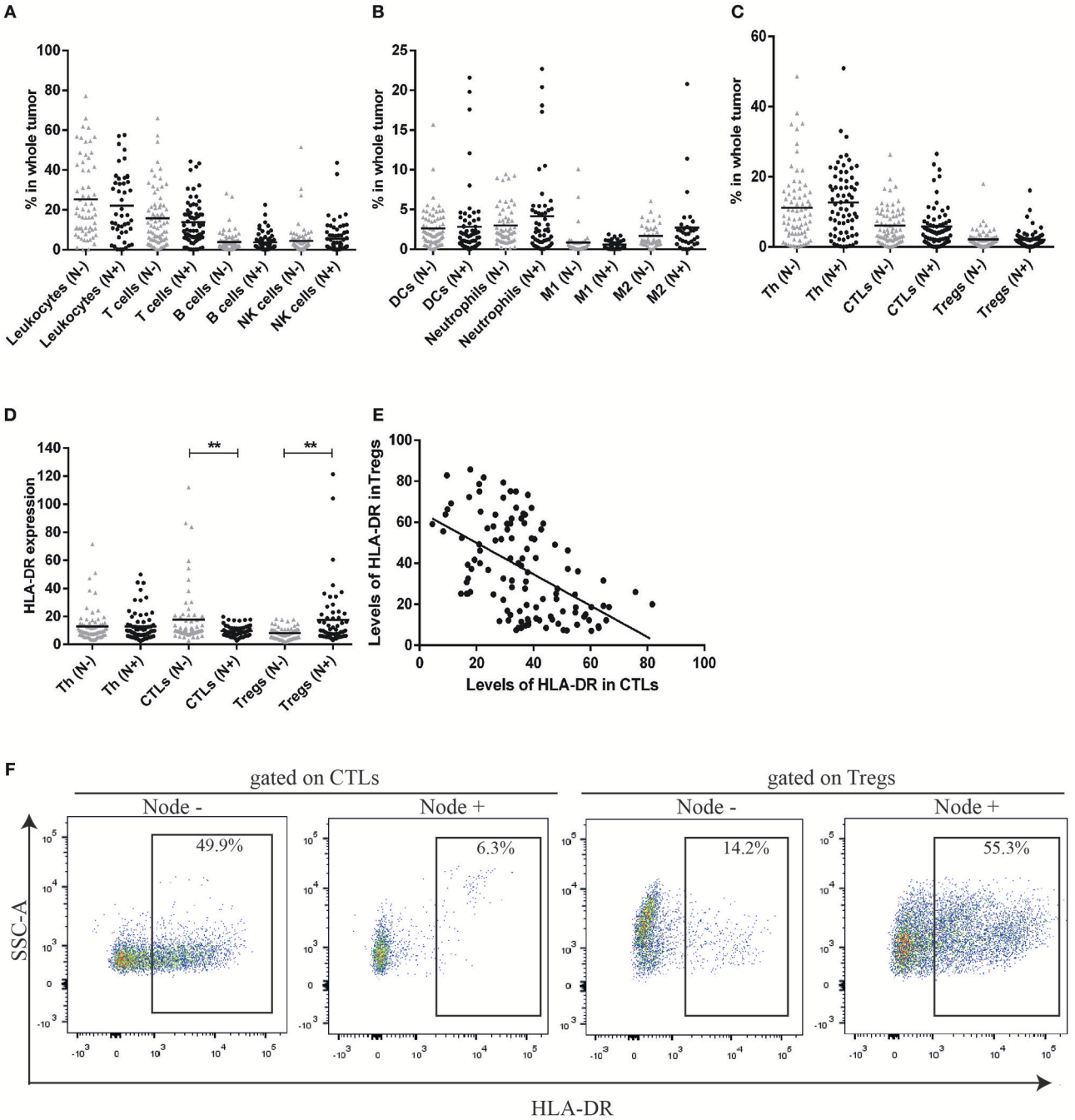

FIGURE 1 | HLA-DR expression in CTLs and Tregs can distinguish between patients without axillary lymph node metastasis (N–) and patients with axillary lymph node metastasis (N+) contrarily to the percentage of distinct cell populations. **(A)** Percentage of leukocytes, T cells, B cells and NK cells in the tumor bulk from patients without axillary lymph node metastasis (N–, gray triangles, $n = 70$, mean) and patients with axillary lymph node metastasis (N+, black dots, $n = 67$, mean). **(B)** Percentage of dendritic cells (DCs), Neutrophils, M1 and M2 macrophages in the same populations. **(C)** Percentage of distinct T cell populations (helper T cells - Th, cytotoxic T cells - CTLs and regulatory T cells - Tregs) in the tumor from both groups of patients. The percentage of each population was obtained in respect to the gate in single cells (**Figure S1**). **(D)** Level of HLA-DR in Th, CTLs and Tregs in the tumor from patients with and without axillary lymph node invasion, expressed as the median fluorescent intensity of positive population normalized relatively to negative population (**$p < 0.01$). **(E)** Correlation between HLA-DR+ CTLs and HLA-DR+ Tregs in breast cancer samples (Spearman $r = -0.54$, $p < 0.0001$). **(F)** Representation of a flow cytometry analysis of HLA-DR expression gated on CTLs and Tregs and differences between a tumor from a patient without axillary lymph node metastasis and a tumor from a patient with axillary lymph node metastasis.

and/or the quantity of immune cells within the tumor immune microenvironment, the quality (i.e., the activation state) of certain immune cells, namely CTLs and Tregs, might be relevant for cancer progression.

HLA-DR^{hi} CTLs Are Associated With Response to Neoadjuvant Chemotherapy

Considering that the HLA-DR expression in T lymphocyte populations allows the distinction of axillary lymph node invasion status, we asked if this trait could also be useful to predict patients' response to NACT. NACT was identical in all patients and was composed of 4 cycles of anthracyclines (doxorubicin) and cyclophosphamide, followed by 12 weeks of paclitaxel. Only the addition of trastuzumab during paclitaxel administration differed in HER2 patients. Biopsies from 30 patients that underwent NACT were here analyzed (pre-treatment) by flow cytometry and, after the treatment, patients were divided in responders and non-responders, according to their radiological and pathological outcome. NACT responders were classified as patients that achieved a pathological complete response (pCR, $n = 6$) or that had a pathological partial response with less than 10% of the initial tumor still present after treatment and without axillary lymph node involvement ($n = 7$). NACT non-responders included patients that still maintained more than 50% of the initial tumor mass after treatment ($n = 6$), or patients that developed brain, liver and/or lung metastasis during NACT ($n = 3$) or patients that had an early relapse after NACT ($n = 8$). Patients considered NACT responders were either ER+ (30.77%), HER2 (38.46%), or TNBC (30.77%). Interestingly, NACT responders showed higher levels of HLA-DR in CTLs ($p = 0.0003$) and lower levels of HLA-DR in Tregs ($p = 0.009$) when compared to the non-responders (**Figure 2B**). Again, the percentage of T lymphocyte populations was not statistically different between the two groups (**Figure 2A**). This finding is independent of BC subtype (**Figure S3**). Indeed, the percentage of CTLs, *per se*, was not sufficient to discriminate responders from non-responders, across all three BC subtypes, while HLA-DR expression levels in CTLs can segregate both groups, especially in ER+ and TNBC (**Figure S3**). In HER2 breast cancer, HLA-DR levels in CTLs was still higher in responders, comparing with non-responders, although not statistically significant. This may be explained by the fact that we only have 3 samples from non-responders, including the only sample where the HLA-DR^{hi} in CTLs did not correspond to a NACT responder.

In order to verify the predictive value of HLA-DR expression in CTLs for NACT response, in general, we plotted the levels of HLA-DR in CTLs and Tregs, of the total 30 biopsies in one single column, regardless of the response (**Figure 2C**). Excitingly, even with a small sample size, a robust separation of responders (black triangles) and non-responders (gray dots) was observed according to the expression level of HLA-DR, especially in CTLs (**Figure 2C**).

Additionally, ROC curve analysis was performed, leading to a statistically valid cut-off point for the HLA-DR expression level in CTLs (8.943 - value above which patients are NACT responders) and in Tregs (5.655 - value beneath which patients are NACT responders). For HLA-DR-expressing CTLs, the area under the ROC curve was 0.959, the sensitivity was 94.12% and the specificity was 100%. For HLA-DR-expressing Tregs, the area under the ROC curve was 0.849, the sensitivity was 81.25% and the specificity was 75%. These results highlighted that, mainly, HLA-DR-expressing CTLs evaluated in biopsies could predict response to NACT with accuracy. To clarify the few borderline cases, the analysis of HLA-DR+ Tregs can also be useful, since there is a strong negative correlation between both markers (**Figure 1E**).

It has been reported that chemotherapeutic agents are able to alter the immune context of breast cancer, usually boosting anti-tumor CTLs' activity (20). Thus, post-NACT surgical samples, non-matched with the pre-NACT samples, were analyzed by flow cytometry. These specimens revealed that non-responders have infiltrating CTLs with low levels of HLA-DR ($p = 0.04$) and Tregs with high levels of HLA-DR (non-statistical) when compared to responders (**Figure S4**), similar to specimens collected before the treatment.

Other immune signatures of cancer immune status, namely PD-1, PD-L1, CTLA4, T cell immunoglobulin and mucin domain 3 (Tim3), IDO and IL-10, that potentially could be used as biomarkers of NACT response were analyzed in the pre-treatment biopsies. Although differences could be observed between biopsies of NACT responders and non-responders, none of them was statistically significant, as was HLA-DR in CTLs. Moreover, we did not observe a segregation of these groups of patients according to the level of expression in any of these molecules in the tumor environment (**Figure 2D**). This emphasizes that HLA-DR^{hi} CTLs (with HLA-DR above the threshold value) could potentially be implemented in the clinic to distinguish NACT responders from non-responders.

HLA-DR-expressing CTLs Are Located Preferably in Intraepithelial Tumor Structures Than in the Surrounding Normal Tissue

Besides the frequency and the type of immune cells in the tumor tissue, their location could also be important to predict patients' clinical outcome (21). For instance, it was found that disease-free survival time is statistically shorter in colorectal carcinoma patients without TILs in the center of the primary tumor mass (22).

Therefore, we have assessed, by immunofluorescence, selected formalin-fixed paraffin embedded (FFPE) tissue samples matched with the previously analyzed by flow cytometry, and evaluated where the HLA-DR+ CTLs are mainly located. We observed that either in surgical samples from patients without axillary lymph node metastasis and in biopsies of patients with good response to NACT (**Figure 3**), HLA-DR-expressing CTLs were mainly present in intraepithelial tumor structures. Representative images in **Figure 3** show a co-localization of anti-CD8 and anti-HLA-DR within the tumor, while no co-localization between these two markers was observed in the tumor surrounding normal tissue. Indeed, outside the tumor

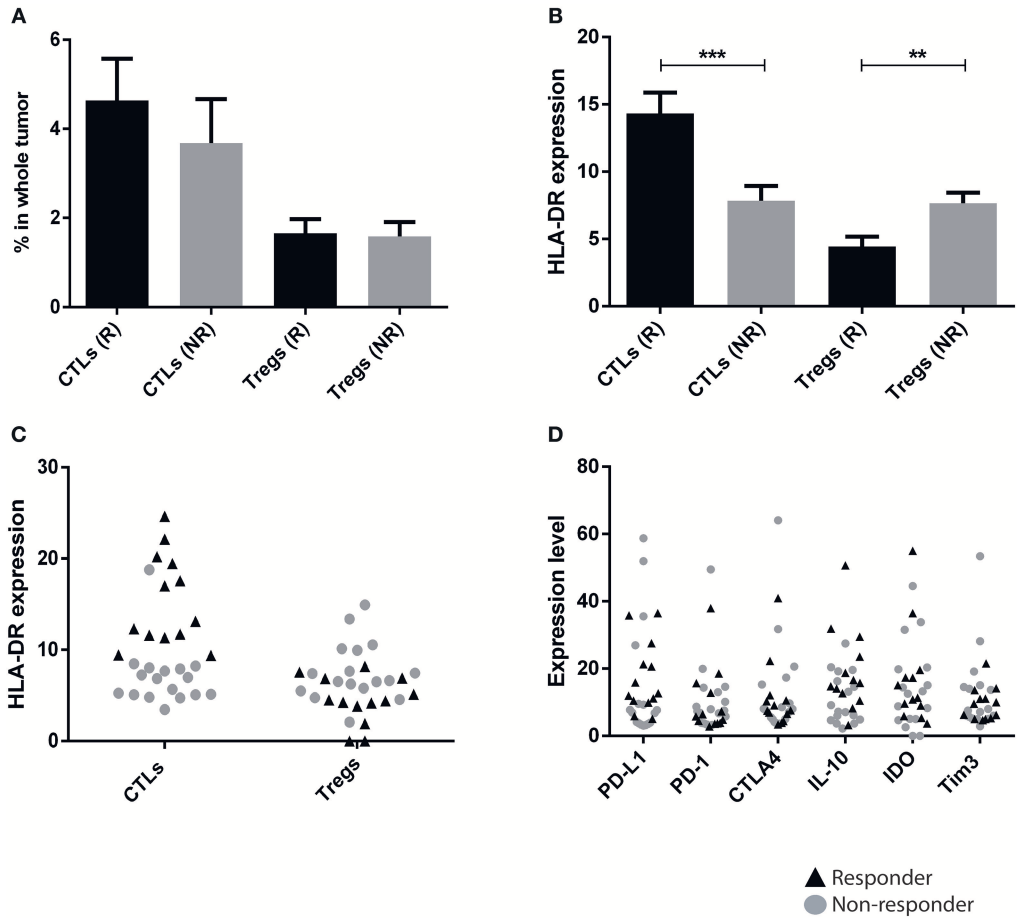

FIGURE 2 | HLA-DR level in CTLs is a predictive factor of patients' response to neoadjuvant chemotherapy (NACT). **(A)** Percentage of CTLs and Tregs in biopsies from breast cancer patients that responded to NACT (R, black bar, $n = 13$, mean ± SD) and that had no response (NR, gray bar, $n = 17$, mean ± SD). The percentage of each population was obtained in respect to the gate in single cells (**Figure S1**). **(B)** Level of HLA-DR in CTLs and Tregs in responders and non-responders, expressed as the median fluorescent intensity of positive population normalized relatively to negative population (**$p < 0.01$, ***$p < 0.001$). **(C)** HLA-DR expression in CTLs and Tregs with data for responders and non-responders plotted in a single column (black triangles and gray dots, respectively). **(D)** The same analysis as in **(C)** was performed for other markers that theoretically could be used in the clinic: PD-L1, PD-1, CTLA4, IL-10, IDO and Tim3.

margins, CTLs were found, but no co-localization with HLA-DR occurred, as HLA-DR staining was more spread throughout the tissue. These results indicate that HLA-DR+ CTLs are located in intraepithelial tumor structures rather than in the surrounding normal tissue. This may suggest that the presence of HLA-DR+ CTLs in a high proximity with tumor antigens and tumor-released soluble factors, could lead to a more efficient anti-tumor activity.

Circulating CTLs Maintain HLA-DR of Tumor Infiltrating CTLs in BC Patients

To verify an association between the tumor immune microenvironment and peripheral blood, blood samples were also collected from 31 patients, prior to treatment implementation, and immunophenotyped by flow cytometry. Intriguingly, we observed that the expression of HLA-DR in tumor infiltrating CTLs correlated with HLA-DR expression in circulating CTLs ($r = 0.58$, $p = 0.001$) (**Figure 4A**). Moreover, circulating CTLs have higher levels of HLA-DR in NACT responders comparing with non-responders and healthy donors (**Figure 4B**, $p < 0.05$), therefore allowing the distinction between responders and non-responders (**Figures 4B,C**). Curiously, non-responders have even lower levels of HLA-DR in circulating CTLs than healthy donors (**Figure 4B**, $p < 0.01$).

IFN-γ and IL-10 levels in pre-treatment patients' plasma, assessed by ELISA, revealed that patients without axillary lymph node invasion, as well as NACT responders, had higher levels of IFN-γ ($p = 0.004$, $p = 0.01$, respectively, **Figures 4D,F**) and lower levels of circulating IL-10 ($p = 0.02$, non-statistical, respectively, **Figures 4E,G**), which is in agreement with the presence of more activated or less activated circulating CTLs, correspondingly.

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of NACT responders and non-responders, at the time of biopsy, and cultured under a canonical stimulus, to clarify if they could recapitulate the characteristics of the tumor infiltrating immune cells *ex vivo*, when exposed exactly to the same conditions. PBMCs isolated from healthy donors

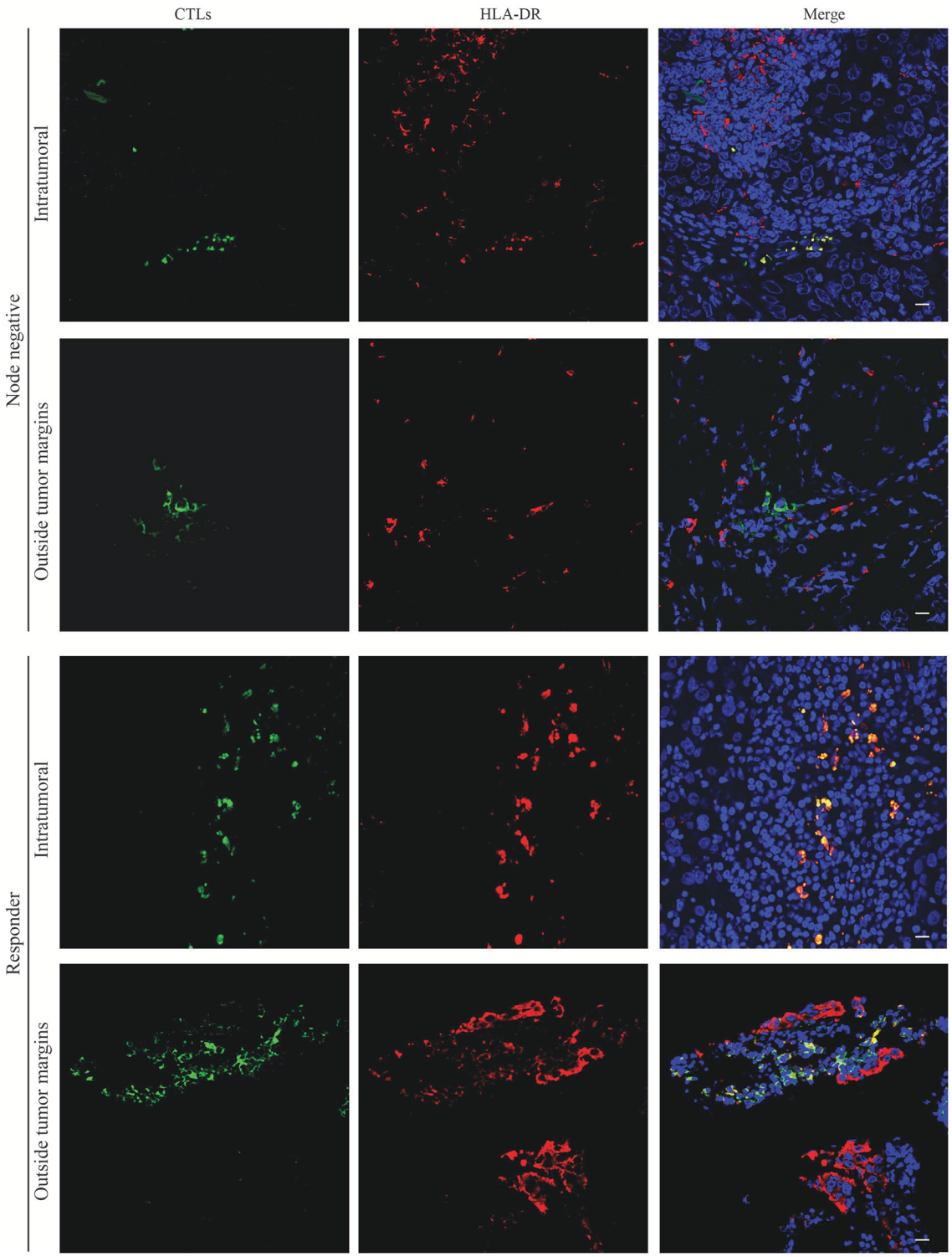

FIGURE 3 | HLA-DR-expressing CTLs are localized in intraepithelial tumor structures of patients without axillary lymph node metastasis and in NACT responders. Representative images of immunofluorescence experiments ($n = 6$) performed in slices of paraffin tissue of surgical BC samples of patients without axillary lymph node metastasis (Node negative) and biopsies of NACT responders (Responders) for CTLs (green, **Left panel**) and HLA-DR (red, **Middle panel**). Nuclei are stained in blue with DAPI and the three staining were merged (**Right panel**). Images from the tumor structures and from the tumor-surrounding tissue were acquired. Scale bars: 20 μm.

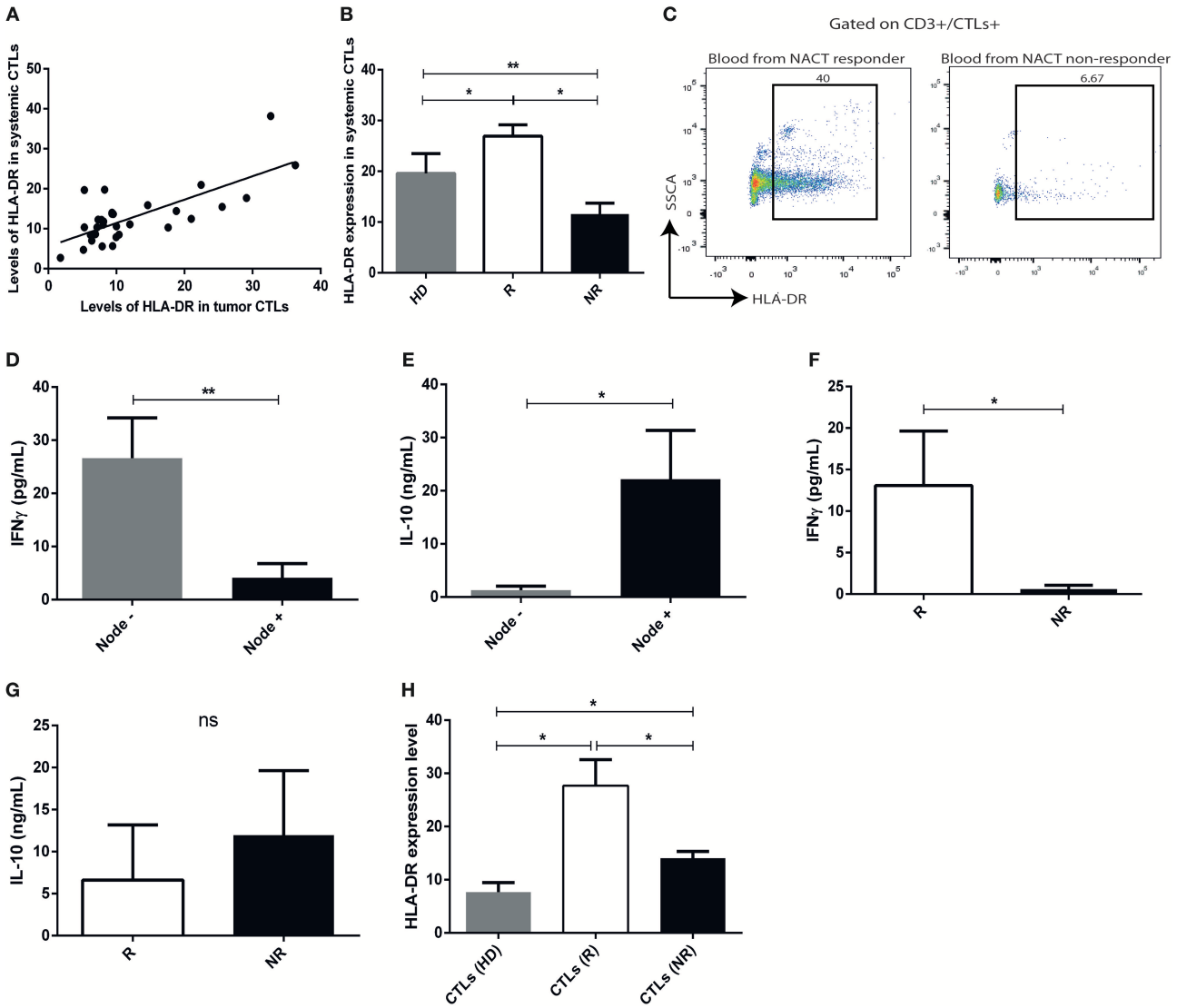

FIGURE 4 | The profile of HLA-DR expression and cytokine production of intratumor CTLs is maintained in systemic CTLs. **(A)** Correlation between expression of HLA-DR in systemic CTLs and tumor infiltrating CTLs (Spearman $r = 0.58$, $p < 0.001$). **(B)** HLA-DR expression in systemic CTLs in healthy donors (HD, gray bar, $n = 13$, mean ± *SD*), NACT responders (R, white bar, $n = 4$, mean ± *SD*) and non-responders (NR, black bar, $n = 5$, mean ± *SD*), assessed by flow cytometry and representing the median fluorescent intensity of positive population normalized relatively to the negative population. **(C)** Representative flow cytometry analysis of HLA-DR in systemic T cells from blood collected at the time of biopsy of responders and non-responders. IFN-γ **(D)** and IL-10 **(E)** levels assessed by ELISA in plasma of patients without axillary lymph node metastasis (gray bar, $n = 9$, mean ± SEM) and with axillary lymph node metastasis (black bar, $n = 12$, mean ± SEM). IFN-γ **(F)** and IL-10 **(G)** levels assessed by ELISA in plasma from responders (R, white bar, $n = 4$, mean ± SEM) and non-responders (NR, black bar, $n = 6$, mean ± SEM). **(H)** Expression of HLA-DR, assessed by flow cytometry, and representing the median fluorescent intensity of positive population normalized relatively to the negative population, in CTLs and Tregs of *ex vivo* stimulated PBMCs isolated from healthy donors (HD, gray bar, $n = 5$, mean ± SEM), from responders (R, white bar, $n = 4$, mean ± SEM) and from non-responders (NR, black bar, $n = 5$, mean ± SEM). $^*p < 0.05$, $^{**}p < 0.01$, ns, non-statistical.

were used as control. Interestingly, the expression of HLA-DR in CTLs was higher in stimulated PBMCs of NACT responders regarding stimulated PBMCs of non-responders ($p = 0.02$, **Figure 4H**). This observation further supports that the tumor immune features are maintained systemically and blood could be used to specifically assess HLA-DR+ CTLs, which is a major advantage for its potential use as a biomarker of response to NACT.

HLA-DR-expressing CTLs Have Increased Expression Levels of Effector Immune Response-Related Molecules

To corroborate that HLA-DR+ CTLs are activated lymphocytes with an anti-tumor activity profile, we assessed in pre-treatment samples, infiltrated HLA-DR+ CTLs and HLA-DR negative CTLs, the expression, by flow cytometry, of other effector immune response-related molecules, such as IFN-γ and

Granzyme B, which are the most important players of activated CTLs. Interestingly, only HLA-DR+ CTLs expressed high levels of IFN-γ and Granzyme B (**Figure 5A**), substantiating their immune competent profile. Furthermore, we have analyzed the expression level of these molecules also in HLA-DR+ CTLs and HLA-DR negative CTLs from patients PBMCs (isolated from pre-treatment blood). Similar to tumor, circulating HLA-DR+ CTLs express more IFN-γ and Granzyme B than HLA-DR negative CTLs (**Figure 5A**). Even if HLA-DR negative CTLs from PBMCs had a higher level of Granzyme B expression when compared to HLA-DR negative CTLs from tumor, the percentage of HLA-DR+ CTLs from PBMCs that express Granzyme B, and the corresponding median fluorescent intensity, is significantly higher than in HLA-DR negative CTLs from PBMCs ($p < 0.01$, data not shown).

Additionally, to further understand differences between HLA-DR+ CTLs and HLA-DR negative CTLs, CTLs with and without HLA-DR expression isolated from the blood were studied by qRT-PCR. In this case, the expression of *Granzyme B, Perforin*, *TNFα* and *Eomes* were assessed. HLA-DR+ CTLs expressed higher levels of the cytolytic proteins *Granzyme B* ($p = 0.02$) and *Perforin* ($p = 0.03$); *Eomes* ($p = 0.03$), involved in differentiation of effector CTLs and the inflammatory cytokine *TNFα* ($p = 0.03$), which also has anti-tumor properties, in comparison with HLA-DR negative CTLs (**Figure 5B**).

Also, *ex vivo* stimulated PBMCs of responders (shown to express more HLA-DR in CTLs than PBMCs of non-responders—**Figure 4H**) have increased levels of IFN-γ ($p = 0.01$) in CTLs and lower levels of IL-10 ($p = 0.01$), produced by Th cells and/or Tregs respectively (**Figures 5C,D**), than stimulated PBMCs of non-responders.

Altogether, these results suggest that CTLs with expression of HLA-DR are active and effector lymphocytes that might have a protective anti-tumor effect, which is in accordance to their higher prevalence in BC without axillary lymph node metastasis and/or in NACT responders.

HLA-DR Expression Level in CTLs Negatively Correlates With the Immunosuppressive and Pro-Tumor Features of the Tumor Milieu

An anti-tumor or a pro-tumor immune response is elicited not only by the immune cells within the tumor and their bioeffector molecules, but also by tumor antigens and the expression of cytokines, chemokines and other immune mediators released by the cancer tissue. Thus, the stimulation of anti-tumor CTLs' activity should, at least in part, rely on the molecules present in the tumor milieu. Interestingly, we have observed, by flow cytometry analysis of pre-treatment BC samples, that CTLs expressing HLA-DR were inversely correlated with immunosuppressive activated Tregs (also expressing HLA-DR, **Figures 1E**, **6**). Moreover, they were also negatively correlated with the expression of molecules from the non-immune compartment that may co-opt the expression of typically innate immune system-associated molecules to squelch the anti-tumor immune program and/or enhance growth and survival of cancer cells (**Figure 6**). Namely, HLA-DR expression level in CTLs was negatively correlated with the expression level of TGF-β ($r = -0.45$, $p < 0.01$), which increases the metastatic profile; PD-L1 ($r = -0.44$, $p < 0.0001$), that inhibits infiltration and/or activation of CTLs (23, 24); IL-6 ($r = -0.51$, $p < 0.001$), IL-8 ($r = -0.56$, $p < 0.001$) and IL-1β ($r = -0.49$, $p < 0.001$) that are inflammatory cytokines that may act as growth factors to sustain cancer cell proliferation and invasion (25–27). It was also negatively correlated with IL-23/IL-12 ($r = -0.56$, $p < 0.0001$) that is pro-inflammatory but can also impair CTLs' activity through the activation of Tregs (28) (**Figure 6**). On the other hand, the expression level of HLA-DR in Tregs was positively correlated with the expression of these pro-tumor molecules - IL-1β ($r = 0.47$, $p < 0.01$), IL-23/IL-12 ($r = 0.43$, $p < 0.01$), IL-6 ($r = 0.48$, $p < 0.01$) and IL-8 ($r = 0.47$, $p < 0.001$) – and, additionally, IL-10 ($r = 0.44$, $p < 0.0001$) and IL-17 ($r = 0.45$, $p < 0.01$), which could also undermine the activation of CTLs and lead to tumor progression (29, 30) (**Figure 6**).

When the same analysis was performed with CTLs or Tregs without accounting for HLA-DR expression, there was no significant correlation between these cells and these molecules of the tumor milieu (results not shown).

To better understand the contribution of cytokines or other soluble factors present in the tumor milieu in the activation of CTLs, we used a co-culture assay of HS 578T breast cancer cell line with peripheral blood mononuclear cells (PBMCs) isolated from patients. The assay was performed in several conditions: PBMCs with HS 578T cell line, PBMCs with the supernatant of the cell line alone, PBMCs plus a canonical stimulus, which served as a positive control, or just PBMCs. Curiously, the supernatant of the cell line alone increased the expression of HLA-DR in CTLs (**Figure S5**).

Altogether, these data are in agreement with the supposition that CTLs (and Tregs) bearing HLA-DR reflect the overall immune status of the BC milieu.

DISCUSSION

Chemotherapy failure is the main reason for disease progression, recurrence and cancer-related death. In the case of breast cancer (BC), less than 50% of the patients have a pathological complete response to neoadjuvant chemotherapy (NACT) (3, 4). Although some efforts have been made in the past years, such as the introduction of tumor infiltrating lymphocytes (TILs) as possible biomarkers of response, especially in TNBC and HER2+ BC (7), there is still no validated biomarker being routinely used in the clinic.

The belief that NACT outcome may depend on the presence of TILs is related to the fact that chemotherapy leads to the release of immunogenic signals, promoting immunogenic cell death, which can potentially boost an anti-tumor immune response (31). However, TILs are functionally heterogenic. For instance, cytotoxic T lymphocytes (CTLs) have been strongly associated with patient survival and response to therapy (7); regulatory T cells (Tregs) have been associated with both good and bad prognosis (6, 9); T helper 1 (Th1) cells have been related with

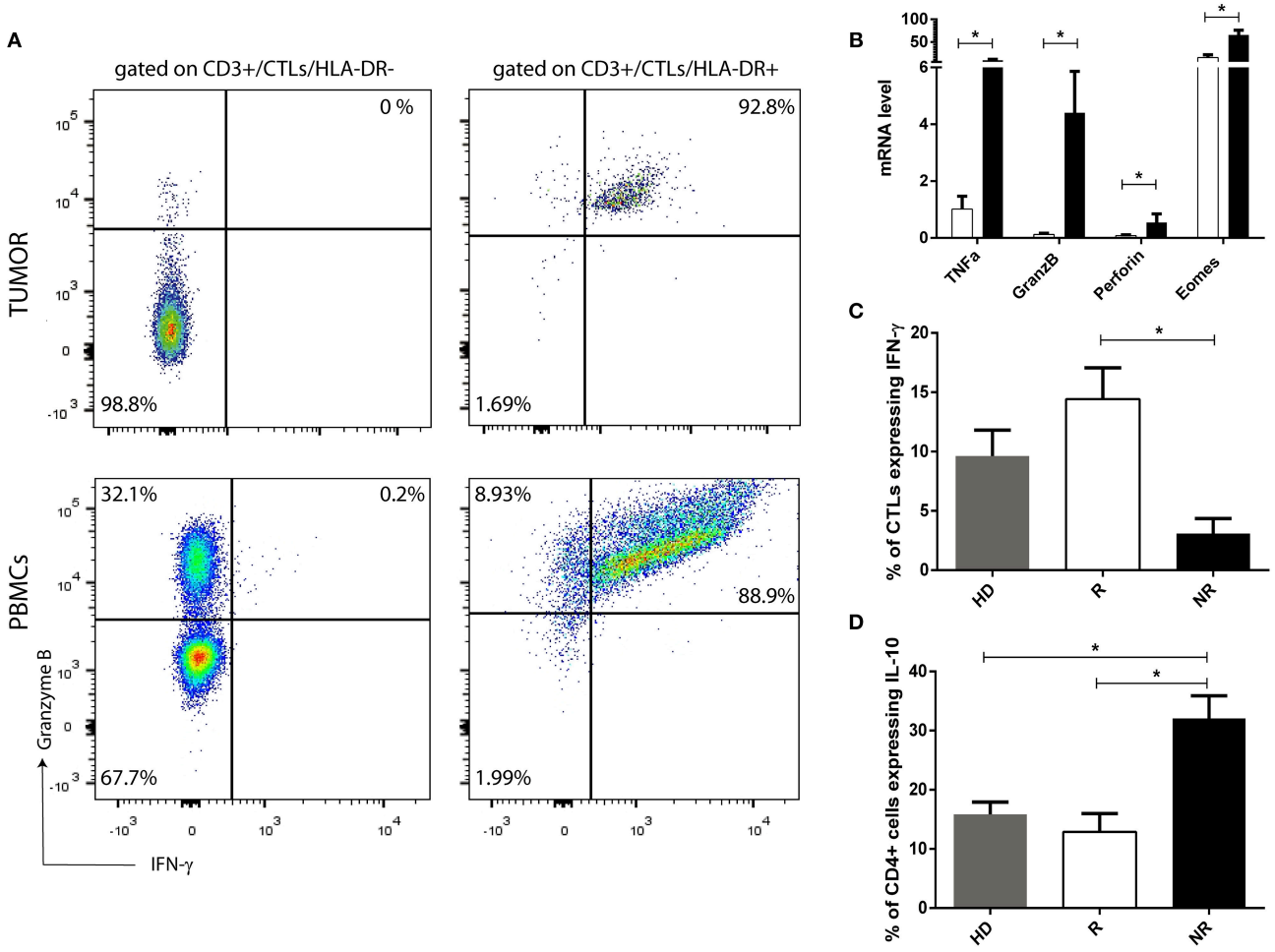

FIGURE 5 | HLA-DR in CTLs reflects their activation and functional status. **(A)** Representation of a flow cytometry analysis of Granzyme B and IFN-γ expression in HLA-DR+ CTLs and in HLA-DR negative CTLs from pre-treatment tumor samples and from PBMCs isolated from patients. **(B)** mRNA level of *TNFα*, *Granzyme B* (*GranzB*), *Perforin* and *Eomes* in HLA-DR+ CTLs (black bars) and HLA-DR negative CTLs (white bars). mRNA level is expressed as the permillage (‰) of the expression of the endogenous positive control ($n = 4$). **(C)** Percentage of CTLs expressing IFN-γ and **(D)** percentage of CD4+ T cells expressing IL-10, assessed by flow cytometry, and representing the median fluorescent intensity of positive population normalized relatively to the negative population, in stimulated PBMCs isolated from healthy donors (HD, gray bar, $n = 5$, mean ± SEM), from NACT responders (R, white bar, $n = 4$, mean ± SEM) and from non-responders (NR, black bar, $n = 5$, mean ± SEM). *$p < 0.05$.

favorable clinical outcomes (32); whereas Th2 cells have been reported to be associated with dampening of the anti-tumor response (33). Therefore, it is controversial that simply the degree of lymphocytic infiltration, assessed by immunohistochemistry in sections of paraffin embedded tissue, has a predictive value of BC patients' response to NACT. Even infiltrating CTLs, *per se*, lack the robustness that a predictive biomarker should have, because their function can be hampered by tumor cells, through several mechanisms. For instance, by expressing inhibitory molecules like PD-L1 or by secreting immunosuppressive molecules such as IL-10, TGF-β and IDO.

Thus, it is still crucial to establish a more accurate BC "immunological status" that reflects the overall strength of individual patients' anti-tumor immune response that will ultimately influence NACT efficiency.

In this study, we went beyond immunohistochemistry evaluation of TILs in BC and analyzed, by flow cytometry, T lymphocyte immune signatures, that could show their activated/exhausted or anergic state and molecules present in the tumor environment that are representative of the global tumor immune status.

We found that CTLs expressing high levels of HLA-DR, mainly located in intraepithelial tumor structures, are characteristic of BC without axillary lymph node metastasis and are strongly associated with good response to NACT treatment, contrasting with CTLs with low or null expression of this molecule. HLA-DR is an antigen presenting molecule expressed at high levels on professional antigen presenting cells, but its expression on effector T lymphocytes upon their activation has also been intensively described in

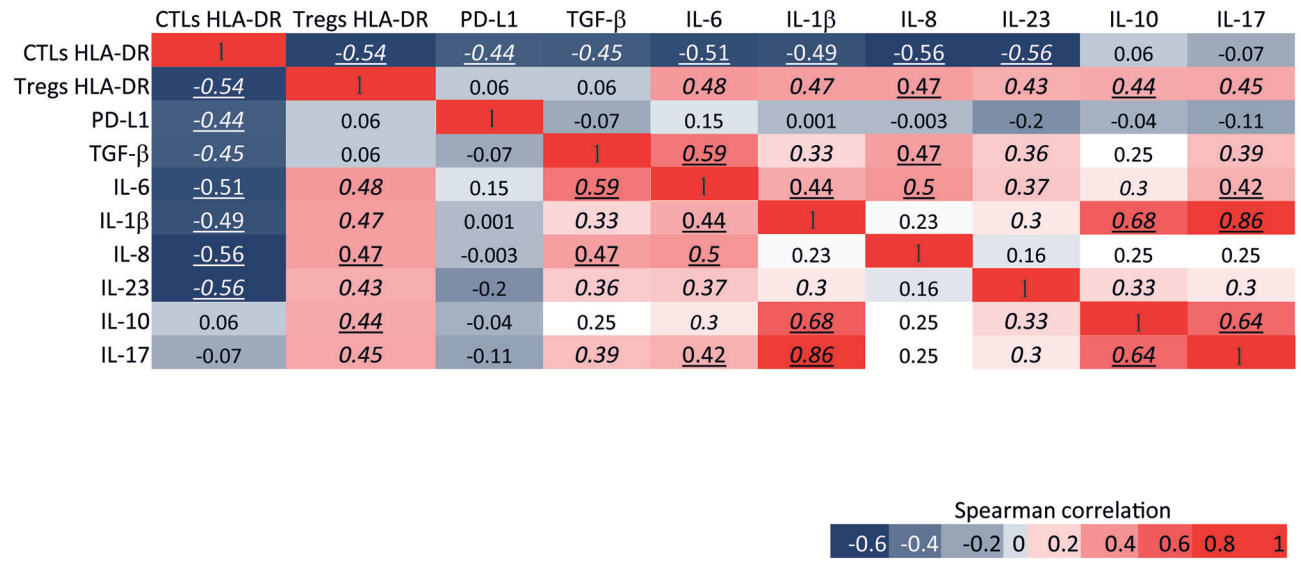

FIGURE 6 | HLA-DR expression level in CTLs and Tregs is differentially correlated with the immunosuppressive and pro-tumor features of the tumor environment. Heat map of Spearman correlations between the expression level of HLA-DR in CTLs and the expression level of HLA-DR in Tregs; and between the expression level of HLA-DR in both of these cells and the expression level of pro-tumor or immunosuppressive molecules from the tumor environment, namely PD-L1, TGF-b, IL-6, IL-1b, IL-8, IL-23/IL-12, IL-10, and IL-17. The expression level of each molecule was assessed by flow cytometry and represents the median fluorescent intensity of positive population normalized relatively to the negative population. The heat map is represented as a gradient from blue (negative correlations) to red (positive correlations). Numbers in italic represent statistical significance of $p < 0.01$, numbers underlined represent $p < 0.001$ and numbers in italic and underlined represent $p < 0.0001$.

some diseases, such as auto-immune diseases and viral infections (14, 15).

In the context of HIV patients, it has been described that these HLA-DR+ CTLs exhibit a decreased proliferative potential although retaining their cytolytic potential (34, 35). Additionally, in severe aplastic anemia, an elevated number of HLA-DR+ CTLs detected in patients' peripheral blood are responsible for the excessive apoptosis of hematopoietic cells (36). However, a suppressive effect of a subset of HLA-DR+ CTLs was also reported (37). Here we confirmed that HLA-DR+ CTLs express immune signatures of functionally activated, and not suppressive CTLs, such as the production of IFN-γ, Granzyme B, Perforin, TNFα, and Eomes. Thus, the infiltrating CTLs bearing HLA-DR found in the analyzed samples should have an active participation in the anti-tumor response, in conformity with the fact that they were more elevated in patients without axillary lymph node metastasis and with a better capacity to respond to NACT.

Tregs are recognized blockers of CTLs' function and it is known that HLA-DR also identifies functionally mature Tregs (13). Thus, not surprisingly, HLA-DR expression level in Tregs negatively correlates with its expression level in CTLs and is associated with BC with axillary lymph node metastasis and more abundant in biopsies of NACT non-responders.

In line with these former results, we also presented data that negatively correlate the level of HLA-DR in CTLs with the level of IL-6, IL-1β, IL-8, and IL-23/IL-12, which are inflammatory cytokines that, at certain level in the tumor microenvironment, could help the anti-tumor immune response (38), but whose upregulation might also favor tumor progression and metastasis (25–28). HLA-DR in CTLs was also inversely correlated with the level of PD-L1 and TGF-β, which are, respectively, a well-known inhibitory immune checkpoint and an anti-inflammatory cytokine, which coordinately work to dampen CTLs' activity, directly or through the activation of Tregs (23, 24). Likewise, HLA-DR expression in Tregs positively correlated with all the aforementioned molecules from the tumor milieu and, additionally, with IL-10 and IL-17, which also have immunosuppressive functions (29, 30, 39).

In vitro experiments, with the supernatant of BC cell lines, highlighted the fact that soluble factors released by tumor cells are important to increase HLA-DR in CTLs, suggesting that these tumor microenvironment molecules can modulate the immune response and influence the activation state of CTLs, without the requirement of contact with tumor cells.

In the other way around, we may say that the HLA-DR expression in CTLs is a reflection of the general tumor immune status. Indeed, an immunosuppressed environment will be unable to stimulate an appropriate immune response and should not give rise to HLA-DR^{hi} CTLs. As cell-to-cell contact and tumor-specific antigen presentation by antigen presenting molecules are required to elicit a specific cytotoxic T cell response against the tumor cells, we may infer that, *in vivo*, the antigens presented by tumor cells, alongside with the soluble factors, contribute to CTLs' effector function.

The increase of HLA-DR at CTLs' surface, upon stimulation, could also be required to boost the anti-tumor immune response. Indeed, HLA-DR+ CTLs were found to have the machinery needed for antigen processing and loading on HLA-DR

molecules and, additionally, could express CD86 and CD80, which are the co-stimulatory molecules of antigen presenting cells that are necessary for the proper T cell effector function (40). Moreover, it was described that T cell-T cell synapsis occur to allow T cells to secrete IFN-γ toward each other, compelling the differentiation of more protective T cells (41). These T cell-T cell interactions and mutual antigen presentation can be essential for mounting a suitable anti-tumor response.

Notably, we observed that HLA-DR expression on tumor infiltrating CTLs correlates to its expression in circulating CTLs and, additionally, the analysis of HLA-DR level on CTLs isolated from the patients' blood could also be associated with BC axillary lymph node invasion status and assist in the prediction of patients' response to NACT. *Ex vivo* assays indeed corroborate that systemic CTLs maintain the profile encountered in the tumor mass, as under similar stimulation, CTLs from NACT responders increase even more their HLA-DR level and produce more IFN-γ than CTLs from NACT non-responders. These results imply that tumor immune status, reflected by the HLA-DR level in CTLs, could also be easily assessed in blood.

HLA-DR molecule, by itself, assessed in tumor cells, has been reported to serve as a favorable prognostic marker for other types of cancers, such as colorectal carcinoma (42) or glioma (43). It was even shown that HLA-DR+ melanoma cells predict the response to anti-PD-1/PD-L1 immunotherapy (44). However, to our knowledge, the expression of HLA-DR in CTLs, besides its extensive application in the study of viral infections and chronic inflammatory diseases, was never recommended as a biomarker in cancer. Our data suggest that HLA-DR+ CTLs, assessed by flow cytometry, in BC patients prior to NACT, is a sensitive and specific potential predictive factor for NACT response. Moreover, this trait can be easily assessed in blood, repeatedly if necessary. Further studies should be conducted in an independent population, in order to validate the predictive performance of HLA-DR+ CTLs in assisting the selection of patients that will truly benefit from NACT, or promptly directing them to alternative therapeutic strategies, such as the combination of immunotherapies with standard chemotherapy. Additionally, this marker also have the potential to be used in clinical trial design to randomize good/bad responders to evaluate novel treatments.

AUTHOR CONTRIBUTIONS

DS conducted all the experiments, analyzed and interpreted the data, performed the statistical analysis, assembled all the figures and wrote the manuscript. AJ contributed to scientific discussion and revised the manuscript. PB helped in the obtainment of patients' samples and clinical data, contributed to scientific discussion and revised the manuscript. SB supervised the study, helped in the obtainment of patients' samples and clinical data, contributed to data interpretation, scientific discussion and revised the manuscript. MC conceived and designed the experiments, supervised the study, analyzed and interpreted the data and wrote the manuscript. All the authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02605/full#supplementary-material

REFERENCES

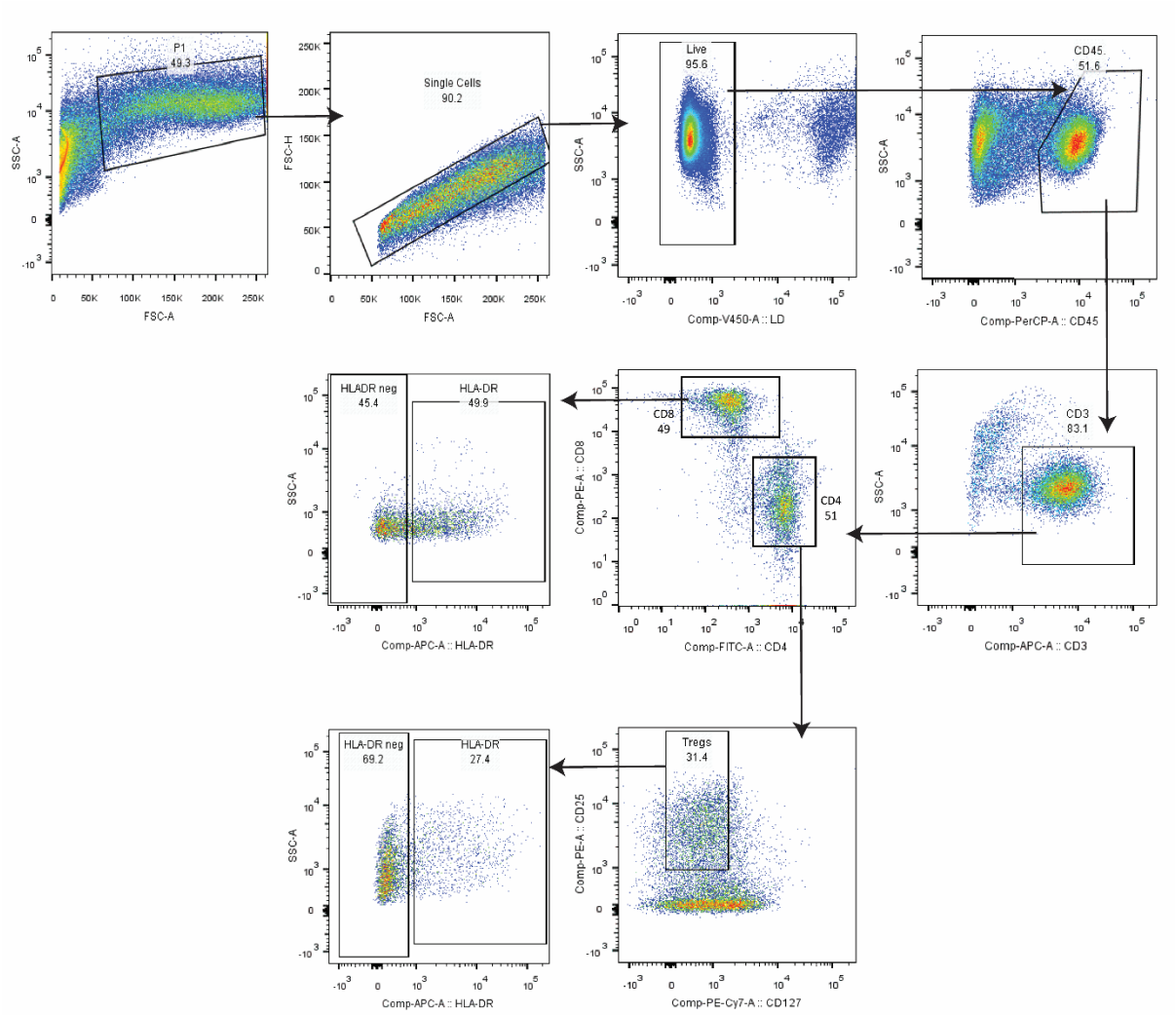
1. Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, et al. The global burden of cancer 2013. *JAMA Oncol*. (2015) 1:505–27. doi: 10.1001/jamaoncol.2015.0735
2. Thompson AM, Moulder-Thompson SL. Neoadjuvant treatment of breast cancer. *Ann Oncol*. (2012) 23(suppl_10):x231–6. doi: 10.1093/annonc/mds324
3. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *Lancet* (2014) 384:164–72. doi: 10.1016/S0140-6736(13)62422-8
4. DeMichele A, Yee D, Esserman L. Mechanisms of resistance to neoadjuvant chemotherapy in breast cancer. *N Engl J Med*. (2017) 377:2287–9. doi: 10.1056/NEJMcibr1711545
5. Teshome M, Hunt KK. Neoadjuvant therapy in the treatment of breast cancer. *Surg Oncol Clin N Am*. (2014) 23:505–23. doi: 10.1016/j.soc.2014.03.006
6. Mao Y, Qu Q, Zhang Y, Liu J, Chen X, Shen K. The value of tumor infiltrating lymphocytes (TILs) for predicting response to neoadjuvant chemotherapy in breast cancer: a systematic review and meta-analysis. *PLoS ONE* (2014) 9:e115103. doi: 10.1371/journal.pone.0115103
7. Hornychova H, Melichar B, Tomsova M, Mergancova J, Urminska H, et al. Tumor-infiltrating lymphocytes predict response to neoadjuvant chemotherapy in patients with breast carcinoma. *Cancer Invest* (2008) 26:1024–31. doi: 10.1080/07357900802098165
8. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol*. (2015) 26:259–71. doi: 10.1093/annonc/mdu450

9. Khaja ASS, Toor SM, El Salhat H, Faour I, Ul Haq N, Ali BR, et al. Preferential accumulation of regulatory T cells with highly immunosuppressive characteristics in breast tumor microenvironment. *Oncotarget* (2017) 8:33159–71. doi: 10.18632/oncotarget.16565
10. Inozume T, Hanada K, Wang QJ, Ahmadzadeh M, Wunderlich JR, Rosenberg SA, et al. Selection of CD8(+)PD-1(+) lymphocytes in fresh human melanomas enriches for tumor-reactive T-cells. *J Immunother*. (2010) 33:956–64. doi: 10.1097/CJI.0b013e3181fad2b0
11. Shin DS, Ribas A. The evolution of checkpoint blockade as a cancer therapy: what's here, what's next? *Curr Opin Immunol*. (2015) 33:23–35. doi: 10.1016/j.coi.2015.01.006
12. Viallard JF, Blanco P, Andre M, Etienne G, Liferman F, Neau D, et al. CD8+HLA-DR+ T lymphocytes are increased in common variable immunodeficiency patients with impaired memory B-cell differentiation. *Clin Immunol*. (2006) 119:51–8. doi: 10.1016/j.clim.2005.11.011
13. Baecher-Allan C, Wolf E, Hafler DA. MHC class II expression identifies functionally distinct human regulatory T cells. *J Immunol*. (2006) 176:4622–31. doi: 10.4049/jimmunol.176.8.4622
14. Viallard JF, Bloch-Michel C, Neau-Cransac M, Taupin JL, Garrigue S, Miossec V, et al. HLA-DR expression on lymphocyte subsets as a marker of disease activity in patients with systemic lupus erythematosus. *Clin Exp Immunol*. (2001) 125:485–91. doi: 10.1046/j.1365-2249.2001.01623.x
15. Saez-Cirion A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, et al. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection *ex vivo* and peculiar cytotoxic T lymphocyte activation phenotype. *Proc Natl Acad Sci USA*. (2007) 104:6776–81. doi: 10.1073/pnas.0611244104
16. Chan LY, Yim EKF, Choo ABH. Normalized median fluorescence: an alternative flow cytometry analysis method for tracking human embryonic stem cell states during differentiation. *Tissue Eng Part C Methods* (2013) 19:156–65. doi: 10.1089/ten.tec.2012.0150
17. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* (2012) 9:676–82. doi: 10.1038/nmeth.2019
18. Biselli R, Matricardi PM, D'Amelio R, Fattorossi A. Multiparametric flow cytometric analysis of the kinetics of surface molecule expression after polyclonal activation of human peripheral blood T lymphocytes. *Scand J Immunol*. (1992) 35:439–47.
19. Rea IM, McNerlan SE, Alexander HD. CD69, CD25, and HLA-DR activation antigen expression on CD3+ lymphocytes and relationship to serum TNF-alpha, IFN-gamma, and sIL-2R levels in aging. *Exp Gerontol*. (1999) 34:79–93. doi: 10.1016/S0531-5565(98)00058-8
20. Medler TR, Cotechini T, Coussens LM. Immune response to cancer therapy: mounting an effective antitumor response and mechanisms of resistance. *Trends Cancer* (2015) 1 66–75. doi: 10.1016/j.trecan.2015.07.008
21. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* (2006) 313:1960 LP−1964. doi: 10.1126/science.1129139
22. Jakubowska K, Kisielewski W, Kanczuga-Koda L, Koda M, Famulski W. Stromal and intraepithelial tumor-infiltrating lymphocytes in colorectal carcinoma. *Oncol Lett*. (2017). 14. 6421–32. doi: 10.3892/ol.2017.7013
23. Jakowlew SB. Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev*. (2006) 25:435–57. doi: 10.1007/s10555-006-9006-2
24. Sun C, Mezzadra R, Schumacher TN. Regulation and Function of the PD-L1 Checkpoint. *Immunity* (2018) 48:434–52. doi: 10.1016/j.immuni.2018.03.014
25. Fisher DT, Appenheimer MM, Evans SS. The two faces of IL-6 in the tumor microenvironment. *Semin Immunol*. (2014) 26:38–47. doi: 10.1016/j.smim.2014.01.008
26. Waugh DJJ, Wilson C. The interleukin-8 pathway in cancer. *Clin Cancer Res*. (2008) 14:6735–41. doi: 10.1158/1078-0432.CCR-07-4843
27. Lewis AM, Varghese S, Xu H, Alexander HR. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. *J Transl Med*. (2006) 4:48. doi: 10.1186/1479-5876-4-48
28. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, et al. IL-23 promotes tumour incidence and growth. *Nature* (2006) 442:461–5. doi: 10.1038/nature04808
29. Benevides L, da Fonseca DM, Donate PB, Tiezzi DG, De Carvalho DD, de Andrade JM, et al. IL17 promotes mammary tumor progression by changing the behavior of tumor cells and eliciting tumorigenic neutrophils recruitment. *Cancer Res*. (2015) 75:3788–99. doi: 10.1158/0008-5472.CAN-15-0054
30. Xiang T, Long H, He L, Han X, Lin K, Liang Z, et al. Interleukin-17 produced by tumor microenvironment promotes self-renewal of CD133+ cancer stem-like cells in ovarian cancer. *Oncogene* (2015) 34:165–76. doi: 10.1038/onc.2013.537
31. Zitvogel L, Apetoh L, Ghiringhelli F, André F, Tesniere A, Kroemer G. The anticancer immune response: indispensable for therapeutic success?. *J Clin Invest*. (2008) 118:1991–2001. doi: 10.1172/JCI35180.
32. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4+ follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest*. (2013) 123:2873–92. doi: 10.1172/JCI67428
33. Teschendorff AE, Gomez S, Arenas A, El-Ashry D, Schmidt M, Gehrmann M, et al. Improved prognostic classification of breast cancer defined by antagonistic activation patterns of immune response pathway modules. *BMC Cancer* (2010) 10:604. doi: 10.1186/1471-2407-10-604
34. Pantaleo G, De Maria A, Koenig S, Butini L, Moss B, Baseler M, et al. CD8+ T lymphocytes of patients with AIDS maintain normal broad cytolytic function despite the loss of human immunodeficiency virus-specific cytotoxicity. *Proc Nat Acad Sci USA*. (1990) 87:4818–22. doi: 10.1073/pnas.87.12.4818
35. Pantaleo G, Koenig S, Baseler M, Lane HC, Fauci AS. Defective clonogenic potential of CD8+ T lymphocytes in patients with AIDS. Expansion *in vivo* of a nonclonogenic CD3+CD8+DR+CD25- T cell population. *J Immunol*. (1990) 144:1696–704.
36. Xing L, Liu C, Fu R, Wang H, Wang J, Liu X, et al. CD8+HLA-DR+ T cells are increased in patients with severe aplastic anemia. *Mol Med Rep*. (2014) 10:1252–8. doi: 10.3892/mmr.2014.2344
37. Arruvito L, Payaslian F, Baz P, Podhorzer A, Billordo A, Pandolfi J, et al. Identification and clinical relevance of naturally occurring human CD8+HLA-DR+ regulatory T cells. *J Immunol*. (2014) 193:4469–76. doi: 10.4049/jimmunol.1401490
38. Allen MD, Jones LJ. The role of inflammation in progression of breast cancer: Friend or foe? (Review). *Int J Oncol*. (2015) 47:797–805. doi: 10.3892/ijo.2015.3075
39. Hamidullah, Changkija B, Konwar R. Role of interleukin-10 in breast cancer. *Breast Cancer Res Treat*. (2012) 133:11–21. doi: 10.1007/s10549-011-1855-x
40. Holling TM, Schooten E, van Den Elsen PJ. Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men. *Hum Immunol*. (2004) 65:282–90. doi: 10.1016/j.humimm.2004.01.005
41. Gerard A, Khan O, Beemiller P, Oswald E, Hu J, Matloubian M, et al. Secondary T cell-T cell synaptic interactions drive the differentiation of protective CD8+ T cells. *Nat Immunol*. (2013) 14:356–63. doi: 10.1038/ni.2547
42. Sconocchia G, Eppenberger-Castori S, Zlobec I, Karamitopoulou E, Arriga R, Coppola A, et al. HLA class II antigen expression in colorectal carcinoma tumors as a favorable prognostic marker. *Neoplasia* (2014) 16:31–42. doi: 10.1593/neo.131568
43. Diao J, Xia T, Zhao H, Liu J, Li B, Zhang Z. Overexpression of HLA-DR is associated with prognosis of glioma patients. *Int J Clin Exp Pathol*. (2015) 8:5485–90.
44. Johnson DB, Estrada M V, Salgado R, Sanchez V, Doxie DB, Opalenik SR, et al. Melanoma-specific MHC-II expression represents a tumour-autonomous phenotype and predicts response to anti-PD-1/PD-L1 therapy. *Nat Commun*. (2016) 7:10582. doi: 10.1038/ncomms10582

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

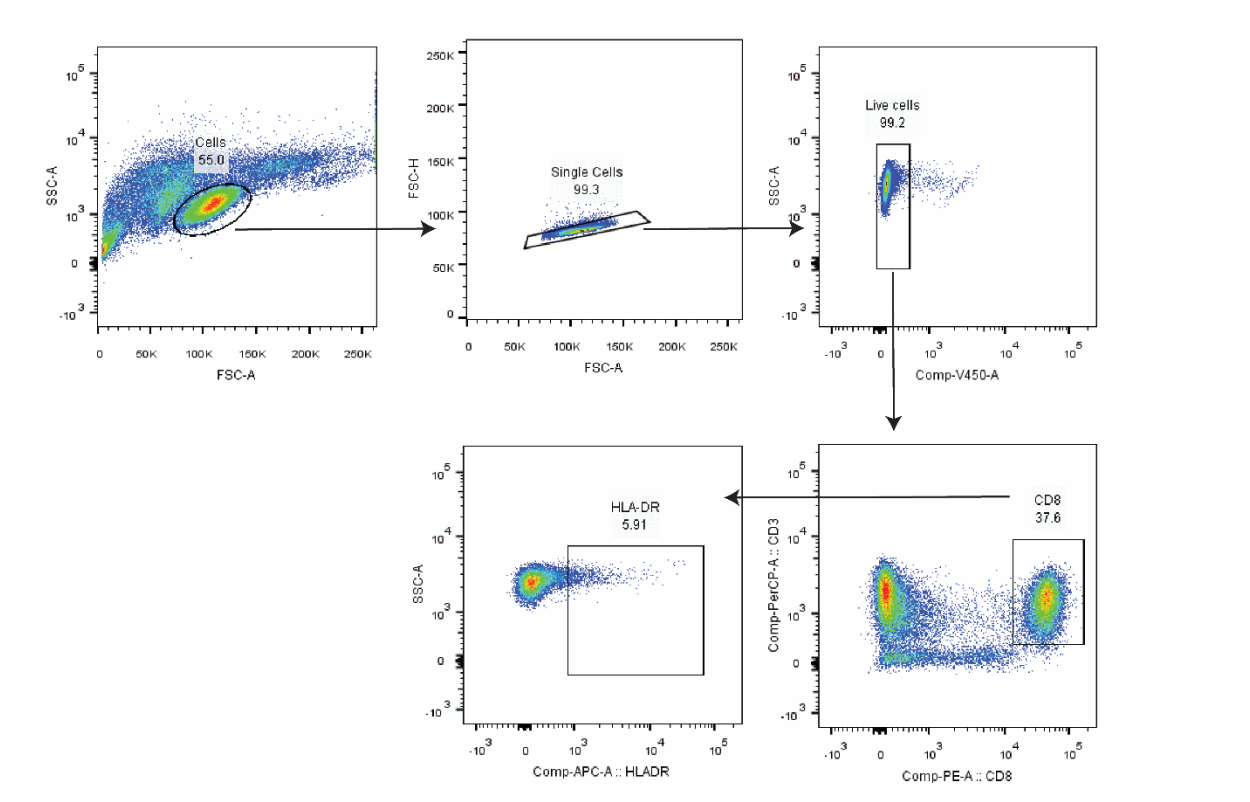
Supplementary material

Fig. S1



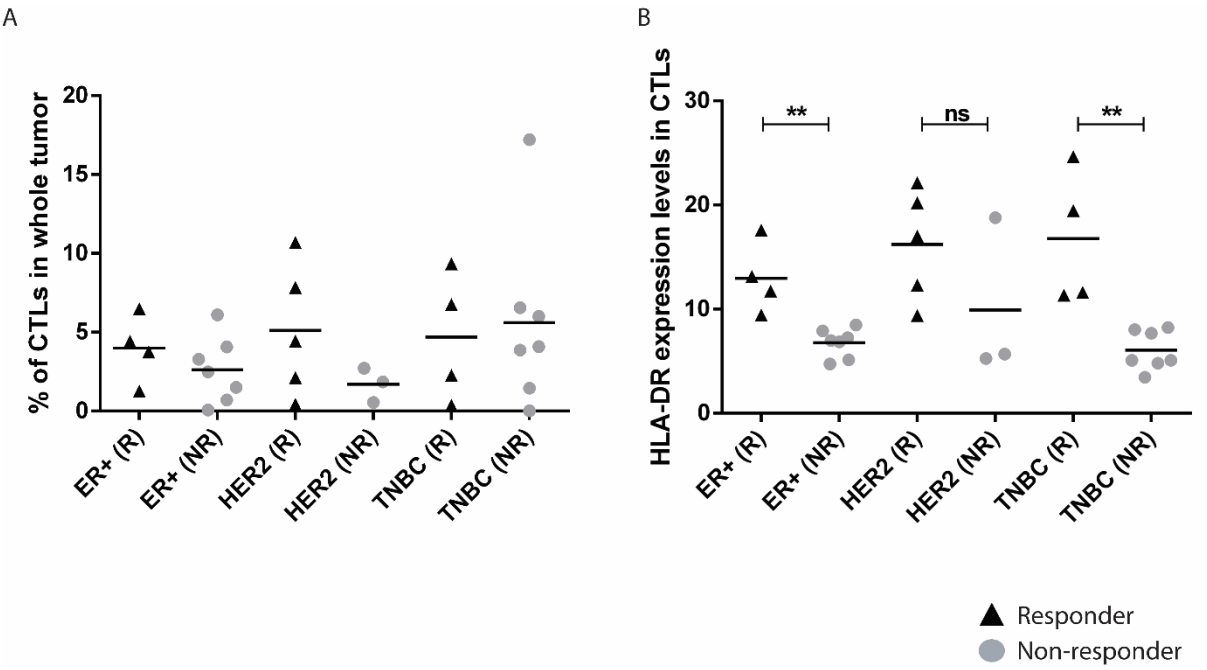
Supplementary figure S1 – Representative dot plots of the gating strategy used in the flow cytometry analysis of breast cancer samples. Positive and negative populations were always selected taken into consideration the negative (unstained) sample.

Fig. S2



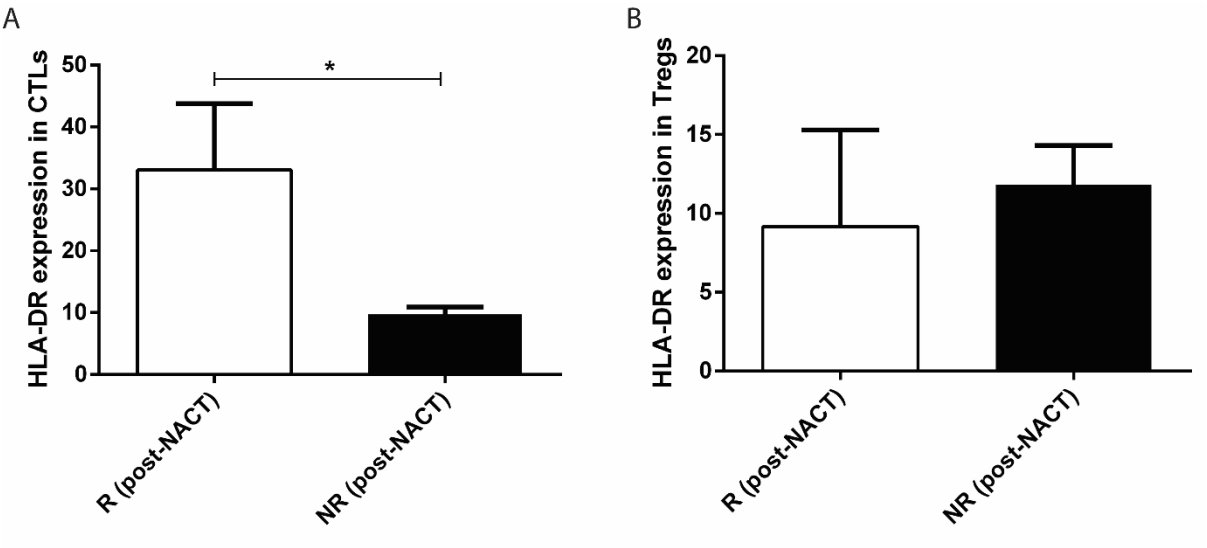
Supplementary figure S2 - Representative dot plots of the gating strategy used for fluorescence activated cell sorting of CTLs positive and negative for HLA-DR. These cells were then used for qRT-PCR analysis.

Fig. S3



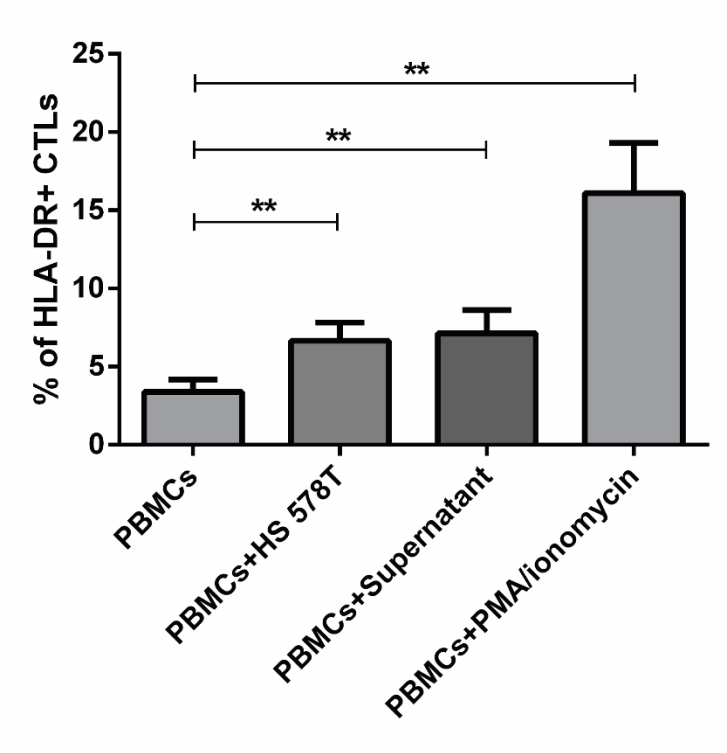
Supplementary figure S3 – HLA-DR levels in CTLs can predict response to NACT, independently of breast cancer subtype. (A) Percentage of CTLs in pre-treatment biopsies of NACT-responders (R, black triangles) and non-responders (NR, grey dots) divided in the three breast cancer subtypes – Estrogen receptor positive (ER+), HER2 amplified (HER2) and triple negative breast cancer (TNBC). **(B)** HLA-DR expression levels in CTLs in responders and non-responders for the three subtypes of breast cancer. The expression level of HLA-DR was assessed by flow cytometry and represents the median fluorescent intensity of positive population normalized relatively to the negative population. **p<0.01, ns – non statistical.

Fig. S4



Supplementary figure S4 – Profile of HLA-DR expression in CTLs and Tregs after neoadjuvant chemotherapy (NACT). Level of HLA-DR in CTLs **(A)** and Tregs **(B)**, assessed by flow cytometry, in responders (R, white bars, n=3, mean ± SEM) and non-responders (NR, black bars, n=4, mean ± SEM), expressed as the median fluorescent intensity of positive population normalized relatively to the negative population. *p<0.05.

Fig. S5



Supplementary figure S5 – HLA-DR expression in CTLs in a co-culture of PBMCs with a breast cancer cell line. HLA-DR+ CTLs, assessed by flow cytometry, in the following conditions: PBMCs in mono-culture, co-culture of PBMCs with HS 578T cell line, PBMCs with the cell line supernatant and PBMCs with the canonical stimulus (PMA/ionomycin). Data are expressed as the percentage of HLA-DR+ CTLs in these conditions (**$p<0.01$, $n=6$).