Extracellular Matrix Regulation of Breast Cancer Immune Microenvironment

Thesis

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Extracellular matrix regulation of breast cancer immune microenvironment

Thesis presented for the degree of Doctor of Philosophy
The Open University, Milton Keynes (UK)

School of Life, Health and Chemical Sciences

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Personal Identifier: G4630052

September 2021
DECLARATION

The data presented in this thesis are original, were not previously used for any other PhD degree and were originated by myself and by the cited below collaborators. During my PhD I worked in the Molecular Immunology Unit, Department of Research, at the Fondazione IRCCS Istituto Nazionale dei Tumori.

My director of studies was Dr. Sangaletti Sabina (PhD) and my Supervisor was Dr. Massimo Di Nicola (MD). My work is supported by Associazione Italiana per la Ricerca sul Cancro (AIRC; Investigator Grant nr. 18425).

Bioinformatic analyses included in this thesis (Figure 26) were performed by Prof. Paolo Verderio and Dr. Mara Lecchi, Bioinformatics and Biostatistics Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan.

Treg suppression assay included in this thesis (Figure 33) was performed by Dr. Massimo Costanza (PhD), Molecular Neuro-Oncology Unit, Fondazione IRCCS Istituto Neurologico Carlo Besta.

Immunohistochemistry included in this thesis (Figures 37A-E, 38A, 39B, 40C, 43A-B-C) were performed by Prof. Tripodo Claudio (MD, PhD) and Dr. Alessandro Gulino, Tumor Immunology Unit, Department of Health Sciences, University of Palermo.

The evaluation of p50 and p65 nuclear translocation (Figure 42A-B) was performed and analysed by Prof. Antonio Sica (PhD) and Dr. Francesca Maria Consonni (PhD), Department of Inflammation and Immunology, Humanitas Clinical and Research Center.

The results reported in one part of Chapter 4.2 (4.2.1-4.2.4) of this thesis are published on Frontiers in Immunology, 2019 (Sangaletti S, Talarico G et al. DOI: 10.3389/fimmu.2019.01369).

The other results collected in this thesis are included in a manuscript under preparation.

# TABLE OF CONTENTS

DECLARATION .................................................................................................................. I

LIST OF ABBREVIATION ................................................................................................ IV

ABSTRACT ......................................................................................................................... 1

1. INTRODUCTION ........................................................................................................ 3

1.1 Breast cancer ............................................................................................................. 3
1.1.1 Epidemiology and risk factors .............................................................................. 3
1.1.2 Preventions and management of breast cancer .................................................. 4
1.1.3 Histological classification .................................................................................... 7
1.1.4 Molecular Classification ....................................................................................... 10

1.2 Preclinical models of breast cancer ........................................................................ 16
1.2.1 Syngeneic models ................................................................................................ 17
1.2.2 Genetically engineered mouse (GEM) models ..................................................... 18

1.3 The Tumor Microenvironment .............................................................................. 19
1.3.1 Cellular components of the TME ......................................................................... 20
1.3.1.1 Immune cells within the TME ........................................................................ 26
1.3.1.2 TME classification according to immune-infiltration .................................... 29
1.3.1.3 Myeloid-derived suppressor cells ..................................................................... 31
1.3.1.4Suppressive mechanisms mediated by MDSCs ................................................ 32
1.3.1.5 Pharmacological targeting of MDSCs .............................................................. 34
1.3.1.5.1 Promotion of myeloid-cell differentiation .................................................. 34
1.3.1.5.2 Drugs inhibiting MDSC expansion ............................................................... 36
1.3.1.5.3 Inhibition of MDSC function ...................................................................... 37
1.3.1.5.4 Reducing MDSC accumulation ................................................................... 38
1.3.1.6 Regulatory T cells ............................................................................................ 39
1.3.1.6.1 Treg cell markers ......................................................................................... 40
1.3.1.6.2 Role of Tregs in the TME ................................................................. 41
1.3.1.6.3 Co-stimulatory and co-inhibitory receptors ............................................... 43
1.3.1.6.4 PD-1 expression on Tregs ........................................................................ 45
1.3.1.6.5 Satb1 restraints PD-1 expression in T cells ................................................. 47
1.3.1.6.6 SATB1 regulations ..................................................................................... 48
1.3.1.7 Immunoediting in cancer ............................................................................... 50

1.3.2. Non-cellular components of the TME ............................................................... 52
1.3.2.1 Extracellular matrix ......................................................................................... 53
1.3.2.2 Matricellular proteins ....................................................................................... 56
1.3.2.2.1 SPARC, one of the matricellular protein .................................................... 56
1.3.2.2.2 Role of SPARC in cancer ........................................................................ 59
1.3.2.2.3 SPARC in high-grade breast cancer .......................................................... 61

2. AIM OF THE THESIS .................................................................................................. 63

3. MATERIALS AND METHODS .................................................................................. 64

3.1 Breast Cancer Patient Samples ............................................................................... 64
3.2 PBMC Flow Cytometry and Cell Sorting .............................................................. 65
3.3 Animals, Cell Lines, and in vivo Experiments ..................................................... 66
3.4 Western blot ............................................................................................................. 67
3.5 Clonogenic assay .................................................................................................... 69
3.6 Mouse Flow Cytometry Analysis ......................................................................... 70
3.7 Regulatory T cells isolation with magnetic cell sorting ......................................... 71
3.8 MDSC Isolation ....................................................................................................... 73
3.9 Total RNA extraction, reverse transcription and quantitative polymerase chain reaction (qPCR) .......................................................... 73
3.10 Treg cell suppression assay ................................................................. 74
3.11 MDSC cell suppression assay ............................................................... 74
3.12 Cytospin ............................................................................................... 75
3.13 Immunohistochemistry and Immunofluorescence .................................. 76
3.14 Evaluation of p50 and p65 Nuclear Translocation.................................. 77
3.15 ROS Detection ...................................................................................... 78
3.16 Statistical analysis ................................................................................ 78

4. RESULTS .................................................................................................... 80
4.1 SPARC drives immune suppression regulating PD-1^{neg} Treg via IL-23 ....... 80
  4.1.1 Treg Ki67+ PD-1^{neg} are enriched in the peripheral blood of ECM3+ patients.... 80
  4.1.2 SPARC is responsible for Treg Ki67+ PD-1^{neg} expansion in high-SPARC BC model ................................................................. 84
  4.1.3 PD1- Treg cells are more suppressive than PD1+ counterpart .................... 89
  4.1.4 Enrichment in PD-1^{neg} Treg in ECM3+ is associated with increase SATB1 expression .................................................................................. 91
  4.1.5 SPARC–mediated IL-23 expression in breast cancer control SATB1 ........... 93
4.2 SPARC Is A New Myeloid-Derived Suppressor Cell Marker Licensing Suppressive Activities ................................................................. 96
  4.2.1 SPARC marks human and murine MDSC.............................................. 96
  4.2.2 Myeloid-derived SPARC is required for EMT....................................... 99
  4.2.3 SPARC from MDSC supports EMT ....................................................... 100
  4.2.4 SPARC-deficient MDSCs are less suppressive than WT counterpart .......... 101
  4.2.5 IHC analysis of CD8 T-cells and PD-1 cells in ECM3 and non- ECM3 tumors .... 107
  4.2.6 ZA treatment alters the TME in BC models ........................................ 109
  4.2.7 Characterization of MDSCS after ZA treatment in HGB patients .......... 111

5. DISCUSSION .............................................................................................. 114
# LIST OF ABBREVIATION

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>A2AR</td>
<td>Adenosine A2A receptor</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium chloride potassium</td>
</tr>
<tr>
<td>AI</td>
<td>Aromatase inhibitors</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARG1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>CAA</td>
<td>Cancer-associated adipocyte</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer-associated fibroblasts</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Succinimidyl ester</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box protein P3</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GEM</td>
<td>Genetically engineered mouse</td>
</tr>
<tr>
<td>GEP</td>
<td>Gene expression profile</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GSS</td>
<td>Glutathione synthetase</td>
</tr>
<tr>
<td>GvHD</td>
<td>Graft versus host disease</td>
</tr>
</tbody>
</table>
HBSS - Hank's balanced salt solution
HER2 - Human epidermal growth factor receptor 2
HGBC - high-grade breast cancer
HGF - hepatocyte growth factor
HIF1α - Hypoxia-inducible factor
IDC - infiltrating ductal carcinoma
ILC - invasive lobular carcinoma
IPEX (immunodisregulatory, polyendocrinopathy and enterophaty, X-linked syndrome)
LCIS - lobular carcinoma in situ
LN - lymph nodes
LOX - lysyl oxidase
M-CSF - macrophage colony-stimulating factor
MDSC - Myeloid-derived suppressor cell
MEM - Minimum Essential Medium
MFI – Mean Fluorescence Intensity
MFP – mammary fat pad
MMP - matrix metalloproteinase
MRI - magnetic resonance imaging
MSC - Mesenchymal stem cell
NAC - antioxidant N-acetylcysteine
NO – nitro
NOD - nonobese diabetic
NOS - nitric oxide synthase
NuRD - nucleosome remodeling deacetylase
OPN - osteopontin
PARP - poly ADP-ribose polymerase
PB - peripheral blood
PBMC - peripheral blood mononuclear cells
PD-1 - Programmed cell death 1
PD-L1 - Programmed cell death receptor ligand 1
PDE-5 - phosphodiesterase-5
PDGF - platelet-derived growth factor
PR - progesterone receptor
qPCR - quantitative polymerase chain reaction
RANKL - receptor activator of nuclear factor ligand
RNI - nitrogen intermediates
ROS - reactive oxygen specie
SATB1 - Special AT-rich binding protein 1
SCF - stem-cell factor
SDF1 - stromal derived factor-1
SERM - selective estrogen receptor modulators
SPARC - Secreted Protein Acidic and Rich in Cysteine
SPL - spleens
TAM - tumor associated macrophage
TBHP - tert-butyl hydroperoxide
TCGA - The Cancer Genome Atlas
TGF-β - transforming growth factor-β
TIL - tumor-infiltrating lymphocyte
TM - tumors
TME - tumor microenvironment
TN - tenascin
TNBC - triple negative breast cancer
Treg - regulatory T cell
TSP - thrombospondin
VEGF - vascular endothelial growth factor
WHO - World Health Organization
ZA - zoledronic acid
ABSTRACT

The pathological grade well defines tumor aggressiveness and, indeed, high-grade breast cancer (HGBC), regardless of molecular findings, is characterized by rapid clinical course and very poor prognosis despite standard chemotherapy regimens and specific-targeted agents.

Both the cellular and the extracellular compartments of the TME can contribute to the evolution of BC by powerful immune escape and immune suppression processes. In this context structural and extracellular components of the TME, namely the extracellular matrix (ECM), has been shown to contribute to many aspects of tumor progression, exerting important regulatory functions on tumor cells. The relevance of the ECM in cancer progression is strengthened by study showing that the ECM composition is a prognostic factor able to identify patients subgroups endowed with a different clinical outcome according to the distinct enrichment in ECM genes leading to four specific ECM signatures (ECM 1-4). Among the different ECM-related signatures, only the ECM3 signature, that characterize about 35 % of HGBC, identifies aggressive tumors with epithelial mesenchymal transition (EMT) features, poor prognosis, T-cell exclusion and increased MDSCs. Data obtained in mouse models over-expressing SPARC, one key gene of the ECM3, in BC cells suggested that the ECM impact on immune infiltration and that the ECM3 is involved in building an immune suppressive environment. We evaluated whether the immune suppressive features of ECM3 tumors can be sensed in the peripheral blood (PB) of HGBC patients. To this aim, we collected 70 consecutive HGBC patients that included 22 ECM3+ and 30 ECM3- and performed multiparametric flow cytometry analysis to define different immune cell populations. This PhD thesis highlights that the influence of ECM3 signature on immune cells is relevant as it is able to modify the composition of the peripheral blood of ECM3+...
patients in favor of an increase in a subset of regulatory T cells (Treg), defined as PD-1\textsuperscript{neg} Treg cells. Notably, we identified PD-1\textsuperscript{neg} Treg being associated to ECM3 patients and provided the mechanisms through which SPARC directly influence the expression of PD-1 on Treg and therefore directly impacting on their suppressive activity. This population together with the increased recruitment in CD33\textsuperscript{+} suppressive myeloid cells might account for the T-cell excluded microenvironment of ECM3 tumors. Furthermore, analyzing SPARC expression in ECM3 tumours along with CD33\textsuperscript{+} recruitment, we provided evidences that SPARC is expressed by MDSCs. Back to mouse model, using Sparc-deficient mice, we demonstrated that SPARC is a new MDSCs marker licensing suppressive activities in these cells.

Considering the peculiar immune suppressive environment of ECM3 tumors, we evaluated whether zoledronic acid (ZA) could be adopted as a strategy to revert the immunosuppression, particularly acting on MDSCs. We observed, in a few BC available patients, a reduction of the frequency of MDSCs and we showed that FACS-sorted CD33\textsuperscript{+} cells had decreased expression of PD-L1 and STAT3 after the administration of ZA.

Data suggest that the PB is a mirror of ECM3\textsuperscript{+} tumors and PD-1\textsuperscript{neg} Treg could be an effective target in ECM3\textsuperscript{+} patients. Therefore, novel therapeutic strategies are an urgent clinical challenge to be addressed in these HGB patients.
1. INTRODUCTION

1.1 Breast cancer

Breast cancer is one of the worldwide leading causes of death in women. It is characterized by high heterogeneity with different histopathological features, treatment sensitivity profiling and clinical outcomes.

1.1.1 Epidemiology and risk factors

Breast cancer (BC) represents 12% of newly diagnosed cancer cases [1]. Although, the mortality decreased in the recent years, still 43,600 women are expected to die in 2021 for BC, which is the second leading cause of cancer death, after lung cancer, in United States. According to the AIOM (Italian Association for Medical Oncology), 53,000 new cases are diagnosed every year in Italy and 12,000 people died of BC in 2020 (www.aiom.it).

The incidence of BC increases with age, especially in premenopausal years, reaching a plateau at 50-55 years old and increasing again after 60 years old [1]. The progressive exposure of mammary epithelium to endocrine proliferative stimulus, the accumulation of DNA damages and/or epigenetic alterations might alter the balance between the expression of oncogenes and tumor suppressor genes (www.aiom.it), thus explaining the relationship with the age.

From a geographical point of view, the disease shows geographical variability and in particular the incidence is ten-fold higher in the most economically developed countries [1]. Indeed, modifiable lifestyle factors have been associated with the development of BC including obesity, excessive consumption of alcohol, physical inactivity, and a high
dietary fat intake [2]. Risk factors that are not modifiable include family history [3], inherited genetic variations in breast cancer susceptibility genes (e.g., BRCA1 or BRCA2) [4], high breast tissue density (the amount of glandular tissue relative to fatty tissue measured on a mammogram) [5]. Reproductive factors such as long menstrual history (menstrual periods that start early and/or end late in life), late age at first pregnancy or not having children [6] not breastfeeding [7], high natural levels of sex hormones can increase the breast cancer risk [8].

1.1.2 Preventions and management of breast cancer

Thus far, great advances have been made in clinical studies of BC. In detail age, family history, reproductive factors, estrogen and lifestyle are important risk factors of BC, represented in the pyramid chart (Figure 1).

![Figure 1 - Schematic diagram of risk factors and preventions of breast cancer][9]

The current prevention methods including screening, chemoprevention and biological prevention are more direct and effective than those in the past [9]. Mammography is an effective screening method to use low energy X-rays to obtain high-resolution images.
of the breast. Magnetic resonance imaging (MRI) is another widely used screening tool for BC. It is more sensitive than mammography in high-risk women, especially in detecting the invasive ductal carcinoma [10]. MRI is not affected by the breast density, compared to mammography, and has advantages in detecting occult primary BC, axillary nodal metastasis, residual tumors after neoadjuvant chemotherapy or other small tumors [11].

Until recently, surgery, radiotherapy and standard chemotherapy were the only treatment options that could be offered to the majority of BC patients. Notably, in early BC without metastases, women with tumors considered operable undergo surgery. However, most women also need systemic therapy that can be given before surgery (neoadjuvant) or after surgery (adjuvant). Neoadjuvant therapy is administered in women with large tumors to reduce the size of the tumor or kill cancer cells that have spread. Adjuvant therapy is delivered after the primary treatment, to destroy remaining cancer cells.

Chemotherapy is defined as the use of pharmacologic or natural agents that inhibit the development of invasive BC either by blocking the DNA damage that initiates carcinogenesis, or by arresting or reversing the progression of premalignant cells in which such damage has already occurred [12]. Given that both radiation and chemotherapy exhibit lack of selectivity and high toxicity, there is an urgent need for novel drugs with more specific, possibly targeted actions [13].

Over the past two decades, there has been a tremendous shift in cancer treatment, from broad-spectrum cytotoxic drugs to targeted drugs [14]. Compared with traditional chemotherapy drugs, targeted drugs can specifically target cancer cells but spare normal cells, thus having high potency and low toxicity. Generally, there are two types of targeted therapy used: one is the small molecular targeted therapy and the other is the
use of monoclonal antibodies [15].

Estrogen receptor (ER) is a major target for chemotherapy because more than 70% of breast cancers are ER-positive breast cancers. Selective estrogen receptor modulators (SERMs) and the aromatase inhibitors (AIs) are two major classes of anti-estrogen drugs. One of the most SERMs is tamoxifen, which has been used to treat BC for more than 30 years [16]. Biological prevention mainly known as the monoclonal antibodies has been developed to improve the quality of life in BC patients.

Human epidermal growth factor receptor 2 (HER2) is one of the major targets of these monoclonal antibodies. Trastuzumab (Herceptin) is a recombinant humanized monoclonal antibody and it is the first HER2-targeted drug to be approved by the FDA. Pertuzumab is another humanized monoclonal antibody able to bind to the extracellular portion of HER2, like trastuzumab, even if the binding domain is different [17].

Recently, immunotherapy becomes a hot spot in cancer therapy, and it shows great potential in clinical use. Programmed cell death 1 (PD-1) is a membrane protein expressed in different immune cells, including T cells, which can be engaged by its specific ligand (Programmed cell death receptor ligand 1, PD-L1) to block the immune system. PD1 inhibitor drugs, nivolumab and pembrolizumab, were approved for the treatment of several solid tumors such as metastatic melanoma and non-small cell lung cancer. PD-L1 is detected in 20% of triple negative breast cancer (TNBC) and in 50% of all breast cancers [18]. In a phase I study, including 54 TNBC patients, atezolizumab, a PD-L1 inhibitor drug, exhibits a 19% of objective response rate [19]. In a phase III trial, atezolizumab plus nab-paclitaxel prolonged progression-free survival among patients with metastatic TNBC [20]. Though TNBC patients typically have poor clinical outcomes, anti-PD-1/PD-L1 drugs might be promising strategies for treating this subtype of BC.
A summary of the targeted therapies used in patients with BC is presented in Table 1.

<table>
<thead>
<tr>
<th>Therapy type</th>
<th>Drug</th>
<th>Type/class</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocrine therapy</td>
<td>Tamoxifen</td>
<td>Selective oestrogen receptor modulator</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td></td>
<td>Letrozole</td>
<td>Aromatase inhibitors</td>
<td>Aromatase enzyme</td>
</tr>
<tr>
<td></td>
<td>Anastrozole</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exemestane</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fulvestrant</td>
<td>Selective oestrogen receptor downregulator</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>HER2 therapy</td>
<td>Trastuzumab</td>
<td>Monoclonal antibody</td>
<td>HER2 receptor</td>
</tr>
<tr>
<td></td>
<td>Lapatinib</td>
<td>Small molecule inhibitor</td>
<td>HER2/EGFR tyrosine kinase pathways</td>
</tr>
<tr>
<td></td>
<td>Pertuzumab</td>
<td>Monoclonal antibody</td>
<td>HER2 receptor</td>
</tr>
<tr>
<td></td>
<td>T-DM1</td>
<td>Antibody-cytotoxic agent</td>
<td>HER2 receptor/tubulin</td>
</tr>
<tr>
<td>PI3K-mTOR inhibitors</td>
<td>Everolimus</td>
<td>Small molecule inhibitor</td>
<td>mTORC1 complex</td>
</tr>
<tr>
<td>BRCA1/2 inhibitors</td>
<td>Platinum analogues</td>
<td>Platinum-based agent (e.g. carboplatin)</td>
<td>DNA</td>
</tr>
<tr>
<td></td>
<td>PARP inhibitors</td>
<td>Small molecule inhibitor (e.g. olaparib)</td>
<td>Poly ADP ribose polymerase</td>
</tr>
<tr>
<td>CDK4/6 inhibitors</td>
<td>Palbociclib</td>
<td>Small molecule inhibitor</td>
<td>Cyclin-dependent kinases 4 and 6</td>
</tr>
<tr>
<td>Skeletal metastasis</td>
<td>Bisphosphonates</td>
<td>Bisphosphonate (e.g. zoledronic acid)</td>
<td>Osteoclast function</td>
</tr>
<tr>
<td></td>
<td>Denosumab</td>
<td>Monoclonal antibody</td>
<td>RANKL</td>
</tr>
</tbody>
</table>

Table 1 – Targeted therapies in BC. Adapted from [15]

1.1.3 Histological classification

The current histological classification of BC was proposed by the World Health Organization (WHO) in their guidelines [21]. Histology classification is based on shape, size and arrangement of breast cancer cells. Breast cancer could arise from different breast anatomical structures, affecting milk ducts (ductal carcinoma or not otherwise specified) or mammary glands (lobular carcinoma). These two types may display
invasive and infiltrating properties (invasive carcinoma) or not (*in situ* carcinoma). Invasive carcinomas are composed by malignant epithelial tumors, characterized by infiltration in surrounding tissues with irregular borders and metastatic spread. Noninvasive tumors display aberrant proliferation, without crossing basal membrane or invading surrounding tissues.

Invasive carcinomas are routinely graded based on the assessment of tubule/gland formation, nuclear pleomorphic and mitotic counts. The histology of the tumor is associated with the clinical outcome and prognosis: grade 1 (low-grade) is associated with a well-differentiated tumor with a good prognosis that demonstrates high homology to the normal breast terminal duct lobular unit, tubule formation (>75%), a mild degree of nuclear pleomorphism, and low mitotic count; grade 2 (intermediate-grade) with a moderately differentiated tumor and grade 3 (high-grade) with a poorly differentiated carcinoma with a marked degree of cellular pleomorphism and frequent mitoses and no tubule formation (<10%) and worse prognosis [22] (Figure 2).

Figure 2 - Histological grade of breast cancer[22]

The stage of BC, as for other tumors, is established using the TNM (tumor, node, metastasis) classification [23]. It takes into account tumor size (T1 to T4), involvement of lymph nodes (N0 to N3) and presence of metastasis (M0 or M1) (cancerresearchuk.org).
According to the histopathological evaluation, based on the microanatomy of the tissue and the organization of the cellular structures, breast tumors are classified as ductal carcinoma in situ (DCIS), infiltrating ductal carcinoma (IDC), lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC) (Figure 3).

DCIS is characterized by the proliferation of malignant cells that accumulate within the lumen of the membrane of the mammary ducts without invading the surrounding tissue [24]. DCIS has been generally recognized as the precursor of IDC type [25]. According to the American Cancer Society (2020), IDC is the most diffuse (81%) invasive carcinoma, which is characterized by an initial hyperplasia of ductal epithelium, followed by the formation of aggregates of malignant cells invading the surrounding stroma, the first step of metastatic dissemination [26].

LCIS and ILC represent a small percentage of BC [27]. Indeed, ILC representing about 15% of all invasive breast cancers (American Cancer Society, 2020). ILC typically grows as single cells, single lines or sheets. ILC is usually larger than IDC, but better differentiated and with a later metastatic spread. ILC might be characterized by bilateral and multifocal growth.

However, this histological classification was unable to reflect the high heterogeneity of BC, because, within each group, tumors displayed different biological and clinical features. Therefore, the histological classification showed poor prognostic and predictive impact.
1.1.4 Molecular Classification

Due to the limited prognostic and predictive power of the histological classification, the molecular profiling of different breast carcinoma has been done to better characterize tumor heterogeneity. Perou and Sorlie, in 2000, proposed “Molecular Classification” terminology in BC for the first time with a study distinguished five different molecular subtypes: Luminal A, Luminal B, HER2-enriched, Basal-like and Normal Breast-like group [31]. These groups of tumors have revealed critical differences in incidence [32], survival [33] and response to treatment.
The presence or absence of estrogen receptor (ER), progesterone receptor (PR) and HER2/neu protein over-expression is commonly used for this classification. Luminal tumors are the most diffuse type of breast carcinoma, displaying hormone receptors expression (ER⁺ PR⁺). Two distinct subtypes are differentially characterized: Luminal A and Luminal B.

- Luminal A subtype is characterized by high expression of ER and/or PR, low expression of genes related to proliferation (Ki67) and negative for HER2 expression (Table 2) [34]. The presence of estrogen and progesterone receptors indicates that the tumor is hormone-sensitive, and they also benefit the most from endocrine-therapy [35] with hormonal aromatase inhibitors or selective estrogen receptor inhibitors (i.e. tamoxifen) and selective ER regulators such as fulvestrant. For this reasons this subtype are characterized by good prognosis and lower rate of relapse.

- Luminal B subtype shows a lower expression of ER and a higher expression of Ki67, compared to Luminal A tumors (Table 2) [36]. Luminal B tumors are more aggressive with a poorer prognosis than Luminal A and are highly proliferative [37]. Endocrine therapy can still be offered to patients affected by Luminal B tumors, even if they have high risk of relapse and death. Indeed, it is associated with a partial response to the endocrine therapy but benefit from neoadjuvant chemotherapy [38].

- HER2 positive subtype is characterized by amplification or overexpression of ERBB2 oncogene (defined as HER2-enriched) [39]. HER2 overexpression is accompanied by lack of ER and PR (Table 2). The therapy of choice for these patients is based on the use of a monoclonal antibody, such as Trastuzumab, raised against the HER2 receptor [40], eventually combined with chemotherapy. Despite the major improvements in survival achieved by the use of the targeted therapy, HER2+ patients have a high relapse rate, a high risk of metastasis and a short overall survival [41,42]. From a
clinical point of view, patients with HER2-enriched tumors have a poor prognosis although they benefit from neoadjuvant treatment with higher response rate than luminal tumors [38].

- Basal-like subtype express genes characteristic of breast basal epithelial cells and it is considered the most aggressive breast cancer subtype, with poor prognosis and high risk of relapse [43]. The tumor lacks HER2 overexpression and ER and PR expression, and for this reason is also called TNBC (Table 2). These tumors stain positive for the basal keratins 5/6 and 17 together with EGFR (Epidermal growth factor receptor) at the IHC analysis. They are characterized by higher expression of proliferation-related genes. Large tumor size, high histological grade and lymph nodes involvement together with high mitotic index are peculiar for basal tumors. Due to the absence of all hormone receptors and HER2, the only therapeutic option for patients with TNBC is chemotherapy [44]. Approximately 75 % of BC patients carrying the BRCA1 mutations have a TNBC indicating that BRCA1 is an important vulnerable gene for this BC subtype [45]. For these patients, platinum based chemotherapies or poly ADP-ribose polymerase (PARP) inhibitor have been offered [46]. TNBCs are clinically relevant due to their lack of responsiveness to standard chemotherapies.

Recently, another BC subtype has been identified, defined as claudin-low, on the basis of the expression of a family of cell adhesion molecules at epithelial tight junctions, the claudins. The claudin-low subtype has been identified in human tumors, in mouse tumors [47] and in a panel of BC cell lines [48]. Like the triple negative subtype, these tumors lack ER and PR expression and HER2 (Table 2). These tumors are characterized by the absence of luminal markers, high genomic instability, expression of epithelial to mesenchymal transition (EMT)-related genes and stem cell-like characteristics [49]. Conversely, these tumors do not display a high expression of the
Ki67 proliferation marker [48]. Clinically, the majority of claudin-low tumors displayed a poor prognosis similar to luminal B or TNBC tumors [50] possibly because the only therapeutic possibilities are chemotherapy and eventually the use of PARP inhibitors for those patients with BRCA1 mutation tumors [49,51].

Tumor proliferation, done by Ki67 staining, as described before, is another important prognostic parameter to be evaluated in molecular classification.

<table>
<thead>
<tr>
<th>Molecular subtype</th>
<th>Biomarker profile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Luminal A</strong></td>
<td>ER+ and/or PR+, HER2- and low Ki67 (&lt;14%)</td>
</tr>
<tr>
<td><strong>Luminal B</strong></td>
<td>ER+ and/or PR+, HER2+</td>
</tr>
<tr>
<td>HER2 positive</td>
<td>ER+ and/or PR+, HER2- and high Ki67 (&gt;14%)</td>
</tr>
<tr>
<td>HER2 negative</td>
<td>ER-, PR- and HER2+</td>
</tr>
<tr>
<td><strong>HER2 enriched</strong></td>
<td>ER-, PR-, HER2-, and CK5/6 and CK17 and/or EGFR+</td>
</tr>
<tr>
<td><strong>Basal-like</strong></td>
<td>ER-, PR-, HER2-, and Claudin-low</td>
</tr>
<tr>
<td><strong>Claudin-low</strong></td>
<td>ER-, PR-, HER2-, and Claudin-low</td>
</tr>
</tbody>
</table>

**Table 2 – Molecular subtypes of breast cancer**

Furthermore, following a large study performed by The Cancer Genome Atlas (TCGA) Project on more than 500 of primary BC, the molecular intrinsic subtypes were refined. The comprehensive analysis at the DNA level (considering methylation pattern, chromosomal copy-number variation and somatic/germline mutation), RNA (i.e. mRNA expression and miRNA) and protein showed the existence of five main BC subgroups that recapitulated the main intrinsic subtypes found by gene expression analysis (**Figure 4**). Hierarchical clustering analysis of 320 human breast tumors and 17 normal breast samples places the claudin-low group next to the basal-like subtype indicating that both types share some gene expression features [49].
The tumor intrinsic subtype-based approach was adopted for the recommendation of systemic adjuvant therapies in early BC (i.e. endocrine, chemotherapy and anti-HER2 treatment) [52]. However, due to the prohibitive costs of gene expression molecular assay, clinical practice uses a surrogate classification of these subtypes on the basis of histological and molecular characteristics by the expression of the four biomarkers through IHC (i.e. ER, PR, HER2 and Ki67) (Figure 5).
Different molecular signatures correspond to distinct tumor severity and prognosis, together with clinic-pathological parameters, such as tumor grade, size and lymph nodes involvement. In terms of outcome, claudin-low tumors are poor outcome compared to luminal A tumors (Figure 6). However, in term of survival there are no differences between claudin-low and the other subtypes (Luminal B, HER2-enriched and Basal-like) or even between claudin-low versus all other tumors combined.
Introduction

1.2 Preclinical models of breast cancer

Animal models are useful in studying breast cancer development and progression, and in testing novel therapeutic approaches. These models ideally possess genetic and abnormalities similar to human counterparts. The most suitable available model is the xenograft, in which human cells or tissue are implanted either heterotopically (subcutaneously) or orthotopically (into mammary fat pad). The principal limitation of xenograft mice is the absence of a functional immune system, which excludes their use in immunological studies.

Many models are also available to analyze the role of immune system on tumor progression, like the syngeneic models or genetically engineered mouse (GEM) models. Each preclinical model presents advantages and limitations, which need to be carefully considered in the choice of the appropriate one.

Figure 6 – Kaplan-Meier relapse-free survival and overall survival curves with Normal Breast-like samples excluded [49]
1.2.1 Syngeneic models

There are different ways to mimic BC growth and metastasis in tumor transplantation experiments. Transplantation of cancer cells from one mouse into another mouse with identical genetic backgrounds (syngeneic transplantation) bypasses the immunologic host-versus-graft reaction and allows the investigation of the contribution of an intact immune system to malignant tumor progression [54]. For example, 4T1 cells, which originally derive from a spontaneous mouse mammary tumor of a BALB/C mouse, grow rapidly when injected into the fat pad of a syngeneic animal and metastasize to lungs, liver, bone, and brain [55]. Syngeneic mouse models are extensively used to analyze the impact of a functional immune system on tumor biology. Recently, they are widely used to develop immunotherapy protocols, able to stimulate immune system against tumor cells.

The principal limitations of these models include the intrinsic differences between the biology of tumors in rodents and humans [56]: i) in the mammary gland of rodents, unlike humans, a full glandular maturation is contingent on pregnancy, generating important differences regarding multipotent stem cells and their role in carcinogenesis; ii) the shorter lifespan of rodents leads to a rapid tumor progression, which may develop very aggressive tumors with multiple genetic alterations; iii) many human BC are hormone responsive, while the vast majority of mouse tumors are hormone independent; iv) BC in humans usually spreads through lymphatic system and the principal sites of distant metastasis are bones, brain, liver and lungs. In contrast, murine BC metastasizes to lungs through blood vessels; profound differences in metabolism between rodents and humans affect tumor behavior and microenvironment.
1.2.2 Genetically engineered mouse (GEM) models

These models are characterized by genetic profile alterations in one or several genes, likely involved in transformation or malignancy of tumor cells. Different genes could be deleted, over-expressed or defective, leading to spontaneous tumor development. GEM models are useful to study the impact of specific alterations in tumor biology and to evaluate in vivo novel therapeutic responses to these tumors.

Several promoters can be used to drive the expression of transgenes in the mammary epithelium and many known oncogenes have been expressed under their control to initiate or modulate breast carcinogenesis in mice, including ErbB2/Neu, polyoma middle T antigen (PyMT), simian virus 40 (SV40) T antigen, Ha-Ras, Wnt-1, TGF-α, and c-Myc. MMTV-Neu and MMTV-PyMT transgenic mice (in which the expression of the oncogene is driven by the Mouse Mammary Tumor Virus promoter) develop metastasis in lung and lymph nodes.

For example, mouse mammary tumor virus (MMTV)/c-neu transgenic mice express an activated rat c-neu oncogene (Erbb2). This alteration mimics the aberrant activation of ErbB2 receptors, a member of the EGF receptor gene family, which in human tumors is associated with 15 to 20% of human BC. MMTV-Neu transgenic mice result in the development of multifocal mammary tumors with metastatic spread to lungs in mice, starting from 5-10 months of age [57].

In this study, we used primary mammary carcinoma cell lines (SN25A) that are previously derived using spontaneous mouse model of mammary carcinomas BALB-neuT mice and SPARC-neuT. BALB-neuT mice, as mentioned, are transgenic for the activated form of the rat c-erB2 oncogene and develop mammary tumors involving all mammary gland [58], while SPARC-neuT mice were derived from BALB-neuT mice that were backcrossed with Sparc<sup>−/−</sup> mice [59].
1.3 The Tumor Microenvironment

The concept of tumor microenvironment (TME) is very old and dates back to the time when the relationship between cancer and inflammation was proposed. It is now increasingly accepted the concept that tumor cells do not manifest disease alone, but the development, progression and aggressiveness of every tumor closely depends on the influence of the different components of the TME [60].

In the year 2000, Hanahan and Weinberg published an influent review [61] in which the hallmarks of cancer were classified into six main hallmarks: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. A decade later, an update review [62] added two emerging features: reprogramming energy metabolism and evading the immune response, and two enabling traits such as genome instability and mutation, and tumor-promoting inflammation. It is clear that even the initial hallmarks list contains tissue-relevant rather than cancer-cell-specific components (e.g. angiogenesis) the last update has a section dealing with the tumor microenvironment.

TME is characterized by the interaction between neoplastic and non-transformed cells [63]. TME consists, in addition, of cellular components such as cancer-associated fibroblasts (CAFs), blood and lymphatic endothelial circulating cells, immune cells, pericytes, and non-cellular components including elements of extracellular matrix (ECM) and soluble factors such as cytokines and growth factors [64,65] (Figure 7).

Several evidences have underlined that the different components of the TME create an intricate network that influence the development, aggressiveness and cancer progression also endowing clinical/prognostic significance [60].
In particular, the function and the recruitment of immune cells change during the evolution of tumor and can limit tumor growth.

Figure 7 - Tumor microenvironment. Adapted from[65]

Indeed, components of TME actively interact with tumor cells but also each-others loading to a tumor-prone or a tumor-suppressive environment [60,66]. Furthermore, different tumor outcomes can be observed under the same tumor-shaping oncogenic drivers microenvironment [67]. Therefore, characterizing the cellular and molecular components of the TME, and understanding how they influence tumor progression, represents a crucial aim for the success of cancer therapies [68].

1.3.1 Cellular components of the TME

On a cellular level, TME comprises a broad spectrum of subpopulations. Hanahan and Coussens, for simplification, have grouped these into three compartments including
angiogenic vascular cells, CAFs and infiltrating immune cells and [69] summarized in

Figure 8.

![Figure 8 - Functions of cell recruited to the TME [69]](image)

**Angiogenic vascular cells**

Specifically, the tumor vasculature network is dynamic and can limit tumor growth [70]. Vascular cell fraction includes pericytes, endothelial cells and smooth muscle cells has been extensively characterized in tumour development [71].

- Vascular endothelium is a thin monolayer of endothelial cells that help to orchestrate the formation of new blood vessels (angiogenesis). Endothelial cells are also critical in promoting cancer cell migration, invasion and metastasis; they are very plastic in nature and can change cell fate. It not only separate circulation blood from tissues, but also contributes to tumor growth providing nutrients and oxygen [72], maintains metabolic
homeostasis, carriers immune cells and facilitates the trafficking of soluble factors or tumor cells important for the dissemination and metastasis [73-75]. Metastasis is a multistep process that involves translocation of cancer cells from primary tumor to distant locations. Tumor cells first need to escape to the primary tumor site to enter into the vasculature during a process known intravasation [76]. During this step, tumor cells adhere to endothelial cells and this interaction changes the endothelial barrier, allowing tumor cells to migrate between two endothelial cells. A hypoxic TME leads to the activation of hypoxia-inducible factors critical for coordinating cellular responses to low O$_2$[77]. Sprouting of vessel is a common mechanism used by tumors to induce the growth of new vessels. In detail, hypoxia-inducible factors initiate vessel sprouting by instructing endothelial cells to secrete pro-angiogenic factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) [78]. VEGF stimulates migration of endothelial cells to form new blood vessel lumens.

- Pericytes are multifunctional cells in the TME that envelop the surface of endothelial cells and along with them they are involved in the basement membrane remodeling during angiogenesis [79] and tumorigenesis [80]. In addition, pericytes have several functions in the immune system including the attraction of inborn leukocytes to exit blood vessels, regulating lymphocyte activation and eliciting direct phagocytic activity [81].

**Cancer-associated fibroblasts**

Among all components in the TME, CAFs not only are one of the most important members but also represent the largest proportion of stroma cells of the TME. These cells are involved in cancer progression, invasion and metastasis [82]. The origin of
CAFs remains unclear but they may be derived from resident fibroblasts, bone marrow derived cells that are actively recruited and/or cancer cells that under-go active EMT [83]. In the TME, cancer and stromal cells secrete factors such as transforming growth factor-β (TGF-β), PDGF and fibroblast growth factor 2 (FGF2) to convert fibroblasts into cancer-associated fibroblasts. CAFs are able to secrete different proteases, which enhance their ability to migrate and remodel ECM [84] and they also produce a variety of soluble factors that can contribute to disease progression such as hepatocyte growth factor (HGF), TGF-β, stromal derived factor-1 (SDF1), VEGF, IL-6 and matrix metalloproteinases (MMPs), thereby affecting several hallmarks of cancer [61,62]. In a meta-analysis involved breast cancer, a high density of fibroblasts was associated with worse clinical outcome, poor tumor differentiation and lymph node metastasis [85]. Moreover, high collagen density can reduce the number of CD8+ tumor-infiltrating lymphocytes (TILs) in breast tumors and modulate T-cell cytotoxic activity [86]. Interestingly, in breast have been identified distinct subsets of CAF (S1-S4) with distinct properties through multicolor flow cytometry and concomitant analysis of the expression level of different markers [87]. CAF-S1 and CAF-S4 are more present in TNBC and, in particular, CAF-S1 promotes an immunosuppressive environment through a multi-step mechanism. By secreting CXCL12, CAF-S1 attracts CD4+CD25+ T lymphocytes and retains them by OX40L, PD-L2, and JAM2. Moreover, CAF-S1 increases T lymphocyte survival and promotes their differentiation into regulatory T cells (Treg), through B7H3, CD73, and DPP4. Finally, in contrast to CAF-S4, CAF-S1 enhances Treg capacity to inhibit T effector proliferation. These data are consistent with Treg accumulation in CAF-S1-enriched TNBC and show how a CAF subset contributes to immunosuppression [87].
Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are mainly composed of stromal cells that reside in mesenchymal tissues such as the bone marrow, fat tissue and cartilage [88]. MSCs are immature cells and potentially differentiate into different cell types. MSCs divide into daughter cells that share the same properties of their mother cell (self-renewal) or differentiate into specialized cells to replace dying cells and repair damaged tissues (multi-lineage differentiation). Notably, these adult stem cells can differentiate into various cell types of the mesodermal lineage, including chondrocytes, osteoblasts, adipocytes and endothelial cells. MSCs express membrane CD90, CD73, and CD105, and are negative for CD45, CD34, CD31, CD14, CD19, and HLA-DR [89]. MSCs migrate toward inflamed areas during pathological immune responses and participate in tumor progression. It was shown that crosstalk between MSCs and cancer cells was crucial for tumor metastasis and promoting EMT [90]. Emerging data suggest that MSCs can promote tumorigenic processes, including malignant transformation, establishment and maintenance of cancer cells, promotion of angiogenesis and neovascularization-sustaining neoplastic tissues, metastasis formation, and chemo resistance to anticancer drugs [91].

Cancer-Associated Adipocytes

Adipocytes represent a relatively abundant component of breast parenchyma. Cancer-associated adipocytes (CAAs) differ from normal adipocytes in size, metabolic activity and adipokines expression [92]. It has been demonstrated that CAAs are involved in tumor progression, metastasis by secretion of adipokines, such as leptin and adiponectin, and different chemokines and interleukins [93]. The increased leptin secretions in CAAs can promote cell proliferation and tumor angiogenesis. Adiponectin,
on the contrary, is decreased in CAAs and it plays an anti-tumor role inducing apoptosis, suppressing tumor growth and invasion of BC cells through AMPK activation and the inhibition of PI3K/AKT pathway [94]. In TNBC patients the serum leptin levels were significantly associated with its severity, while the adiponectin levels were comparative [95]. Moreover, IL-6 produced by adipose tissues is more secreted in CAAs compared with other adipocytes and it has been shown to promote cancer progression when adipocytes are co-cultured with BC cells [96].

Therefore, blocking IL-6 signaling might be a potential therapeutic strategy for breast DCIS characterized by pathological IL-6 overproduction [96].

In detail, components, functions and classification of TME are summarized in Table 3.

<table>
<thead>
<tr>
<th>Component</th>
<th>Function</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer-associated fibroblasts (CAFs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages (M2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulatory T cells (Treg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myeloid-derived suppressor cells (MDSCs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages (M1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
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<tr>
<td>Adipocytes</td>
<td></td>
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<tr>
<td>Neutrophils</td>
<td></td>
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<tr>
<td>Vascular network</td>
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<tr>
<td>Extracellular vesicles (EVs)</td>
<td></td>
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<tr>
<td>Extracellular matrix (ECM)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Function</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer-associated fibroblasts (CAFs)</td>
<td>Sequestering profibrotic signaling; activating angiogenesis and metastasis; tumor-promoting inflammations; evading immune destruction; reprogramming cellular metabolism; promoting genomic instability and mutation.</td>
<td>Tumor promoting; less known to be tumor inhibiting. at low levels in TME</td>
</tr>
<tr>
<td>Macrophages (M2)</td>
<td>Enhancement of angiogenesis and metastasis; associated with poor prognosis.</td>
<td>Tumor promoting (N2): tumor inhibiting (N1); increased levels in the colon, stomach, and lung cancer patients.</td>
</tr>
<tr>
<td>Regulatory T cells (Treg)</td>
<td>Promoting degradation of the extracellular matrix; aiding the expansion of inflammatory cytokines, such as TNF-α, enhancement of angiogenesis and remodeling.</td>
<td>Tumor promoting (M2); tumor inhibiting (M1); the major proinflammatory component in TME; the first nonproliferative cells infiltrating the tumor, attracted by chemokines secreted by both macrophage and stromal cells.</td>
</tr>
<tr>
<td>Myeloid-derived suppressor cells (MDSCs)</td>
<td>Induce apoptosis, necrosis, and growth arrest by inhibiting TGF-β and other cytokine cytokines; establishing an antiinflammatory environment.</td>
<td>Tumor inhibiting; the major antimetastatic component in TME.</td>
</tr>
<tr>
<td>Macrophages (M1)</td>
<td>Secreting cytokines such as IL-10, TGF-β, establishing an immunosuppressive environment; associated with poor prognosis.</td>
<td>Tumor promoting; promoting tumor maintenance.</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Associated with tumor progression and neovascularization; suppressing T cells and NK cells; differentiating into TAMs under hypoxic conditions.</td>
<td>Tumor promoting; increased in almost all patients with cancer; including primary glioblastoma, macroglia, dendritic cells, and myeloid precursors.</td>
</tr>
<tr>
<td>Adipocytes</td>
<td>Differentiating into mammary tissues; home, cartilage, and fat tissues; vasculogenic mimicry; forming the perivasculature niche; promoting cancer initiation and malignancy.</td>
<td>Tumor promoting; the major component of stromal cells in TME.</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Consisting of tumor blood vessels; secreting angiogenic factors such as adhesion molecules; intercommunicating with tumor cells (via secreting EVs including CD68, CD40L).</td>
<td>Tumor promoting.</td>
</tr>
<tr>
<td>Vascular network</td>
<td>Regulating the balance of systemic energy and metabolism; secreting enzymes, cytokines, chemokines, and hormones; promoting cancer progression.</td>
<td>Tumor promoting.</td>
</tr>
<tr>
<td>Extracellular vesicles (EVs)</td>
<td>Promoting proangiogenic; secreting neurotrophic factors, including CA9, chondrocytic and vascular progenitors; regulating NK cell migration and toxicity ability.</td>
<td>Tumor promoting; all malignant tissues are angiogenesis-dependent.</td>
</tr>
<tr>
<td>Extracellular matrix (ECM)</td>
<td>Providing oxygen, clearing carbon dioxide, and metabolizing wastes; providing nutrients support for cancer cells; promoting angiogenesis and metastasis.</td>
<td>Tumor promoting.</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Helping immune cell avoid immunity and dissemination; providing a physical link between lymph nodes and tumor.</td>
<td>Tumor promoting.</td>
</tr>
</tbody>
</table>

Table 3 – Summary of components, functions and classifications of TME [97]
1.3.1.1 Immune cells within the TME

Immune cells are critical component of the TME and are included in two categories: adaptive immune cells and innate immune cells. Innate immunity is characterized by a non-specific defense mechanism that occurs rapidly (minutes to hour) and lacks of the capacity to recognize individual pathogen species and strains, and next is not able to mount immunological memory. Innate immune cells include macrophages, neutrophils, eosinophils, basophils, dendritic cells, mast cells and natural killer cells. Recently, several studies have demonstrated that innate immune cells, such as monocytes, macrophages or natural killer cells, can develop an immunological memory to an initial insult (priming), which results in an enhanced immune response to a secondary challenge [98]. This property has been termed “trained immunity” or “innate immune memory” and appears nonspecific, because exposure to one pathogen can afford better protection to a second, unrelated pathogen [99] and is mediated by epigenetic and metabolic reprogramming [100]. On the other side, the adaptive immunity is activated by exposure to specific antigens and builds an immunological memory to evaluate the threat and enhance the effectively of the immune response. Adaptive immunity comprises T and B-lymphocytes derived from lymphoid lineage. Immune cells could play a critical role as promoting or suppressing tumor growth (Figure 9); depending on the context they can be either pro- or anti-tumorigenic [101].
In particular, active immunosuppression depends on the recruitment and activation of different cell types, including Tregs, Myeloid-derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) [102] (Figure 10). Tumors carry out different mechanisms for evading the immune system. One simple method is the defects in the cellular machinery responsible for MHC class I (MHC-I) antigen processing and presentation [103,104]. Cytotoxic T-lymphocytes (CTLs) cannot recognize and eliminate transformed cells if tumor peptide antigens are not presented by MHC-I. Blocking the effector function of CTLs through various mechanisms is perhaps the most common and effective means of interfering with antitumor immunity. Tumors promote an immunosuppressive microenvironment by recruiting Treg and myeloid elements, such as and MDSC that produce anti-inflammatory cytokines, IL-10 and TGF-β and inhibit the cytolitic activity of CTLs [105,106]. Increased metabolism of arginase, nitric oxide synthase (NOS) inhibits T-lymphocyte responses through L-arginine depletion, an important metabolite for T-cell function [107]. MDSC also produce reactive oxygen species (ROS) and nitrogen intermediates (RNI) [108]. Moreover, TAM and MDSC can
express on the surface inhibitory proteins such as PD-L1 and PD-L2, which inhibits CTL function through the expression of PD-1 receptor on the surface of T-lymphocytes [109]. Hypoxia-inducible factor (HIF1α) expression, induced by low level of oxygen in tumor, stimulates the production of SDF-1, a chemokine that recruits myeloid-derived cells through the chemokine receptor CXCR4 [110].

Figure 10 – Mechanism for evading immune system utilized by tumors [102]
1.3.1.2 TME classification according to immune-infiltration

Understanding the composition, proportion, activation, or functional states of immune infiltrates within the TME are critical for both diagnosis and therapeutic intervention.

The density and diversity of tumor-infiltrating immune cells are closely related to prognosis and prediction of treatment efficacy. The TME could be simply characterized into “cold” (immunologically ignorant/immune-desert), immune-excluded or “hot” (preexisting immunity/immune-inflamed) [111] (Figure 11).

- The “cold” tumors are characterized by a paucity of T cells in either the parenchyma or the stroma of the tumor with highly proliferating tumor cells with low mutational burden and low expression of antigen presentation machinery markers including MHC class I [112].

- The immune-excluded tumors are characterized by the presence of abundant immune cells. However, immune cells do not penetrate the parenchyma of these tumors but are retained in the stroma that surrounds nests of tumor cells [112,113]. The stroma may be limited to the tumor capsule or might penetrate the tumor itself, making it seem that the immune cells are actually inside the tumor. After treatment with anti-PD-L1/PD-1 agents, stroma-associated T cells can show evidence of activation and proliferation but not infiltration, and clinical responses are uncommon. A pre-existing anti-tumor response might have been present but was rendered ineffective by a block in tumor penetration through the stroma or by the retention of immune cells in the stroma. Cold tumor and immune-excluded tumors can both be considered as non-inflamed tumors.

- The immune-inflamed tumors are characterized by the presence in the tumor parenchyma of both CD4- and CD8-expressing T cells, high density of IFN-\(\gamma\)-producing CD8+ T cells, often accompanied by myeloid cells and monocytic cells;
the immune cells are positioned in proximity to the tumor cells [113]. Samples from inflamed tumors may exhibit staining for PD-L1 on infiltrating immune cells and, in some cases, tumor cells [114]. Indeed, clinical responses to anti-PD-L1/PD-1 therapy occur most often in patients with inflamed tumors [115]. However, a response is not assured in these individuals, which indicates that immune-cell infiltration is necessary but insufficient for inducing a response. In general, the hot tumors present higher response rates to immunotherapy, such as PD-L1/PD-1 therapy. Therefore, various studies have focused on converting non-inflamed cold tumors into hot ones using the combination of radiotherapy with immunotherapy to improve the effectiveness of the latter [116].

Figure 11 - Anti-cancer immunity in humans is segregated into three main phenotypes[112]
1.3.1.3 Myeloid-derived suppressor cells

MDSCs are a very heterogeneous population of cells comprising myeloid progenitor cells and immature myeloid cells (immature macrophages, immature granulocytes and immature dendritic cells). In healthy individuals, immature myeloid cells that are generated in the bone marrow quickly differentiate in mature cells. It is known that MDSCs expand during cancer [117,118], acute inflammation and infection by suppressing the antitumor immune response [119]. Indeed, in pathological conditions a partial block in the differentiation of immature myeloid cells into mature myeloid cells results in the expansion of this population. MDSCs are characterized by the co-expression of the myeloid differentiation markers Gr-1 and CD11b in mouse, and/or of the common myeloid markers CD33 and CD11b in human (see Table 4) [120]. Gr-1 is composed by two different cell membrane molecules Ly6C and Ly6G and according to their expression levels, MDSCs are classified in mouse in two subtypes: granulocytic MDSCs (PMN-MDSCs CD11b+ Ly6G+ Ly6C\text{low}) and monocytic MDSCs (M-MDSCs CD11b+ Ly6G\text{−} Ly6C\text{high}) [121]. Human PMN-MDSCs are defined as CD11b+ CD33+ CD15+ HLA-DR\text{−} or CD11b+ CD14- CD66b+ and M-MDSCs as CD11b+ CD33+ CD14+ HLA-DR\text{−} CD15- [121,122]. However, another subset of myeloid cells is proposed [121], defined as “early-stage MDSC” (eMDSC) and characterized as Lin- (including CD3, CD14, CD15, CD19, CD56) HLA-DR\text{−} CD33+. Immature myeloid cells, in healthy individuals, constitute ~0.5% of peripheral blood mononuclear cells [123]. There have been studies investigating circulating MDSCs in peripheral blood of cancer patients. In BC patients, it was shown that the amount of circulating MDSCs in peripheral blood of patients was associated with metastasis and impaired clinical outcome [124,125].
Introduction

<table>
<thead>
<tr>
<th>MDSCs</th>
<th>Mouse Phenotype</th>
<th>Human Phenotype</th>
</tr>
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<tbody>
<tr>
<td>PMN-MDSC</td>
<td>CD11b+ Ly6C&lt;sup&gt;low&lt;/sup&gt; Ly6G+</td>
<td>CD14- CD11b+ CD15+ (or CD66b+)</td>
</tr>
<tr>
<td>M-MDSC</td>
<td>CD11b+ Ly6C&lt;sup&gt;high&lt;/sup&gt; Ly6G-</td>
<td>CD11b+ CD14+ HLA-DR- CD15-</td>
</tr>
<tr>
<td>eMDSC</td>
<td>Not determined</td>
<td>Lin- (CD3/14/15/19/56) HLA-DR- CD15+</td>
</tr>
</tbody>
</table>

Table 4 – Immune phenotype identify MDSC populations

1.3.1.4 Suppressive mechanisms mediated by MDSCs

MDSCs carry out their immunosuppressive functions through direct cell-to-cell contact, which suggests that they act either through cell-surface receptors and/or through the release of short-lived soluble mediators. Mechanisms used by MDSCs to inhibit antitumor T cell response can be classified into four classes.

The first type of mechanism is associated with the metabolism of L-arginine and therefore with lymphocyte nutrient depletion through ARG1-dependent consumption and L-cysteine deprivation via its consumption and sequestration [126,127]. Specifically, L-arginine is a substrate for two enzymes, iNOS (generates NO) and arginase 1 (converts L-arginine to urea and L-ornithine). These depletions cause down-regulation of the CD3 ζ-chain in the T cell receptor (TCR) complex and proliferative arrest of antigen-activated T cells [128].

The second type of mechanism is the generation of oxidative stress, which is caused by their production of ROS and reactive nitrogen species. Production of ROS has emerged as one of the main characteristics of MDSCs isolated from tumour-bearing mice and patients with cancer. The production of ROS by MDSC can be induced by several tumor-derived factors, such as TGF-β, IL-3, IL-6, IL-10, PDGF and granulocyte-macrophage colony-stimulating factor (GM-CSF) [129]. Peroxynitrite is a product of a
reaction between NO and superoxide anion and high level are present at sites in which MDSCs and inflammatory cells accumulate [130]. Hydrogen peroxide are produced by the combined and cooperative activity of phagocytic oxidase, ARG1 and iNOS in different MDSC subsets and they drive a number of molecular blocks in T cells, ranging from the loss of ζ-chain expression and interference with IL-2 receptor signaling to nitration and subsequent desensitization of the TCR [131,132].

The third set of mechanism interferes with lymphocyte trafficking and viability. Plasma membrane expression of ADAM17 (a disintegrin and metalloproteinase domain 17) by MDSCs decreases L-selectin expression on the surface of naïve CD4+ and CD8+ T cells, thereby limiting T cell recirculation to lymph nodes [133]. Another example is the modification of CCL2 by MDSC-derived peroxynitrite, a process that impairs migration of effector CD8+ T cells to the tumour core. MDSCs express galectin 9, which binds to T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) on lymphocytes and induces T cell apoptosis [134]. MDSCs mostly through membrane contactdependent mechanisms, i.e. membrane bound TGF-β (mouse MDSCs) and interaction with the NK receptor NKp30 decrease the number and inhibit function of mouse and human NK cells [135,136].

The fourth mechanism is the activation and expansion of Treg cells. MDSCs expand antigen-specific natural Treg (nTReg) cells and also promote conversion of naïve CD4+ T cells into induced Treg (iTReg) cells. The mechanisms are not completely understood, but may involve cell-to cell contact, including CD40–CD40L interactions, production of soluble factors by MDSCs, such as IFNγ, IL-10, and TGFβ, and possibly also MDSC expression of ARG1 [137]. Human CD14+ HLA-DRlow MDSCs promote the transdifferentiation of Foxp3+ iTReg from Th17 lymphocytes by producing TGF-β and retinoic acid [138] (Figure 12).
1.3.1.5 Pharmacological targeting of MDSCs

The acknowledgment that immune suppression plays a crucial role in promoting tumor progression and the recognition that MDSCs are one of the main immunosuppressive factors in cancer and other pathological conditions, several therapeutic strategies that target these cells are currently explored.

1.3.1.5.1 Promotion of myeloid-cell differentiation

To target MDSCs one of the most promising approaches for therapy is to promote their differentiation into mature myeloid cells that do not have suppressive activity. This possibility can be mediated using retinoic acid, a vitamin A metabolites, that have been found to stimulate the differentiation of myeloid progenitors into dendritic cells (DCs).
and macrophages [140]. Numbers of precursor cells are expanded in the bone marrow and spleen of mice treated with retinoic acid receptor antagonist [141]. Differently, all-
trans retinoic acid (ATRA) administration, a derivate of vitamin A, results in a decrease of MDSCs number both in tumor-bearing mice and patients with cancer. MDSCs were differentiated into DCs and macrophages in vitro and in vivo using ATRA [142]. ATRA mediates the differentiation of the monocytic subset of MDSCs and the apoptosis of the granulocytic subset up-regulating glutathione synthetase (GSS), responsible for the accumulation of glutathione. ATRA induce MDSC differentiation by neutralizing high ROS production, essential for maintaining the immature state of MDSC [143]. Decreasing number of MDSCs in tumor-bearing mice enhanced CD4+ and CD8+ T cell-dependent, tumor-specific immune responses. Studies showed that the combination of ATRA and two different types of cancer vaccines prolonged the antitumor effect of the treatment, suggesting that ATRA could be a possible candidate for enhancing the effectiveness of active immunotherapy of cancer [144]. Another agent with the potential to decrease MDSC numbers and also to promote myeloid-cell differentiation is vitamin D3.

Head and neck squamous cell carcinoma patients treated with the differentiation-inducer 25-hydroxyvitamin D(3) have a reduction in the number of immune suppressive CD34+ cells and an increase of HLA-DR expression, IL-12 and IFN-γ levels in plasma [145].
1.3.1.5.2 Drugs inhibiting MDSC expansion

Several studies have focused on neutralizing the effects of tumor-derived soluble factors responsible for MDSC expansion and recruitment. These factors include: prostaglandins; growth factors like granulocyte-macrophage colony-stimulating factor GM-CSF, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), stem-cell factor (SCF), VEGF; TGF-β, IL-1b, IL-6, IL-10, IL-12, IL-13; and chemokine, like CCL2, CXCL2 and CXCL12. SCF is shown to be involved in MDSC expansion, mice bearing tumor cells with SCF siRNA knockdown reduced MDSC expansion and restored proliferative responses of tumor-infiltrating T cells. Additional possible target to block MDSC expansion is the matrix metalloproteinases, a family of closely related, zinc-dependent proteolytic enzymes able to degrade all the components of the extracellular matrix. In particular, inhibition of MMP-9 function decreased the number of MDSCs in the spleen and tumor tissues, resulting in a delay in the growth of spontaneous NeuT tumors in transgenic BALB/c mice [146]. Pamidronate and zoledronate, two different amino-bisphosphonates, interfere with the conversion of mevalonate to geranylgeranylpyrophosphate necessary for cholesterol synthesis. In detail, zoledronic acid (ZA) treatment has been shown to impair tumor growth, significantly decrease MMP-9 expression and the number of macrophages into tumor stroma. Importantly, treatment with amino-bisphosphonates reduced MDSC expansion both in bone marrow and peripheral blood [146]. A single dose of pamidronate and zoledronic acid, in cancer patients, decreased VEGF, TGF-β and MMP-2 serum levels [147]. The effect of ZA was also studied in pancreatic mouse model. In this study, mice treated with ZA showed less intra-tumoral MDSC accumulation, reduced tumor growth with a prolonged median survival and increased T
cells infiltrating tumor. In addition, ZA-treated mice demonstrated increased levels of IFN-γ and decreased levels of IL-10 within the tumors [148].

**1.3.1.5.3 Inhibition of MDSC function**

Another way to inhibit MDSCs is to block the signaling pathways that regulate MDSC production of suppressive factors. This might be achieved by targeting COX2, required for the production of prostaglandin E2. In 3LL lung carcinoma, COX2 has been shown to induce the up-regulation of Arginase 1 (ARG1) expression by MDSCs, thereby inducing their suppressive function [149]. Accordingly, COX2 inhibitors (Colecxib) were found to down regulate the expression of ARG1 by MDSCs, which improved antitumor T-cell responses and enhanced the therapeutic efficacy of immunotherapy [107]. COX2 inhibitors may have more than one mechanism of suppressing MDSC, namely they can block their activation and also reduce their numbers [150]. Similarly, phosphodiesterase-5 (PDE-5) inhibitors, such as sildenafil and tadalafil, are able to down regulate the expression of ARG1 and NOS2 by MDSCs inhibiting the degradation of cyclic guanosine monophosphate (cGMP). It is not completely clear how this occurs but it has been proposed that high cGMP levels could interfere with the expression of IL-4Ra on myeloid cells leading to decreased STAT6 activity and down regulation of both ARG1 and NOS2 [151]. Another help against nitric oxide metabolism of MDSCs are nitro-aspirins (NO-aspirin). NO-aspirins suppress the production of ROS, provide feedback inhibition of NOS2 catalytic activity and decrease of pro inflammatory cytokine production by monocytes [152]. The *in vivo* adjuvant effect of nitroaspirin was demonstrated in both a colon cancer and a mammary carcinoma model. C26 colon carcinoma-bearing mice treated with a nitro-aspirin, NCX-4016 improved T cell
proliferation, decreased numbers of MDSC within the tumor, and retarded tumor growth compared to untreated mice [153].

1.3.1.5.4 Reducing MDSC accumulation

Some strategies have been found to cause MDSC depletion through unknown mechanisms. Either chemotherapy or host irradiation has been used to eliminate MDSCs. Effectively, the administration of chemotherapy drugs, such as gemcitabine, in tumor bearing mice reduced selectively the number of splenic MDSCs, while the number of CD4+ or CD8+ T cells or, NK cells was not affected [154]. The mechanism by which gemcitabine eliminates splenic CD11b+ Gr-1+ cells are unknown. One attractive possibility is the selective killing of MDSCs. In fact after in vivo treatment with gemcitabine an increased rate of apoptosis in splenocytes indicates, together with in vitro studies, that gemcitabine accelerates the death of MDSCs without affecting other cells [154]. Gemcitabine, used either as a single agent or in combination with cisplatin or paclitaxel has been tested in different clinical trials. In a study of 17 patients with early-stage breast cancer that were treated with doxorubicin–cyclophosphamide chemotherapy, a decrease in the level of MDSCs in the peripheral blood was observed [155]. Another chemotherapy agent, 5-Fluorouracil (5-FU), has been found to lead to a reduction in splenic and tumor MDSC. This drug had no significant effect on other immune cells except for an increase in the number of B cells. As a pyrimidine analog, 5-FU is used for the treatment of colon, breast, stomach and pancreas cancer and it induced cell cycle arrest and apoptosis.

Controlling MDSC recruitment as well as interfering with the molecular pathways of MDSC differentiation/expansion, or blocking MDSC suppressive functions represents an important approach for cancer therapy. The main categories of targeting agents used to inhibit MDSC activity are summarized in Figure 13.
1.3.1.6 Regulatory T cells

Treg cells are a highly immunosuppressive subset of CD4+ T cells characterized by the expression of the master transcription factor forkhead box protein P3 (FOXP3). Tregs are tolerogenic subsets that mediate peripheral self-tolerance by suppression of effector cell function.
T cells and other immune cells. Sakaguchi et al. [157] identified Treg cells as CD4+CD25+ (IL-2 receptor α chain) T cells with crucial roles in maintaining immune homeostasis and thus preventing autoimmune disease. Now, Tregs are established as the primary mediator of peripheral tolerance crucial for the aversion of autoimmune disease, allergies, transplant rejection and graft versus host disease (GvHD). Mice (known as scurfy mice) and individuals that lack FOXP3 develop a profound autoimmune-like lymphoproliferative disease that emphasizes the importance of Treg cells in the maintenance of peripheral tolerance. Indeed, human lack functional FOXP3 develop IPEX (immunodisregulatory, polyendocrinopathy and enteropathy, X-linked syndrome), which is a severe autoimmune disease that develops early in infancy.

1.3.1.6.1 Treg cell markers

Identifying discriminatory markers for the characterization and isolation of regulatory T cells has always been a crucial goal. Treg cells were originally identified as CD25-expressing CD4+ T cells, although CD25 is a general marker of T cell activation and expression of this protein is not exclusive to Treg cells, thus highlighting the need for additional specific markers of this cell type. Expression of FOXP3 is mostly limited to the Treg cell population in mice [158]. By contrast, FOXP3+ T cells in humans are heterogeneous with respect to their phenotype and function, with immunosuppressive subpopulations as well as non-immunosuppressive subpopulations generated through up regulation of FOXP3 expression upon TCR stimulation of Tconv cells [159,160]. Consequently, it is important to search Treg cell specific markers, particularly in humans. For instance, it was shown that CD127 expression (also known as IL-7 receptor subunit-α) is down regulate by Treg cells and that this could be used to increase the purity of human Treg cell isolation [161]. Indeed, there is a 90% correlation
Introduction

between CD4+ CD25+ CD127\text{low} T cells and FOXP3 expression [162]. In addition, it was found that Treg cells expressed a higher level of folate receptor 4 compared with activated effector T cells [163]. An alternative classification of human Treg cells has been proposed according to the expression levels of a marker of naive T cells, CD45RA, as well as CD25 and FOXP3, with a strong association between immunological phenotypes and functions. Using this classification, FOXP3+ CD25+ CD4+ T cells can be classified into three fractions: naive Treg cells (CD45RA+ FOXP3\text{low} CD25\text{low} CD4+); effector Treg (eTreg) cells (CD45RA- FOXP3\text{high} CD25\text{high} CD4+); and non-Treg cells (CD45RA- FOXP3\text{low} CD25\text{low} CD4+). Upon TCR stimulation, naive Treg cells proliferate rapidly and differentiate into highly immunosuppressive eTreg cells. By contrast, FOXP3+ non-Treg cells are not immunosuppressive but rather are immunomodulatory. Thus, this classification of FOXP3+ CD4+ T cells reflects the pathophysiology of autoimmune and inflammatory diseases. Specifically, highly immunosuppressive eTreg cells have been implicated as the dominant FOXP3+ CD4+ T cell subpopulation in patients with inflammatory disease, whereas FOXP3+ non-Treg cells have been implicated as the predominant subpopulation in those with autoimmune diseases.

1.3.1.6.2 Role of Tregs in the TME

As mentioned before, Treg cells are key players within the tumor immune suppressive environment. High number of Treg in the TME is correlated with a bad prognosis in multiple cancer types [164]. Therefore, developing effective strategies for Treg depletion is an important consideration in current immunotherapy approaches. Treg cells act in a number of ways to suppress the immune response against cancer, summarized in Figure 14 below.
Firstly, Tregs compete for IL-2 binding, as well as its consumption, thereby limiting the availability of IL-2 to other cells, such as Tconv cells [165]. This is due to the fact that Tregs are highly dependent on IL-2 for survival and proliferation, but are unable to produce it in enough quantities to sustain themselves. However, as they constitutively express high levels of CD25, they are able to bind and compete for IL-2 with neighboring effector cells.

Evidence for IL-2 depletion as a mechanism for Treg suppression came from a few studies which showed that the depletion of IL-2 or blocking of its binding replicated the effect of Treg cells on activated T cells. Moreover, adding IL-2 or blocking its uptake by Treg cells was shown to be enough to remove the suppressive capacities of Tregs in vitro [166].

Other mechanisms include Tregs inhibiting the maturation of antigen presenting cells (APCs) and therefore the consequent activation of T cells [167]. This is due to the expression of high levels of CTLA4 which binds to the B7 molecules B7-1 and B7-2 (also known as CD80 and CD86, respectively) on APCs with a higher affinity than that of CD28, thereby competing with this co-stimulatory molecule for ligand binding, and induces inhibitory signaling in APCs [168]. Moreover, Tregs produce immunosuppressive cytokines such as IL-10, IL-35 and TGF-β [169-171] suppressing the immune response.

Furthermore, Tregs produce high amounts of ATP through which they are able to convert into adenosine via the expression of CD39 and CD73 [172] inhibiting proper T cell activation by binding to the adenosine A2A receptor (A2AR), leading to the immune suppression of Teff cells as well as APCs. CD39 is an ectoenzyme constitutively expressed on the surface of murine Tregs and approximately 50% of human Tregs that converts ATP or ADP to AMP [173,174]. A proportion of Tregs co-express another ectoenzyme, CD73, able to degrade AMP to Adenosine, a nucleoside with direct immunosuppressive effects on T cells [175].
Tregs are also able to secrete granzyme and/or perforin, important mechanism by which Tregs destroy effector T cells [176].

![Figure 14 – Suppressive mechanisms of Tregs](image)

**1.3.1.6 Co-stimulatory and co-inhibitory receptors**

Co-stimulatory and co-inhibitory signaling mechanisms control T cell activation to prevent excessive immune responses.

The first co-stimulatory receptor described on T cells was CD28, constitutively expressed on the surface of T cells. This receptor binds to B7-1 and B7-2 ligands, which are expressed on B cells, monocytes, dendritic cells and T cells [178]. There are many other co-stimulatory receptors on T cells including ICOS, 4-1BB (CD137), OX40, GITR (Figure 15). The latter three are part of the TNF superfamily, which upon high affinity binding to their ligands leads to receptor clustering and initiation of signal transduction pathways leading to various cellular responses, including survival, death or
Amongst co-inhibitory molecules, CTLA-4 and PD-1 are the most characterized targets. They belong to the B7-CD28 family of receptors. CTLA-4 is a cell surface receptor that rapidly upregulated following T cell activation and binds the B7-1 and B7-2 ligands with much greater affinity than the CD28 receptor, leading to down regulation or termination of T cell responses. CTLA4 ligation by B7 ligands leads to a decreased secretion of cytokines as well as the inhibition of proliferation of T cells. It therefore acts as a regulator of naive and effector antigen-specific T cell activation to prevent aberrant effector responses [180]. PD-1 is expressed on T cells after activation, and its ligands (PD-L1 and PD-L2) are expressed on the surface of APC as well as tumour cells. Ligation of the PD-1 receptor with its ligands results in inhibitory signalling in T cells and thus limits their effector functions [181]. Antibodies blocking the interaction of these receptors with their natural ligands, known as immune checkpoint inhibitors, are now routinely used in the treatment of several malignancies.

Figure 15 – Co-stimulatory and co-inhibitory receptors in the immune synapse [182]
1.3.1.6.4 PD-1 expression on Tregs

PD-1 pathway delivers inhibitory signals that control the relevance of adaptive immune responses and tolerance [183]. The PD-1 receptor is expressed on conventional CD4+ and CD8+ T cells as well as Tregs during immune activation and chronic inflammation [184]. The PD-1 pathway has proven an attractive target for cancer immunotherapy. PD-1 or PD-L1 blockade is approved for the treatment of melanoma, non-small cell lung cancer and renal cell carcinoma. The functions of PD-1 inhibitory signals are best understood in CD4+ and CD8+ effector T cells. It is not completely clear the role of expression of PD-1 on Tregs and, therefore, how PD-1 impacts on Treg cell activation and functions. Lowther et al. demonstrated, in malignant gliomas, that high PD-1 expression on human Tregs identifies a population of dysfunctional, exhausted Tregs secreting IFN-γ [185]. Indeed, the authors describe a decrease in the suppressive function of circulating and tumor-infiltrating Tregs from patients with glioblastoma puntiforme associated with PD-1<sup>high</sup> expression. They found a total of 791 differentially expressed genes, including TIM3, LAG3, CXCR3, CCR6, CD226 and CCR5 distinguished PD-1<sup>high</sup> and PD-1<sup>−</sup> Tregs. Another recently paper showed that PD-1 restrains regulatory T-cell suppressive activity (Figure 16) [186]. To investigate the role of PD-1 signals in controlling Treg cell activation and function the authors, in this study, used Treg cell-specific PD-1 conditional knockout strategies. The potent suppressive capacity of PD-1-deficient Treg cells was illustrated by EAE (ameliorated experimental autoimmune encephalomyelitis), that is the most commonly experimental model used for the human inflammatory demyelinating disease, multiple sclerosis, and protection from diabetes in nonobese diabetic (NOD) mice lacking PD-1 selectively in Treg cells. Their findings establish PD-1 restraint of Treg cells as a new mechanism by
which PD-1 inhibitory signals regulate T cell tolerance and autoimmunity. Actually, results showed that selective PD-1 deficiency in Treg cells increase their suppressive function, resulting in attenuated autoimmunity in EAE and protection from type 1 diabetes. Both studies identified reduced signaling through the PI3K-AKT pathway as one mechanism underlying the increased suppressive activity of PD-1 deleted Treg cells. Down-regulation of the PI3K-AKT pathway is critical for Treg cell development and optimal Treg cell suppressive capacity in vitro and in vivo [187-189]. pAKT was reduced in PD-1 deficient Treg cells and by using AKT activator, they demonstrated that the enhanced suppressive function could be overcome in vitro by increasing AKT signaling in PD-1 deficient Tregs. Findings underline the importance of understanding the consequences of PD-1 inhibitory signals in different cell types. PD-1 immunotherapy inhibits PD-1 signals in all cell types, but the integrated outcome is enhanced T cell functions in tumors that respond to PD-1 immunotherapy. Even more effective it could be the development of immunotherapy agents such as specific antibodies that target selectively PD-1 on Teff cells, but not Tregs.

**Figure 16 – Proposed model for the function of PD-1 expressed on Treg** [186]
1.3.1.6.5 Satb1 restraints PD-1 expression in T cells

The inhibitory co-receptor PD-1, encoded by the *Pdcd1* gene, is induced transiently upon TCR engagement and T cell activation. Mechanisms that govern PD-1 expression and exhaustion in T cells are not fully understood. PD-1 expression is regulated through epigenetic mechanisms that involve two regulatory regions upstream of the *Pdcd1* promoter [190,191]. Epigenetic changes in T cell precursors are controlled throughout thymic development by the chromatin organizer SATB1 (Special AT-rich binding protein 1), demonstrated by Stephen et al. The authors revealed that Satb1 plays a critical role in the control of both PD-1 expression and anti-tumor T cell responses [192]. They find that TCR and co-stimulatory signals induce Satb1 expression and that Satb1 suppresses PD-1 expression in CD8+ T cells through the recruitment of the nucleosome remodeling deacetylase (NuRD) repressive complex to regulatory elements upstream of the *Pdcd1* locus. In addition to TCR and co-stimulatory signals, cytokines also modulate PD-1 expression. When T cell activation occurs in the presence of TGF-β, an immunoregulatory cytokine, SATB1 expression is down regulated because it is non available to interact with the NuRD complex required for transcription of *Pdcd1* gene, which leads to T cell exhaustion. In Satb1-deficient setting, PD-1 is expressed at high levels and tumor immunity is impaired. CR-B and CR-C, two conserved regulatory regions, are important for *Pdcd1* expression in activated CD8+ T cells. Therefore, SATB1 regulated pathways in CTLs and also in tumor epithelial cells and Treg (Figure 17) [193]. In particular, the suppressive functions of Treg cells were slightly impaired in the absence of SATB1 [194]. Moreover, elevated levels of SATB1 are known to be associated with tumorigenesis, tumor metastasis and progression. SATB1 regulates tumor progression in epithelial cells via Wnt/β-catenin signaling [195] and EGFR-
STAT3 signaling. SATB1 mediates cellular transformation through the Rb-E2F pathway and promotes metastasis via the EMT associated transcription factors [196].

Figure 17 – Schematic representation of pathway regulated by SATB1 [193]

1.3.1.6.6 SATB1 regulations

The chromatin organizer and transcription factor, SATB1 plays a pivotal role in the regulation of global gene networks through the activation or the repression of gene transcription in mice and humans. It is indispensable for the development of multiple cell types, including mature CD4+ and CD8+ T cells and Tregs in the thymus. Indeed,
SATB1-knockout mice had small thyme and spleens and were fatal by the age of 3 weeks. Thymocyte development was blocked at the CD4+ CD8+ double-positive stage as only a few CD4+ and CD8+ single-positive T cells survive and migrate to the periphery in SATB1-null mice [197]. SATB1 is differentially expressed during thymocyte development and it is down regulated in peripheral CD4+ T cells after thymic exit [198]. However, it remains unknown how SATB1 regulates the differentiation and effector program of the Th subsets in the periphery. Yasuda et al. demonstrated that SATB1 differentially regulates gene expression profiles in non-pathogenic and pathogenic Th17 cells and promotes the pathogenic effector program of encephalitogenic Th17 cells by regulating GM-CSF via Bhlhe40 and inhibiting PD-1 expression [199]. However, they showed that SATB1 is dispensable for the differentiation and non-pathogenic functions of Th17 cells. Data indicated that SATB1 regulates the specific gene expression and function of effector Th17 cells in tissue inflammation. Because SATB1 alone is not sufficient to change the expression levels of PD-1, external environmental cues such as IL-23 signaling in inflamed tissue may be necessary not only to up-regulate the expression of SATB1, but also to modulate its function and dynamic chromatin architecture. Collectively, in this study it has been reported that in vitro re-stimulation of draining LN Th17 cells from control EAE mice with IL-23, but not IL-6 or IL-1β increased SATB1 expression, whereas TGF-β restrained its effect (Figure 18). Furthermore, SATB1 is an attractive therapeutic target for cancer therapy and although a couple of drugs have been explored to exhibit anticancer activity via down regulation of SATB1, the underlying molecular mechanisms are still unclear. Hydrophobic statins such as simvastatin and fluvastatin have been shown to down regulate SATB1 possibly acting at the post-translational level [200].
1.3.1.7 Immunoediting in cancer

The immune system has an inherent capacity to function as tumor suppressor by destroying and eliminating tumor cells spontaneously. This concept is termed “cancer immunosurveillance“ and is now known as the elimination phase of a process that has been termed cancer immunoediting [201]. Cancer immunoediting takes into account that the immune system not only protects the host from tumor development but also it can promote tumor growth itself. It is now considered as a process composed of three phases known as the “three Es” [202]: elimination (i.e., cancer immunosurveillance), equilibrium (a phase of tumor dormancy where tumor cells and immunity enter into a dynamic equilibrium that keeps the expansion of tumor), and escape (where tumor cells emerge that either display reduced immunogenicity or engage immunosuppressive mechanisms to attenuate the antitumor immune responses leading to progressively growing tumors, as illustrated in Figure 19.

Figure 18 – SATB1 controls the pathogenic function of Th17 cells via regulation of Bhlhe40 and PD-1 expression (eurekalert.org)
During the elimination phase, both the innate and the adaptive arm of the immune system cooperate in the process of tumor eradication [203-206]. The exact signals that attract the immune system to the tumor are not fully elucidated but likely involve the release of so-called “danger signals” and stress ligands which are also involved in the initiation of an immune response. In case of tumor elimination, cancer cells are destroyed before they become clinically relevant. However, cancer cells cannot be eradicated in all cases. Cells that are not destroyed in the elimination phase by the immune system can be kept under control in the equilibrium state. T cells, IL-12, and
IFN-γ are required to maintain tumor cells in a state of functional dormancy, whereas NK cells and molecules that participate in the recognition or effector function of cells of innate immunity are not required; this indicates that equilibrium is a function of adaptive immunity only [207,208]. In this stage of carcinogenesis, tumor cells are kept in a state of dormancy, preventing the outgrowth of these tumor cells into established tumors. However, the way in which the immune system functions to control latent tumor cells, or a change in the controlling capacity of the immune system can change the characteristics of the tumor cells, which may allow them to escape from immune control. In this stage of tumor immune escape, the immune system is no longer capable to control tumor, causing clinically apparent established [209]. The development of immune escape mechanisms is a crucial process in tumorigenesis and can involve the recruitment of immunosuppressive cells and/or the regulation of various immunosuppressive molecules, including the immune-checkpoint proteins cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death 1 (PD-1), and programmed cell death 1 ligand 1 (PD-L1).

1.3.2. Non-cellular components of the TME
Dynamic interactions of cancer cells with their microenvironment consisting not only of stromal cells (cellular part) but also of non-cellular components including cytokines, growth factors, soluble factors, exosomes and extracellular matrix that exert an important role for tumor cell heterogeneity, clonal evolution and the multidrug resistance.
1.3.2.1 Extracellular matrix

ECM is a fundamental component of all tissues and organs; it regulates organism development, tissue repair and homeostasis. In addition to provide architectural and mechanical support, the ECM controls every cellular process, including cell proliferation and survival, cell fate determination, cell migration and invasion and tissue morphogenesis [210]. In cancer, the ECM is altered at the biochemical, biomechanical, architectural and topographical levels, and recent years have seen an exponential increase in the study and recognition of the importance of the ECM in solid tumors. Importantly, the composition and the mechanical properties of the ECM characterize the cancer hallmarks [211]. ECM molecules bind to cell surface receptors, which activates intracellular signaling pathways. ECM adhesion-induced signals through ERK and PI3K promote self-sufficient growth [212]. FAK signaling inhibits growth suppressors p15 and p21 and limits the induction of apoptosis through p53 [213]. ECM components and biophysical properties promote EMT induction and enhance pro-migratory pathways, particularly TGF-b and RhoA/Rac signaling [214]. ECM stiffness also enhances angiogenesis and increases VEGF signaling in endothelial cells [215]. At each phase of tumorigenesis, the ECM adapts to reinforce the progression of the disease through promotion of the hallmarks (Figure 20). This suggests that biochemical and biophysical properties of the ECM should be considered when examining tumor behavior and therapeutic interventions [216].
The ECM is recognized as an important regulator in BC. It is composed of a complex network that contributes to ECM structure and function such as collagens, fibronectin, laminins, glycosaminoglycans, proteoglycans, matricellular proteins and ECM remodeling enzymes [217]. BC is characterized by significant changes in ECM composition (Figure 21). A desmoplastic reaction is associated with BC development, due to the increased production of fibrous ECM by activated fibroblasts and cancer cells [218]. The increased collagen deposition and crosslinking by lysyl oxidase (LOX) enzymes, together with the high production of fibronectin and other ECM components, promotes tumor aggressiveness [219]. ECM remodeling enzymes like MMPs, heparanase and others break down membrane surrounding the mammary gland epithelium. Evidence indicates that ECM composition continues to change during cancer progression and may promote metastatic spread [220].
Proteomics analysis are been used to investigate the ECM of human mammary carcinoma xenografts and showed that primary tumors with high metastatic potential differ in the composition of the ECM [221].

Modifications in mammographic density reflect variations in the amounts of collagen, fat and the number of epithelial and non-epithelial cells in breast [222]. A complex interplay of hormonal, inflammatory and environmental factors may all influence breast density [223]. Mammographic density is associated with a marked increase in risk of invasive BC and its potential applications include scheduling of mammographic screening, risk prediction in individuals, breast cancer prevention research, and clinical decision [224]. The finding that stiffness and composition act together to regulate malignant phenotypes in the mammary epithelium highlights the importance of considering specific receptor-ligand interactions in *in vitro* studies of mechanotransduction. The combination of stiffness and composition is sensed through β4 integrin, Rac1, and the PI3K pathway, and suggest a mechanism in which an increase in ECM stiffness, without an increase in basement membrane ligands, prevents normal α6β4 integrin clustering into hemidesmosomes [225]. Indeed, in the context of breast cancer, the TME exhibits higher stiffness, and the ability of mammography and palpation to identify regions of dense tissue is typically used as an initial screen [226].
1.3.2. Matricellular proteins

Matricellular proteins are a group of ECM proteins, unlike collagens, that do not have a primary structural role, but rather function as modulators of cell-matrix interactions. Members of the group, such as thrombospondin (TSP)-1, TSP-2, SPARC, tenasin (TN)-C, and osteopontin (OPN), have been shown to participate in a number of processes related to tissue repair [227]. Certainly, understanding how matricellular proteins are involved in wound healing will allow us to evaluate their role in cancer and probably to investigate the link of inflammation and cancer.

1.3.2.1 SPARC, one of the matricellular protein

SPARC (Secreted Protein Acidic and Rich in Cysteine) is a 32-kDa multifunctional
glycoprotein that belongs to the matricellular group of proteins [228]. It is also known as osteonectin or basement-membrane-40, BM40. SPARC contains a calcium-binding C-terminal extracellular module, follistatin-like module, and N-terminal acidic module, as shown in Figure 22 [229]. It modulates cellular interaction with the ECM by its binding to structural matrix proteins, such as collagen and vitronectin, and to surface receptors and growth factors. Its expression is spatially and temporally confined during embryogenesis, tissue remodeling, repair and tumorigenesis. SPARC functions to regulate cell–matrix interactions and thereby influences many important physiological and pathological processes [230].

![Figure 22 – Structure of SPARC protein [229]](image)

SPARC can modulate cellular interactions with ECM by its binding to structural ECM proteins, such as collagen and fibronectin, and by abrogation of focal adhesions, features contributing to a counter-adhesive effect on cells [231]. Accordingly, SPARC can work as a chaperon, particularly on collagen [232]. It plays an important role in the regulation of tissue remodelling and plasticity in physiological and pathological processes, including ECM assembly and regulation, cell proliferation, cell
Introduction
differentiation, cell migration, EMT and angiogenesis [229,233,234]. Several studies indicate that SPARC activates down-stream components of integrin signaling and in particular integrin linked kinase after binding integrin β1 with its copper-binding domain [235,236]. SPARC regulates the activity of growth factors, such as PDGF, FGF or VEGF [237] and, in addition, it can also inhibit cellular proliferation by an arrest of cells in the G1 phase of the cell cycle [238]. Moreover, SPARC is also implicated in the regulation of TGF-β through a reciprocal regulatory feedback loop in which TGF-β induces SPARC expression and vice versa [239,240]. Notably, all these growth factors have an important role in tumor progression, angiogenesis and metastasis. The roles of SPARC are illustrated in Figure 23.

Figure 23 – Schematic illustration of the roles of SPARC
1.3.2.2 Role of SPARC in cancer

SPARC expression could be having a pro-tumorigenic or anti-tumorigenic function depending on cell type and its interactions, by the tumor grade and the composition of the surrounding TME [241-244]. Generally, SPARC has been identified as a pro-tumorigenic marker of poor prognosis and aggressiveness in the most majority of human cancer types but published evidence also reported that it could also have an anti-tumorigenic function [245]. In particular, its pro-tumorigenic function has been showed in gliomas, astrocytomas, melanomas, breast ductal carcinoma, colorectal carcinoma, clear-cell renal cell carcinoma, pancreatic ductal adenocarcinoma and prostate cancer but other evidence also reported an anti-tumorigenic properties mainly in acute myeloid leukemia, neuroblastoma, breast cancer, colorectal adenocarcinoma, hepatocellular carcinoma, non-small cell and small cell lung cancer, ovarian cancer and pancreatic ductal adenocarcinoma [245].

The reasons for such differences in SPARC expression are unknown. This complexity arises from the cell-origin and the fact that tumor and/or stromal cells can produce SPARC, although the role of stromal-SPARC in tumor growth is debated as exemplified in the studies on melanoma cells. For instance, evidence published in literature highlight that SPARC overexpression in the tumor has been associated with EMT and aggressiveness of human melanomas [246,247]. The presence of high levels of SPARC and glypican-3 in the sera of melanoma patients was proposed as a useful marker for early melanoma detection [248]. Serial analysis of gene expression identified SPARC as a member of a cluster of genes associated with breast cancer cell invasive capacity [249]. SPARC expression correlated with less differentiated and more aggressive BC tumors [250]. Furthermore, SPARC expression was also significantly associated with poor survival of BC patients [251]. As to the underlying mechanisms by
\textit{Introduction}

which SPARC might favor metastatic dissemination, it was shown that SPARC expression in human melanoma cells directly correlated with MMP-2 and MMP-9 levels and activity [252]. SPARC might enhance the invasive capacity of malignant cells through the activation of matrix-degrading enzymes. On the other hand, increased expression of SPARC and VE-cadherin was observed in the tumor vasculature and stromal cells of human BC in correlation with the progression from carcinoma in situ to invasive carcinoma [253] suggesting that SPARC might promote angiogenesis.

Moreover, it was showed that genetic deletion of SPARC in mice seems to favor the ectopic growth of lymphoma cells, of lung and pancreatic cancer when compared to wild-type animals [254]. On the contrary, in our laboratory has been demonstrated that transplantation of BC cells in a SPARC knockout host inhibits its growth [255]. Indeed, breast cancer cell lines derived from Her-2/Neu transgenic mice when injected in SPARC knockout mice show a reduced tumor growth, with high immune infiltrate, necrosis and reduced vascularization compared to the same cell lines injected in wild type mice [255].

Contrary to the evidence accumulated in the vast majority of human tumors (as described above), SPARC expression was associated with good prognosis in different types of human cancer such as ovarian [256], colorectal [257], pancreatic [258] and acute myeloid leukemia [259].

These given evidence reveal the multi-faceted role of this protein in different tumor settings and within the same single tumor, supporting the need to clarify its different contribution in tumors.
1.3.2.2.3 **SPARC in high-grade breast cancer**

The key role of ECM in cancer progression is strengthened by its prognostic relevance, such as in the case of high-grade breast cancers where the ECM-gene profile classification correlates with clinical outcome and response to therapy [260]. A signature termed ECM3, characterized by up-regulation of SPARC, COL1A1, COL5A2, LAMA4, COL6A3, and MMP11 genes, delineates the most robust cluster with stable tumor partition within 6 independent datasets of more than 600 samples, allowing the re-classification of breast cancers into two groups of ECM3 or non-ECM3 type [261]. The ECM3 signature characterizes approximately 35% of breast carcinomas including high-grade cases, which undergo EMT and respond poorly to therapy [262]. Prognostic relevance of the ECM3 signature applies only to grade III tumors and it is not associated to a particular intrinsic molecular subtype of BC. ECM3 signature is characterized by genes related to TGF-β pathway, integrin signaling, cell adhesion, and the lack of NK, T and B cell-related gens, which were enriched in their non-ECM3 counterparts. High-grade ECM3 tumors are infiltrated by a higher number of CD33+ myeloid cells that are in close contact to the tumor cells [262]. Notably, the prognostic value of this signature is conserved among all breast cancer molecular subtypes suggesting its relevance in providing information related to the biology of these tumors beyond cancer cell-intrinsic characteristics. Similar ECM gene cluster have been described in different tumors including metastatic BC [263], ovarian [264], colon [265], Hodgkin’s lymphoma [266] and diffuse large B cell lymphoma [267] that correlated with clinical outcome and response to therapy.

A recent study investigated the usefulness of combining two relevant molecular signatures, which reflect different important aspects of the TME that are the ECM composition and immune cells infiltration. The ECM3 (eight genes) and the IFN
signature (four genes) were combined in analysis of patients with early high-grade breast cancer (HGBBC) in order to investigate a novel molecular classifier with prognostic ability, which reflects the structural and immunological aspects of the TME [268] Table 5.

<table>
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<th>Signature</th>
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<th>Number of genes</th>
</tr>
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<tbody>
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<td>IFN</td>
<td>OAS1, OAS3, MX1, IFI44L</td>
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</table>

Table 5 – Genes included in ECM3 and IFN reduced signature [268]

It has been defined as ECM3+/IFN− the high-risk patients with worst prognosis in terms of OS, compared to patients classified in other subgroups. Although ECM3 BCs express lower levels of immune genes than non-ECM3 BCs [261], those classified as ECM3+/IFN− could be considered “cold” tumors. Based on these immunosuppressive functional and phenotypic features of TME, the HGBBC ECM3+/IFN− might have a very low chance of responding to therapy with immune checkpoint inhibitors.

This study could be generated a novel prognostic clinical applicable classifier able to not only identify patients with HGBCs who have the worst prognosis but also to identify those requiring a more aggressive treatment as well as those who should be excluded from treatment with single-agent PD-1/PD-L1 blockade due to the low expression of PD-1/PD-L1 and the high myeloid infiltration [268].
2. AIM OF THE THESIS

The ECM influences the hallmarks of cancer including inflammation and immune suppression. However, in the era of immunotherapy defining how the ECM might have an impact on immune cell composition and on the expression of immune checkpoints, such PD-1 is of outstanding relevance.

In this context, the aims of this PhD thesis are:

1. To define whether the ECM3 signature also affects adaptive immune cells, and whether SPARC, a key orchestrator of the ECM, and a key gene of the prognostic ECM3 signature, is responsible for such a regulation.

2. To evaluate the functional role of SPARC when produced by MDSCs and to test aminobisphosphonates activity in resetting the immune suppressive environment engendered by the ECM, via SPARC.
3. MATERIALS AND METHODS

3.1 Breast Cancer Patient Samples

Peripheral Blood was obtained from consecutive high-grade breast cancer patients (70 cases) and collected one day before surgery. The study was approved by the Medical Ethics Committee (Auth. Number 167/17), and all clinical data were obtained after receiving informed consent, according to institutional rules (Table 6).

Peripheral Blood of 4 patients (Auth. Number 151/15) with a newly diagnosed metastatic HGBC with bone lesions or with a recurrence in bone in which ZA administration is planned as standard care, were collected the day before (Day 0), 28-days and 56 days (Day 28 and Day 56, respectively) after ZA administration (Table 7).

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<th>ER status</th>
<th>PR status</th>
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Materials and Methods

### Table 6 - Clinical information of the patients enrolled in the study (Auth. Number 167/17)

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### Table 7 - Information of the patients enrolled in the study (Auth. Number 151/15)

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### 3.2 PBMC Flow Cytometry and Cell Sorting

Blood samples (6 ml) were collected in heparin and peripheral blood mononuclear cells (PBMCs) were obtained by diluting whole blood samples patients 1:2 with PBS 1X and subsequently subjected to a density gradient stratification. Diluted whole blood samples was carefully layered onto Histopaque-1077 Ficoll (Sigma- Aldrich) and centrifuged at
1,800 rpm for 30min at room temperature without brake. Finally, the lymphocyte-enriched ring at the interface was transferred into a new collection tube and washed with PBS 1X by centrifugation at 1,200 rpm for 5min. PBMCs were then stained with a cocktail of antibodies to characterize different immune cell populations and analyzed by BD Celesta Instrument (Panel 1).

Total MDSC were sorted from PBMC according to HLA-DR, CD33 and CD11b expression. Cells were sorted using a FACSaria BD Instrument.

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<th>Clone</th>
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Panel 1 – Antibodies used for flow cytometry

3.3 Animals, Cell Lines, and in vivo Experiments

BALB/cAnNCrl mice (BALB/c) were purchased from Charles River Laboratories (Calco). All experiments involving animals were approved by the Ministry of Health (INT 16_2016, authorization number 288/2017-PR). Sparc−/− mice on a BALB/c background were previously obtained in our laboratory [59]. The mammary carcinoma
Materials and Methods

cell line SN25A was obtained from SPARC-deficient mice that spontaneously developed mammary tumors due to the expression of the rat HER2/neu oncogene (BALB/c; SPARC < tm1Hwe > Tg(MMTV-Erbb2)NK1Mul/J), whereas the N3D cell line was derived from transgenic Her2/Neu mice (BALB/c-Tg(MMTV-Erbb2)NK1Mul/J). Both cell lines were infected with the retroviral vector LXSPARCSH to over-express SPARC and the co-isogenic cell lines, SN25ASPARC and N3DSPARC, were obtained [262]. Mice were injected into the mammary fat pad with SN25A, N3D, N3DSPARC (all at the dose of 2x10^5 cells) and SN25ASP (10^6 cells) cell lines. Another BC cells, 4T1c15 (SPARC-high) were obtained previously in our laboratory sub-cloning 4T1 cells; its SPARC-negative counterpart was obtained through lentiviral delivery of SPARC shRNA (4T1c15SP548). 4T1 is a TNBC cell line derived from the mammary gland tissue of a mouse BALB/c strain; they was selected without mutagenesis for its resistance to 6-thioguanine [269].

Primary tumor growth was measured twice a week with a caliper, and the volume was calculated using the formula d^2XD/2, where d and D are the short and long diameters, respectively. Tumors were collected when they reached a 10 mm diameter.

All breast cancer cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % fetal bovine serum (FBS; Thermo Fisher Scientific), 1 % antibiotics (Thermo Fisher Scientific), 2 mM glutamine, 1 mM sodium pyruvate, 1 mM HEPES and 1X Minimum Essential Medium (MEM) Non-Essential Amino Acids Solution, in a humidified atmosphere containing 5 % CO2 at 37°C.

3.4 Western blot

Cells were trypsinized and collected in the falcon, centrifuged at 1500 rpm for 5 min at RT. Cells were lysed in a RIPA buffer containing 50 mM Tris, pH 7.4, 1% NP-40, 150
mM NaCl, 1 mM EDTA, 1 mM Na$_3$VO$_4$ and protease inhibitor cocktail (Roche). Cell lysates were microcentrifuged at 14000 rpm for 20 minutes at 4°C and supernatants collected and stored at −20°C. For the protein quantitation, Bradford assay was used (Bradford, 1976). 5 µl of lysate or bovine serum albumin (BSA) were diluted in 195 µl of the Bradford reagent and incubated for 5 min at the room temperature. Serial dilutions of BSA were used as standard protein, in a 96-well plate. After incubation, protein concentration was estimated from the absorbance at 595 nm. Protein concentration was determined by interpolation with the curve obtained with the standard BSA.

30 µg of the total protein was separated on 4-12 Bis-Tris NuPAGE gels (Thermo Fisher Scientific). Cell lysates were mixed with 4 x reducing SDS-Sample buffer and heated for 10 min at 99 °C. Before loading, protein samples were prepared by adding ZAP solution to 20-50 µg of proteins and samples were denatured for 10 min at 99 °C. As a molecular weight standard, Page Ruler Plus Pre-Stained Protein Ladder (EuroClone) was used. The electrophoretic separation was achieved by applying a constant voltage in MOPS buffer. As suggested by the manufacturer, 500 µl of NuPAGE antioxidant were added to the chamber to protect reduced disulfide bonds and sensitive amino acids from oxidation, thus allowing proper protein migration in reducing conditions. Proteins within the gels were then transferred onto nitrocellulose membranes (Amersham, Biosciences). Following blocking with 5 % bovine serum albumin (BSA) and 0.1 % Tween-20, the membranes were incubated overnight at 4°C with the following antibodies: goat anti-mouse SPARC IgG (catalog #AF942, R&D Systems; dilution 1:500) and monoclonal mouse anti-mouse/human vinculin (clone hVIN-1, catalog #V9131 Sigma-Aldrich; dilution 1:5000). After rinsing in tris-buffered saline (TBS) 0.1 % Tween-20, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody: rabbit anti-goat IgG (catalog #R21459, Invitrogen;
Materials and Methods
dilution 1:2000) and sheep anti-mouse IgG (catalog #NA931, Ge Healthcare; dilution 1:2000) 1h at room temperature. The membranes were incubated for one minute with ECL Plus Western Blotting Substrate (Thermo Scientific) and then developed of an X-ray film.

3.5 Clonogenic assay
Quantification of metastatic cells in distant organs, for most tumor models, is difficult because the tumor cells may be dispersed and embedded within normal tissue. Because 4T1 tumor cells are 6-thioguanine-resistant, 4T1 metastatic cells can be detected and quantified by explanting organs, plating dissociated cells in medium supplemented with 6-thioguanine, and counting the number of 6-thioguanine-resistant clonogenic tumor cells [269]. In detail, for evaluation of lung micrometastases, clonogenic assays were performed on day 28: lungs were removed from each mouse, minced into small pieces, digested in 5 ml of an enzyme mix containing 1× Hank's balanced salt solution (HBSS, Lonza, Basel, Switzerland), 1 mg/ml collagenase type IV (Worthington, Lakewood, NJ), and 6 U/ml elastase (Roche), and incubated for 75 minutes at 4°C on a rotating wheel. After incubation, samples were filtered through and plated at different dilutions (1:2, 1:10, and 1:100) onto 10-cm tissue culture dishes in DMEM–10% FBS containing 10µg/ml thioguanine (Sigma, Saint Louis, MO) for clonogenic growth. After 14 days of incubation at 37°C, tumor cells, which are thioguanine-resistant, formed individual colonies representing micro-metastases that were fixed with methanol and stained with Giemsa (Sigma). Presence of disseminated or metastatic tumor cells was determined by growth of tumor colonies on tissue culture plates and colonies were detected with 0.5%
Materials and Methods

crystal violet staining. Each colony was counted to quantify the number of disseminated cells for each individual mouse.

3.6 Mouse Flow Cytometry Analysis

For FACS analysis primary tumors (TM) and spleens (SPL) were collected and maintained in DMEM–10%FBS, then minced and filtered through a 40 µm for TM and 70 µm-pores cell strainer for SPL (BD). Red blood cells were removed using ACK lysis buffer (ammonium chloride potassium) containing 150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA resuspended in distilled H₂O and filtered before using. Cells were stained with different anti-mouse monoclonal antibodies (Panel 2 results session 4.1, Panel 3 results session 4.2).

For the intracellular staining, the Foxp3/Transcription Factor Staining Buffer Kit (Tonbo Biosciences). Briefly, after surface marker staining, cells were washed with FACS buffer (PBS 1 X, 0.5 % EDTA, 3 % FBS) and fixed and permeabilized for 30 minutes using Foxp3 / Transcription Factor Fix/Perm buffer provided by the kit. The following antibodies were used: anti-Foxp3, anti-IL17A, anti-Ki-67, anti-TNFa, anti-IFNg and anti-SATB1.

Samples were analysed with the BD LSR II Fortessa instrument. Flow cytometry data analyses were performed using FlowJo software (v10.2).
3.7 Regulatory T cells isolation with magnetic cell sorting

CD4+ CD25+ regulatory T cells were isolated from single-cell suspensions of SPL, lymph nodes (LN) and TM of mice injected with SPARC-low/null (4T1 or 4T1cl5sp548) and SPARC-high (4T1SPARC and 4T1cl5) cells. Spleen, lymph nodes and tumors were disrupted with the plunger of a 1 ml syringe in a petri dish filled with 2
Materials and Methods

72

ml of DMEM and then passed through a cell strainer. Cell suspensions were lysed with ACK and after centrifugation resuspended in a MACS buffer solution containing PBS, pH 7.2, 0.5% BSA and 2 mM EDTA. The isolation of the mouse Treg cells was performed using CD4+ CD25+ Regulatory T Cell Isolation Kit mouse (Miltenyi Biotec, #130-091-041). Isolation was performed in a two-step procedure. First, the non-CD4+ T cells are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies (CD4+ CD25+), as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads and CD25-PE, as secondary labeling reagent. After 20 min incubation under 4 °C, and 1500 r/min centrifugation for 10 min, we discarded the supernatant, re-suspended it by adding MACS solution put an LD separation column in MACS separator device. The LD separation column was washed twice with 1.0 ml of MACS solution, and then added the cell suspension to the separation column to obtain the cells flowing from the sorting column.

The magnetically labeled non-CD4+ T cells are retained in the column, while the unlabeled CD4+ T cells run through. In the second step, the CD25+ PE-labeled cells are magnetically labeled with Anti-PE MicroBeads and isolated by positive selection from the pre-enriched CD4+ cell fraction by separation over a MS MACS Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD4+ CD25+ cells are retained in the column, while the unlabeled CD4+ CD25− cells run through and used for suppression assays as responder T cells. After removing the column from the magnetic field, the magnetically retained CD4+ CD25+ cells can be eluted as the positively selected cell fraction. Finally, we checked purity with flow cytometry using and CD4 APC (clone GK1.5) CD25 PE (Clone PC61.5) antibodies.
3.8 MDSC Isolation

For MDSC isolation, spleens and mammary lesions from tumor bearing mice (or naive mice as controls for spleen MDSC), were, minced and filtered to obtain a single cell suspension. Red blood cells were lysed by ACK lysis buffer and MDSC were sorted with FACSaria BD Instrument with the following antibodies: CD45, CD11b, Ly6G, and Ly6C (all from Ebioscience).

3.9 Total RNA extraction, reverse transcription and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from different murine tissues (spleen, tumor, LNs) using the Quick RNA micro prep kit (Zymo Research) and subsequently quantified by NanoDrop 2000c Spectrophotometer (Thermo Scientific). 1 µg of total RNA was reverse-transcribed into cDNA using the MultiScribe-Reverse Transcriptase kit (Applied Biosystems). Quantitative PCR was performed using the following Taqman Probes: Arg1 (Mm00475988_m1), Tnf (Mm00443258_m1), Sparc (Mm00486332_m1), Vegf (Mm01281449_m1), Pd-l1 (Mm00452054_m1), Nos2 (Mm00440502_m1), Stat3 (Mm01219775_m1), Il-23a (Mm00518984_m1), Il-10 (Mm01288386_m1), Satb1 (Mm01268937_m1), Pdcd1 (Mm01285677_g1) Gapdh (Mm99999915_g1), beta-actin (Mm02619580_g1), IL-23A (Hs00900828_g1), BETA-ACTIN (Hs01060665_g1), PDL1 (Hs00204257_m1) STAT3 (Hs00374280_m1) and the Taqman Universal PCR Master Mix (Applied Biosystems). qPCR was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems).
3.10 Treg cell suppression assay

To assess suppressive potential of PD-1\(^\text{high}\) or PD-1\(^{\text{neg}}\) Tregs, LNs and SPL were isolated from BALB/c mice injected with 4T1SPARC BC cells. Tissues were pooled, homogenized and sorted based upon CD4\(^+\) (APC, clone GK1.5) CD25\(^+\) (PE, Clone PC61.5) and PD-1 (FITC, clone RMP1-30) surface expression into Treg PD-1\(^{\text{high}}\) and Treg PD-1\(^{\text{neg}}\) populations. CD4 effector T cells and Tregs of naïve mice were isolated through a magnetic separation (Miltenyi Biotec, described in Material and Methods section 3.5). Isolated Teff and Treg cells (ratio 1:1) were stimulated using mitomycin C-treated spleen cells from TCR-deficient BALB/c mice as APC and 1 \(\mu\)g/ml anti-CD3 mAb. Cells were cultured in round-bottom 96-well plate at a density of 4 x 10\(^5\) cells/well in 200 \(\mu\)l of RPMI 1640 (EuroClone) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5 x 105 M), HEPES buffer (0.01 M) and 10% FBS (enriched RPMI 1640). After 72 h of incubation at 37°C with 5% CO2, cultures were pulsed for 9 h with 0.5 \(\mu\)Ci of [3H]-thymidine per well and proliferation was measured from triplicate harvest cultures with a commercial cell harvester and determine with a \(\beta\)-counter (PerkinElmer). Data are expressed as mean count per minute (c.p.m).

3.11 MDSC cell suppression assay

Myeloid derived suppressor cells were purified using CD11b-conjugated microbeads (for overall population) and Myeloid-DerivedSuppressorCellIsolationKit for separation of the two subsets (Miltenyi Biotec) following the manufacturer's instructions. For in vitro suppression assay, 4 x 10\(^5\) naïve BALB/c splenocytes have
been labeled with CFSE (Carboxyfluorescein Succinimidy1 ester; SIGMA Aldrich) and co-cultured with the different MDSC population at different ratio in presence of 2µg/ml of soluble anti-CD3 and 1µg/ml of anti-CD28 to activate lymphocytes. Each sample was seeded in triplicate. Proliferation of CD4 and CD8 T cells has been assessed 2 and 3 days later, by flow cytometry evaluating CFSE dilution in the CD4+ and CD8+ gated populations. Results are shown as percentage of proliferated cells.

3.12 Cytospin

SN25A, SN25ASPARC, 4T1, 4T1SPARC cells were detached, and suspended at 5x10^5/200 µl. Glass slides were mounted with paper pad and cuvettes with a metal holder, loaded with 200 µl of cell suspension and then spinned 3 minutes at 800 rpm with a cytocentrifuge. After detaching of cuvettes and filters, slides were dried overnight and then fixed for 10 minutes with 4% PFA. For immunofluorescence, after permeabilization for 5 minutes with PBS containing 0.1% Triton X-100 (Sigma) and blocking with PBS containing 5% BSA we followed the incubation with anti-IL23 antibody (Ab45420, abcam) at 1:250 dilution in PBS containing 2% BSA for 1 h at room temperature. An Alexa Fluor 488 goat anti-rabbit polyclonal IgG (Invitrogen, 1:500) was used as the secondary antibody for 45 minutes at room temperature. Slides were mounted with ProLong Diamond Antifade Mountant with DAPI (ThermoFisher Scientific), and acquired with a Leica DM4 B microscope equipped with a Leica DFC450 C digital camera, utilizing the LAS X software (Leica Biosystems).
3.13 Immunohistochemistry and Immunofluorescence

Histological and immunohistochemistry analyses of human and mouse tissues were performed. Human and mouse tissues were fixed in formalin and embedded in paraffin. For double-marker immunofluorescence staining in which primary antibodies of the same made were adopted, the tyramide signal amplification system Opal multiplex IHC kit (Lot number 2395285, PerkinElmer Inc.) was adopted. In detail, after de-paraffinization, antigen retrieval was performed using microwave heating and a pH 9 buffer and the first primary antibody was incubated overnight at 4°C (monoclonal anti-Human Osteonectin/Sparc, Clone ON1-1, 1:500, Life technologies). Immunofluorescence labeling was achieved by incubating with a specific secondary antibody, followed by the addition of one selected Opal fluorophore and microwave treatment in pH 9 buffer. The same procedure was repeated for the second primary antibody for 90 min at room temperature (monoclonal anti-Human CD33, Clone PWS44, 1:100, Novocastra), using a different Opal fluorophore and DAPI nuclear counterstain.

For IHC on mouse tissues samples were fixed in 10% buffered formalin and paraffin embedded. Four-micrometers thick sections were de-paraffined and re-hydrated. The antigen unmasking technique was performed using Novocastra Epitope Retrieval Solutions pH 9 (Novocastra, Leica Biosystems), in a PT Link Dako pre-treatment module at 98°C for 30 minutes. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H₂O₂ and Fc blocking by a specific protein block (Novocastra UK) the samples were incubated with the primary antibodies. All anti-mouse antibodies that have been used are listed in Panel 4. Staining was revealed by polymer detection kit (Novocastra, Leica Biosystems) and either 3-3’-diaminobenzidine (DAB) or 3-amino-9-
ethylcarbazole (AEC) as chromogenic substrate or DAKO (catalog. #GV800, EnVision flex by Dako Omnis) followed by counterstaining with Harris haematoxylin (Novocastra, Leica Biosystems). Slides were evaluated under a Zeiss Axioscope A1 and microphotographs were collected using a Zeiss Axiocam 503 Color with the Zen 2.0 Software (Zeiss). For the quantification of nuclear transcription factors Zeb-1 (on murine samples) in malignant cells, the average percentage of cells displaying nuclear reactivity was calculated.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZEB1 (NBP1-05987)</td>
<td>Rabbit</td>
<td>Novus</td>
</tr>
<tr>
<td>SPARC</td>
<td>Goat</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>E-Cadherin (ECCD-2)</td>
<td>Rat</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Ly6C (ER-MP20)</td>
<td>Rat</td>
<td>Acris</td>
</tr>
<tr>
<td>Gr-1 (RB6-8C5)</td>
<td>Rat</td>
<td>BD</td>
</tr>
<tr>
<td>Collagen type-IV</td>
<td>Rabbit</td>
<td>Merck</td>
</tr>
</tbody>
</table>

**Panel 4 - Antibodies used for IF and IHC**

### 3.14 Evaluation of p50 and p65 Nuclear Translocation

To assess p50 and p65 nuclear translocation BM-derived MDSC were seeded onto poly-D-lysine coated glasses for 2 h in presence of TM supernatants or LPS (10 ng/ml). Cell permeabilization was obtained after 1 h incubation with PBS 0.1% Triton-X100 (Sigma-Aldrich) plus 5% normal goat serum (Dako Cytomation, Carpinteria, CA USA) and 2% BSA, (Amersham Biosciences, Piscataway Township, NJ USA). Cells were then incubated with rabbit anti-mouse p50 NF-kB (NLS, sc-114; Santa Cruz) or rabbit anti-mouse p65 NF-kB (c-20, sc-372; Santa Cruz). After 1h of incubation at RT, goat anti rabbit AlexaFluor 488 conjugated (LifeTechnologies) were used as secondary antibodies. Nuclei were counterstained with DAPI (Invitrogen, Molecular Probes). Samples were mounted with FluorPreserve Reagent (Calbiochem San Diego, CA USA) and analyzed with a Leica SP8 I laser scanning confocal microscope using a fine
focusing oil immersion lens (40X, N.A. 1.3) at 1 Airy Unit resolution and operating in channel mode with 405 and 633 nm excitations. The mean fluorescence intensity of the nucleus was quantified after a freehand drawing considering the nucleus as regions of interest using Image-pro Premium 9.2.

### 3.15 ROS Detection

The detection of ROS was performed on the overall population of myeloid derived suppressor cells purified using CD11b conjugated microbeads using the CellROX® Green Flow Cytometry Assay kit (catalog #C10492 Life technologies) a fluorogenic probe for measuring ROS in live cells. In detail, the cell-permeable CellROX reagent (component A) is essentially non-fluorescent while in a reduced state, but exhibits a strong fluorogenic signal upon oxidation, providing a reliable measure of ROS in live cells. In addition to the CellROX® ROS detection reagent, kit provides the common inducer of ROS production tert-butyl hydroperoxide (TBHP) as a positive control, the antioxidant N-acetylcysteine (NAC) as a negative control and the red-fluorescent, cell-impermeant SYTOX® Red Dead Cell stain (Component B). Oxidation of the cells was detected by flow cytometry, using 488-nm excitation.

### 3.16 Statistical analysis

For the analysis of the prospective cohort, the qPCR expression levels of the 8 genes included in the ECM3 reduced signature [268]together with the housekeeping gene RPLP1 were used to classify patients as ECM3 and non-ECM3. All the available specific immune cell populations were analyzed in their original scales as well as with appropriate transformations (ie logarithmic, arcsin transformation) applied to the
original values. Firstly, the association between the ECM3 status and each immune
cell populations was investigated by resorting to the on parametric Kruskal Wallis
test in order to identify populations differently distributed between EMC3+ and
ECM3-. Populations resulted associated to the ECM3 status by adopting a
significance level of \( a = 0.10 \), were deeply investigated by applying a t-test on the
transformed values to confirm the previous result at a significance level of 0.05. All
statistical analyses were carried out with SAS software (Version 9.4.; SAS Institute,
Inc., Cary, NC, USA).

Statistical analyses were performed with the GraphPad Prism software (version 6.0).
For in vitro and ex-vivo experiments histograms report mean ± standard deviation,
and data were analyzed using Ordinary One-Way ANOVA followed by Turkey’s
tests for multiple comparisons. Statistical analysis of single treatments was performed
using the Mann-Whitney t-test. For ethical reasons, the number of animals used for in
vivo studies was the minimum necessary to ensure the significance of the results. In
all statistical comparison, differences were considered significant when \( P < 0.05 \), and
were indicated as: * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), **** \( P < 0.0001 \). All
experiments were performed at least two times and for in each in vitro experiment
two biological replicates were used.
4. RESULTS

4.1 SPARC drives immune suppression regulating PD-1<sup>neg</sup> Treg via IL-23

4.1.1 Treg Ki67+ PD-1<sup>neg</sup> are enriched in the peripheral blood of ECM3+ patients

Data generated in our laboratory have shown that ECM3+ tumors are defined by the peculiar immune suppressive microenvironment enriched in CD33+ MDSCs but devoid of T, B-cells and NKs [262]. To challenge the hypothesis that immune suppressive features of ECM3+ tumors can be intercepted in the peripheral blood (PB) of HGBC patients, as changes in specific immune cell populations, collaborating with the medical oncology unit, we set up a clinical study based on the prospective enrollment of HGBC patients. Overall, we collected blood samples from 70 consecutive BC patients. Meanwhile, Dr. Cappelletti, from our institute, collected tumor biopsy from the same patients to perform a semiquantitative real-time PCR analysis and subdivide patients in ECM3 and non-ECM3. We subdivided our cohort in 22 ECM3+ and 30 ECM3- patients, according to the ECM3 signature [268].

On PB samples we performed a multiparameter flow cytometry analysis (FACS) using different panels allowing the identification of MDSC, Treg, Teff and exhausted CD8 T-cells. In detail, panels that included HLA-DR, CD33, CD11b, CD14, and CD15 were used to define PMN and M-MDSC subsets in patients, being PMN-MDSC HLA-DR-CD33+CD11b+CD15+ and M-MDSC HLA-DR-CD33+CD11b+CD14+ (Figure 24A) [121,270]. Early-stage MDSCs (e-MDSCs) were identified as Lin- HLA-DR-CD33+ CD11b+ (as described in [121]) (Figure 24B). T-cell panel comprised different markers able to better characterize CD8 T cells, such as PD-1, LAG-3, TIM-
3. Treg cells were defined as CD4+ CD25+ FOXP3+ CD127- and in addition, we used PD-1 and Ki-67, a proliferation marker, for better characterization (Figure 25).

Figure 24 – A. Gating strategy for the identification of PMN-MDSC (HLA-DR- CD33+ CD11b+ CD15+) and M-MDSC (HLA-DR-CD33+ CD11b+ CD14+) subset. B. Gating strategy for the identification of eMDSC subset in the peripheral blood of HGBC patients. CD11b+ CD33+ eMDSCs were defined within the gate of HLA-DR- Lin- cells. HLA-DR-Lin- cells were identified within the CD8- cells gate. CD8+ gate was determined on live cells after the exclusion of doublets.
Results

Figure 25 - Gating strategy to identify Treg populations in the peripheral blood of HGBC patients. PBMC were stained with a combination of different antibodies as described in Materials and methods. Lymphocytes were identified based on their forward- and side-scatter properties. CD4 T cells and CD8 T cells were identified as uniquely expressing CD4 or CD8 antigens. Treg cells were defined as CD4+ T expressing CD25 and negative for the expression of CD127. FOXP3 expression was measured in the gate represented by CD4+ CD25+ CD127- . We further characterized Treg using Ki67, proliferation marker, and PD-1.

Statistical analysis, performed on the different immune cell populations, showed a significant association between ECM3 status and Tregs (Figure 26). Specifically, we observed higher distributions of Treg (p-value 0.017), Treg Ki67- (p-value 0.034) and Treg Ki67+ PD-1\textsuperscript{neg} (p-value 0.04) in the ECM3+ group of patients with respect to ECM3-. Furthermore, ECM3+ group had lower distributions of Treg Ki67+ (p-value 0.043) and Treg Ki67+ PD-1+ (p-value 0.037) compared to the ECM3-.

Notably, the most relevant association was found between ECM3+ tumors and Ki67+ PD-1\textsuperscript{neg} Treg subset. On the other hand, Teff subsets, myeloid cells or CD8 T cells were not apparently associated with the ECM3 signature.
Overall these data suggest that ECM3+ tumors might impact on the expansion of PD-1\textsuperscript{neg} Tregs.

\textbf{Figure 26} - Distributions of immune cell populations resulted significantly associated to the ECM3 status. Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median, and whiskers indicate the extreme measured values.
4.1.2 SPARC is responsible for Treg Ki67+ PD-1neg expansion in high-SPARC BC model

To define how ECM3 characteristics might have an impact on the generation of PD-1neg Treg, we took advantages from the availability of a mouse models, mimicking ECM3 tumors [262] and consisting of BC cell lines forced to overexpress SPARC, a key gene of the ECM3 signature. De facto the up-modulation or the genetic knock-down of SPARC in murine HGBC cells can reproduce ECM3+ and ECM3- tumors, respectively, also reflecting some peculiarity of ECM3 tumors, such as the association with the EMT phenotype [262]. In detail, in our experiments, we used SPARC-low, 4T1, and SPARC-null, SN25A, cells and their co-isogenic SPARC-overexpressing counterpart, 4T1SPARC and SN25ASPARC. Moreover, we used the more aggressive 4T1cl5 cell line that spontaneously overexpressed SPARC, as shown by Western Blot (WB) analysis (Figure 27). The clonogenic assay performed on lung of 4T1 vs 4T1cl5 injected BALB/c mice showed that 4T1cl5 was more aggressive with higher number of metastasis compared to its parental 4T1 cells (Figure 28A). As the previous model, we also generated the SPARC-negative counterpart through lentiviral delivery of SPARC shRNA (4T1cl5SP548). In line with a role of SPARC in EMT and metastasis, the silenced clone (4T1cl5sp548) was unable to generate metastases when injected in vivo (Figure 28B). A WB analysis showed the level of SPARC expression in all different BC cells (Figure 27).

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**Figure 27** - Western blot analysis showed the expression of SPARC in different breast cancer cells, using vinculin as a loading control.
**Results**

Figure 28 - Clonogenic assay was performed 28 days after mammary fat pad injection of 4T1 and 4T1cl5 cells (A) and 4T1cl5 vs 4T1cl5sp548 cells (B). Colonies formed from the meshed lung after 14 days of incubation. Results are expressed as mean ± SD (**p< 0.01; ****p<0.0001).

To study the activity of SPARC on immune cells and particularly on Tregs, 4T1 and 4T1SPARC BC cells were injected into the mammary fat pad (mfp) of immune competent BALB/c mice. At the end point, established on the basis of tumor growth, mice were sacrificed and we evaluated the immune infiltration by FACS analysis performed onto primary tumors. FACS analysis, performed on tumors of mice previously injected with 4T1 and 4T1SPARC cells, showed a significant expansion of CD4+ CD25+ Foxp3+ Ki-67+ PD-1neg Treg cells in 4T1SPARC comparing to the wt counterpart (Figure 29A). Treg expanded in SPARC+ tumors also showed higher expression of CD73 (Figure 29B), a cell-surface ecto-enzyme (ecto-5’-nucleotidase) that catalyzed the conversion of extracellular ATP and ADP to adenosine and that was associated with Treg suppressive activities [172], and of *il-10*, an important suppressive cytokine, as detected through semiquantitative real-time PCR analysis on both intra-tumor and LNs isolated Treg (Figure 30). Interestingly SPARC+ tumors also expanded a population of IL-17 secreting Treg (Figure 29B), a subset of
regulatory T cells with the same suppressive characteristic of Treg, which were involved in tumor growth [271].

**Figure 29** - A. FACS analysis showed the frequency of Treg (defined as CD4+ CD25+ Foxp3+), Treg Ki67+ PD1+/PD1- in 4T1 and 4T1SPARC injected mice. B. FACS analysis showed the expression of CD73 (MFI expression) and the frequency of IL-17 on Treg performed onto primary tumors of 4T1 and 4T1SPARC injected mice. Results are expressed as mean ± SD (*p< 0.05; ****p<0.0001).

**Figure 30** - Semiquantitative real-time PCR on Tregs isolated through magnetic separation from LN and TM of mice injected with 4T1 and 4T1SPARC showed Il-10 levels. Results are expressed as mean ± SD (*p< 0.05; **p< 0.01)
Consistently with the enrichment in suppressive Treg cells, 4T1SPARC tumors has significant decrease in CD8+ PD1+ cells, paralleled by a reduction in IFNγ production and a significant reduction in CD4+ T cells producing IFN-γ and TNF in comparison to low-SPARC cell lines (Figure 31). Furthermore, in the group of mice injected with 4T1SPARC cells, we observed high production of IL-17 by CD4 T cells (Figure 31).

Figure 31 – FACS analysis showed the frequency of CD8+ PD1+, CD8+ PD1+ proliferating and activated (Ki67+ IFN-γ+) T-lymphocytes within the tumor of 4T1 and 4T1SPARC injected mice. Frequencies of activated CD4+ lymphocytes producing IFN-γ and TNF type I cytokines. Frequencies of CD4+ cell producing IL-17. Results are expressed as mean ± SD (***p<0.001; ****p<0.0001).
To confirm our data we also injected 4T1cl5 and 4T1cl5sp548 BC cells. Again, tumors obtained from mice injected with 4T1cl5 (SPARC high) were characterized for the expansion of PD-$1^{\text{neg}}$ Treg also expressing higher level of CD73 and of $\text{il-10}$, detected through semiquantitative real-time PCR analysis, on both intra-tumor and LN isolated Treg (Figure 32).

**Figure 32-** A. FACS analysis showed the frequency of Treg (CD4+ CD25+ Foxp3+), the MFI of CD73 on Treg cells, Treg PD1+/PD1- within the tumor of 4T1cl5 (high SPARC) and 4T1cl5SP548 (low SPARC) injected mice. B. Semiquantitative real-time PCR on Tregs isolated through magnetic separation from LN and TM of mice injected with 4T1 and 4T1SPARC showed $\text{il-10}$ levels. Results are expressed as mean ± SD (**p< 0.01; ****p<0.0001).

Overall this data suggest that SPARC over-expression in BC cells is responsible for the enrichment in PD-$1^{\text{neg}}$ Treg cells.
4.1.3 PD1- Treg cells are more suppressive than PD1+ counterpart

To functionally characterize PD-1<sup>neg</sup> Treg we performed an *in vitro* Treg cell suppression assay (Figure 33A, for schematic representation). To this end, Treg cells were isolated from a pull of LN and spleen of mice injected with 4T1SPARC cell and the WT counterpart. Treg were previously checked for purity and cultured for 72h in presence of naïve T effector cells isolated from naïve mice (CD4+ CD25-), APC, using splenocytes treated with mitomycin C and soluble anti-CD3. Data show that PD-1<sup>neg</sup> Treg cells were more efficient in suppressing Teff cells than the PD1+ counterpart (Figure 33B).

**Figure 33** - A. Schematic representation of Treg suppression assay performed on Treg PD-1<sup>high</sup> and Treg PD-1<sup>neg</sup> isolated from FACS-sorted. B. Suppression assay showed that PD-1<sup>neg</sup> Treg cells were more efficient in suppressing Teff cells than the PD-1<sup>pos</sup> counterpart. Results are expressed as mean ± SD of at least two independent experiments (*p< 0.05)
Notably, PD-1\textsuperscript{neg} Treg display low expression of the exhaustion markers TIM-3 and LAG-3 compared to PD-1\textsuperscript{high} Treg, in both 4T1cl5 and SN25ASP (high-SPARC model) \textbf{(Figure 34A and Figure 34B, respectively)}. In 4T1SPARC model, we observed a reduced TNF expression (MFI) in PD-1\textsuperscript{neg} Treg paralleled by an increase in the expression of the MFI of IL-17A \textbf{(Figure 34C)}, supporting the high suppressive function of this subset of regulatory T cells [271-273].

\textbf{Figure 34 - A.} FACS analysis showed TIM-3 and LAG-3 (MFI expression) on Treg PD-1\textsuperscript{high} and Treg PD-1\textsuperscript{neg} derived from tumor of 4T1cl5 and \textbf{(B)} SN25ASP injected mice. \textbf{C.} MFI expression of TNF and IL-17A on Treg PD-1\textsuperscript{high} and Treg PD-1\textsuperscript{neg} derived from tumors of
mice injected with 4T1SPARC. Results are expressed as mean ± SD (*p< 0.05; **p< 0.01; ***p<0.001)

4.1.4 Enrichment in PD-1neg Treg in ECM3+ is associated with increase SATB1 expression

To gain insight into mechanisms linking SPARC to the down-modulation of PD-1 on Treg we focused on SATB1, a nuclear protein relevant for T-cell development and Treg suppressive activity, which notably has been shown to play a role in restraining PD-1 expression in CD8 T cells [192]. We hypothesized that a deregulated expression of SATB1 might be responsible for PD-1 down-modulation on Treg in SPARC+ tumors. In line with this hypothesis qPCR analysis, performed on Tregs isolated from tumor, spleen and lymphonodes of 4T1 and 4T1SPARC injected mice, showed increases satb1 expression along with decreased pd-1 in Treg isolated from mice carrying 4T1SPARC tumors in comparison to the 4T1 counterpart (Figure 35A). Results were confirmed also at protein levels as FACS analysis revealed increase SATB1+ cells expression in Treg from 4T1SP tumors, along with a reduction in PD-1 (MFI of expression) (Figure 35B). To confirm the direct correlation between SPARC and SATB1 in Treg we used the alternative 4T1cl5 (SPARC+) and 4T1cl5sp548 model (SPARC-). Also in this case, Tregs isolated from LN of mice injected with 4T1cl5 express high level of SATB1 and low level of PD-1 (also on Treg isolated from spleen and tumor), compared to Tregs isolated from 4T1cl5sp548 bearing mice (Figure 35C).

Overall these data prove a direct role of SPARC in modulating SATB1 expression in Treg and therefore of PD-1 down-regulation.
**Figure 35 - A.** Semiquantitative real-time PCR on Tregs isolated from lymphonodes, spleen and tumors of mice injected with 4T1 and 4T1SPARC showed Satb1 and Pd-1 levels. **B.** FACS analysis showed the MFI expression of SATB1 and PD-1 on Treg isolated from tumors of mice injected with 4T1 and 4T1SPARC. **C.** Semiquantitative real-time PCR on Tregs
isolated from lymphonodes, spleen and tumors of mice injected with 4T1cl5 and 4T1cl5sp548 showed Satb1 and Pd-1 levels. Results are expressed as mean ± SD (*p< 0.05; **p< 0.01; ***p<0.001)

4.1.5 SPARC–mediated IL-23 expression in breast cancer control SATB1

To analyze more in depth mechanism underscoring SPARC-regulation of SATB1 expression, we took advantages from data derived from a GEP analysis previously performed in our laboratory onto SN25A and SN25ASP tumors. This analysis revealed a significantly increased expression of IL-23 (Figure 36A), a key cytokine that has been involved in the regulation of Satb1 expression [199]. Different cells such as dendritic cells, activated macrophages, distinct subpopulation of gamma/delta T cells and innate lymphoid cells along with different tumor cells secrete IL-23 [274,275]. Given the combined evidence of a causal relationship between chronic inflammation and cancer as well as the pivotal role played by IL-23 in autoimmunity [276], we hypothesized that IL-23 is an important molecular link between high SPARC BC cells and SATB1. We focused on the production of IL-23 by BC tumor cells. To this aim, we performed il-23 semiquantitative real-time PCR analysis on all available SPARC+ and SPARC- BC models showing a direct correlation between SPARC level and IL-23 (Figure 36B). This data on mRNA expression was confirmed at protein level both through FACS (Figure 36C) and IF analysis onto cytospin of BC cells (Figure 36D).

Collectively, these results suggest IL-23 as the key mediator of SPARC-related effects over SATB1 expression the consequent down-modulation in PD-1 expression on Treg. To provide confirmatory data on the link between SPARC and IL-23, we evaluated IL-23 mRNA expression on tumor biopsies from our prospective BC cohort. Data show
that *IL-23* expression is mostly associated to ECM3, with a significant increased expression in ECM3+ tumors (Figure 36E).
Results

Figure 36 – A. Boxplot shows a significant up-modulation (FDR < 0.05) of IL-23a mRNA expression in SN25ASP tumor versus SN25A, each point corresponds to Il23a expression within each sample. P-value of class comparison between those classes was calculated through limma package and FDR was obtained with the Benjamini and Hockeberg correction. B. Semiquantitative real-time PCR of different BC cell lines (low and high SPARC) showed Il-23 levels. C. FACS analysis performed on BC cell lines showed the expression (MFI) of IL-23. D. IF analysis for IL-23 onto cytospin of BC cell lines. E. Semiquantitative real-time PCR analysis showed the expression of IL-23 on tumor biopsies of ECM3+ and ECM3- BC patients. Results are expressed as mean ± SD (***p<0.001; ****p<0.0001).
4.2 SPARC Is A New Myeloid-Derived Suppressor Cell Marker Licensing Suppressive Activities

4.2.1 SPARC marks human and murine MDSC

Comparing ECM3+ tumors vs ECM3- tumors, in our laboratory it has been shown an increased local recruitment of myeloid cells in ECM3 tumors, which were also in close contact with tumor cells [262]. Confocal microscopy analysis performed to analyze more in depth the contact between SPARC+ tumor cells and CD33+ cells revealed a co-expression of CD33 with SPARC in ECM3 tumors, a data that suggested the possibility that MDSCs could express SPARC (Figure 37A).

To assess whether human MDSCs truly express SPARC, HLA-DR-CD33+CD11b+ MDSC were FACS-sorted from the PB of BC patients and evaluated for the expression of SPARC through RT-PCR and confocal microscopy (Figure 37B-C). RT-PCR analysis showed that the expression of SPARC and STAT3 was higher in MDSCs obtained from BC patients compared to HD (Figure 37B). In line, confocal microscopy analysis confirmed SPARC expression in MDSC of BC patients and less on the fewer HLA-DR-CD33+CD11b+ cells obtained from pooled HD (Figure 37C).

To study the role of SPARC in regulating MDSC suppressive activity, we moved to mouse models and firstly assessed whether also murine MDSC express SPARC. To perform this analysis we used MDSCs expanded from mice bearing 4 different mammary tumors, previously characterized for their different capacity to promote MDSC expansion and activation [262]. Indeed, these models were used to demonstrate that SPARC over-expression in BC cells support MDSC development and expansion. In detail, as before, SN25A (SPARC-deficient) or N3D (low expressing) did not promoted MDSC expansion, whereas SN25ASP and N3DSP (SPARC-transduced counterparts) strongly supported MDSC recruitment and suppressive capacity [262].
Results

RT-PCR (Figure 37D) and IF (Figure 37E) analysis shown that SPARC is expressed by both PMN- and M-MDSC according to tumor expression of SPARC, being associate to N3DSP and SN25ASP, but not to N3D and SN25A tumors. These data point to SPARC as a potential new marker for MDSC. Notably, human BC samples in which SPARC was absent on tumor cells were also devoid of CD33+ cells expressing SPARC, in parallel with mouse results (Figure 37F).
Figure 37 - A. Representative confocal microscopy analysis showing SPARC (red) expression in FACS-sorted HLA-DR- CD33+ CD11b+ cells from two representative BC patients and one healthy control. B. Semiquantitative real-time PCR analysis for SPARC and STAT3 performed on FACS-sorted MDSC isolated from breast cancer patients (BC PT; n = 5) compared to healthy donors (HD; n = 6). C. Representative confocal microscopy analysis showing SPARC (red) expression in FACS-sorted HLA-DR-CD33+CD11b+ cells from two representative BC patients and one healthy control. D. Semiquantitative real-time PCR analysis for Sparc performed on murine MDSC subsets sorted from SN25A, SN25ASP, N3D and N3DSP tumors. E. Cytospin preparations of FACS-sorted PMN-MDSC and M-MDSC isolated from SN25ASP tumors and stained for Gr1 (green) and SPARC (red). F. Representative confocal microscopy analysis showing the increased recruitment of CD33+ cells in human BC tumors in which SPARC was highly expressed by the tumor cells. On the contrary TM characterized for low SPARC expression were associated to a reduced recruitment of CD33+ cells that never
express SPARC. On the contrary in SPARC-high tumors associated CD33+ cells, at least in part, express SPARC (white arrows). Results are expressed as mean ± SD (*p < 0.05; **p < 0.01).

4.2.2 Myeloid-derived SPARC is required for EMT

To determine the functional relevance of SPARC when directly produced by MDSC we used SN25ASP and N3DSP models in which it has been shown that the recruitment of MDSCs promotes EMT in vivo [262]. This phenotype was dependent on a preserved suppressive function of MDSCs, indeed ZA treatment that impairs MDSCs suppressive function [146] reverted the EMT phenotype. Therefore, whether SPARC-expressed by MDSCs is involved in regulating their suppressive activity could be tested injecting SN25ASP cells into Sparc-deficient mice and evaluating EMT. SPARC-producing SN25ASP cells were injected into SPARC-competent (WT) and SPARC-deficient (Sparc−/−) mice. Histopathological analysis showed that tumors developing into Sparc−/− mice had reduced EMT features than those growing into WT recipients (Figure 38A-B). Indeed, in WT mice the tumor mass was composed mainly by cells with spindle morphology intermingled with abundant collagenic matrix forming ill-defined nest-like infiltrates. On the contrary, the EMT phenotype was almost entirely reverted in tumors grown into Sparc−/− hosts that showed well-formed nest-like tumor structures stained for membrane-expressed E-cadherin and reduced frequency of ZEB-1+ nuclei (Figure 38A-B). These data demonstrated that the robust EMT observed in SN25ASP tumors grown in WT mice was likely dependent on microenvironment-derived SPARC. Notably, SN25ASP tumors grew significantly less in Sparc−/− than WT mice (Figure 38C), although in presence of reduced EMT.
Results

Figure 38 – A. H&E and IHC analysis for E-cadherin and Zeb-1 markers performed in SN25ASP tumors obtained from WT and Sparc−/− mice. Scale bars, 100µm. B. Quantitative IHC data for EMT markers are shown as the fraction of positive nuclei for Zeb-1 in tumors. C. Mean tumor volume of SN25ASP tumors injected in WT and Sparc−/− mice. Results are expressed as mean ± SD (*p < 0.05).

4.2.3 SPARC from MDSC supports EMT

To test whether SPARC produced endogenously by MDSC contributed to EMT, 10⁶ MDSC isolated from the spleen of SN25ASP tumor-bearing WT or Sparc−/− mice, were injected, once a week for 4 consecutive weeks (Figure 39A), intra-tumorally into SN25ASP lesions grown in Sparc−/− mice. Results show that SN25ASP tumors gained the EMT marker ZEB-1 and lost E-cadherin, thanks to the supplement of SPARC-producing MDSC, despite the SPARC-deficiency in the host (Figure 39B-C). In presence of WT MDSC we observed also an increased tumor growth (Figure 39D). The data support the hypothesis that SPARC from MDSC is required for immune-mediated EMT.
Results

Figure 39 – A. Schematic representation for the MDSC transfer experiment. B. H&E and IHC analysis for E-Cad and ZEB-1 showing the increased expression of EMT markers in SN25ASP tumors grown in Sparc−/− mice transferred with WT but not Sparc−/− MDSC. C. Quantitative IHC data for EMT markers are shown as the fraction of positive nuclei for Zeb-1. D. Tumor volume of SN25ASP tumors grown in Sparc−/− mice transferred with MDSC from WT and SPARC-deficient mice.

4.2.4 SPARC-deficient MDSCs are less suppressive than WT counterpart

To test the functional relevance of SPARC expressed by MDSC, PMN- and M-MDSC subsets were purified from the spleen of tumor-bearing WT or Sparc−/− mice and evaluated for their capacity to inhibit T cell proliferation in vitro. To this end, we performed an in vitro suppressive assay. In detail, naïve splenocytes labeled with the CFSE (a fluorescent cell staining dye) were co-cultured with the different MDSC population at different ratio also in presence of soluble anti-CD3 and anti-CD-28.
Proliferation was evaluated 72h later, as CFSE dilution through FACS analysis. Results show that PMN-MDSC from Sparc<sup>−/−</sup> mice had a marked reduced ability to suppress T cell proliferation (Figure 40A). Comparison between PMN- and M-MDSC subsets, in this model, was however cumbersome being PMN-MDSC the population that expands mostly in the spleen of tumor-bearing mice, accounting for nearly 80% (77.8 ± 9.6) of the CD11b<sup>+</sup> cells, in comparison to the M-MDSC that account for 1% (1.10 ± 0.3). Additionally, the expansion of PMN-MDSC (Ly6G<sup>high</sup> cells) in Sparc<sup>−/−</sup> hosts was even higher than in WT counterparts, whereas the M-MDSC (Ly6C<sup>high</sup> cells) fraction was reduced (Figure 40B–C).

**Figure 40 – A.** Immunosuppressive activity of PMN-MDSC isolated from the spleens of WT and Sparc<sup>−/−</sup> tumor-bearing mice evaluated as the ability to suppress a-CD3/a-CD28-induced CD4 and CD8 T cell proliferation in vitro. **B.** FACS analysis of CD11b+, PMN- and M-MDSC performed on peripheral blood of WT and Sparc<sup>−/−</sup> mice injected with the SN25ASP cell line. Results are expressed as mean ± SD (*p < 0.05). **C.** IHC analysis of the myeloid markers Gr-1 and Ly-6C performed on WT and Sparc<sup>−/−</sup> tumors, showing the enrichment in Gr-1+ cells in Sparc<sup>−/−</sup> tumors. Scale bars, 100mm.
To explain the reduced suppressive capacity of PMN-MDSC from Sparc−/− versus WT mice, we evaluated the expression of genes that are involved in MDSC suppressive activity in FACS-sorted MDSC from both tumors and spleens. Despite their paucity, we also included FACS-sorted M-MDSC obtaining enough material at least for RT-PCR analysis. Expecting differences, we were surprised of finding similar or higher expression of Stat3 and Arginase-I in Sparc−/− MDSC (both from spleen and tumor) (Figure 41A-B). Different was Nos2 that was higher in Sparc−/− than WT PMN-MDSC. NO, the product of NOS2 activity is a well-recognized pro-inflammatory agent involved, for example, in ulcerative colitis. In support of this idea, Tnf mRNA level was higher in Sparc−/− than WT MDSC (Figure 41A). Trying to explain the reduced suppressive activities of Sparc−/− MDSC, we evaluate ROS expression in total MDSC (CD11b+ fraction, as described in [146] isolated from the spleen of WT and Sparc−/− tumor-bearing mice. We found a significantly decreased ROS expression by MDSC isolated from Sparc−/− hosts (Figure 41C-D).
Results

Figure 41 – A. Semiquantitative real-time PCR analysis for Stat3, Arginase1, Nos2 and Tnf genes performed on PMN-MDSC and M-MDSC subsets sorted from SN25ASP tumors grown in WT and Sparc−/− mice (n = 4 for per group). B. Semiquantitative real-time PCR analysis for Stat3, Arginase1, Nos2 and Tnf genes performed on PMN-MDSC and M-MDSC subsets sorted from the spleen of WT and Sparc−/− mice bearing SN25ASP tumors (n = 4 for per group). C. Representative histogram plots for ROS detection in WT and Sparc−/− MDSC. Oxidation of the cell-permeant dye by ROS generates a bright green fluorescence detectable at FACS in the FITC channel. D. Cumulative day showing ROS production by MDSC in terms
of percentage of cells oxidating the dye and therefore expressing ROS or the MFI of expression of the oxidated permanent dye. Results are expressed as mean ± SD (*p < 0.05; **p < 0.01).

To further study the mechanisms behind SPARC induction of a pro-tumoral phenotype in MDSC, we look at NF-κB signaling, as this pathway is involved in monocytes to M-MDSC reprogramming [277-279]. To test whether Sparc<sup>−/−</sup> MDSC have defective NF-κB activation, we evaluated p65 and p50 translocation into the nucleus of BM-differentiated MDSC [270] from WT and Sparc<sup>−/−</sup> mice, after exposure to tumor supernatants or, as control, to LPS. Confocal microscopy analysis revealed that SPARC-deficient MDSC showed a significantly lower amount of p50 but not of p65 into the nucleus (Figure 42A) than SPARC-competent MDSC, at baseline or when in culture with SN25ASP tumor supernatant (Figure 42B). Overall these data suggested that SPARC-deficient PMN-MDSCs are less suppressive and skewed toward an inflammatory phenotype.
Results

Figure 42  – A. Quantitative data showing p50 and p65 nuclear translocation in MDSC differentiated in presence of G-CSF, GM-CSF, and IL-6 from the BM of WT and Sparc−/− mice. MDSC were culture for 2 h in presence of SN25ASP tumor supernatants or LPS B. Representative confocal microscopy analysis for p50 and p65 performed on BM-derived MDSC obtained from WT and Sparc−/− mice and cultured 2h in presence of SN25ASP tumor
Results

supernatants or LPS (10 ng/ml). Results are expressed as mean ± SD (*p < 0.05; ***p < 0.001).

4.2.5 IHC analysis of CD8 T-cells and PD-1 cells in ECM3 and non-ECM3 tumors

The activity of SPARC in generating PD-1\textsuperscript{neg} Treg as well as its regulatory activity on MDSCs suggested evaluating whether the microenvironment of ECM3 tumors were excluded from T-cell infiltration, according to the increased immune suppression. Collaborating with Prof. Tripodo from the University of Palermo, we performed an IHC for CD33, CD8 and PD1 on representative TM sample collected from high-grade ECM3 and non-ECM3 patients. The representative analysis shows that the increased CD33\textsuperscript{+} cell recruitment (Figure 43A) in ECM3\textsuperscript{+} patients is paralleled by a decreased infiltration in CD8 T-cells (Figure 43B) and an overall reduced expression of PD-1 cells (Figure 43C).

A

![Image of IHC analysis](image-url)
Results

Figure 43 – Representative IHC analysis for CD33+ (A), CD8+ (B) and PD-1+ (C) cells infiltrating high-grade ECM3 and non-ECM3 tumors. Scale bars, 100 µm.
4.2.6 ZA treatment alters the TME in BC models

The T-cell excluded microenvironment of ECM3 tumors suggested to adopt drugs able to revert the immune suppression. Data obtained in preclinical models suggested that ZA could be an effective strategy to revert some feature of immune suppression of ECM3 tumors, and particularly those related to MDSCs, which might be responsible for the lack of T-cell infiltration [280].

According to these data, we evaluated whether ZA treatment could overall affect the suppressive microenvironment of ECM3 tumors. To this end, we injected SN25ASP (SPARC+) BC cells into the mfp of BALB/c mice. At 30 mm³ of tumor size, mice were randomized into different groups (CTRL VS ZA treated mice). Mice were treated with ZA daily (0.1 mg/kg). Histological analysis performed onto tumors highlights a reduction in collagen type I and IV deposition in tumor treated with ZA compared with untreated mice; furthermore IHC analysis for PD1 showed an increased infiltration by PD1+ cells in ZA treated tumors (Figure 44).
Results

Figure 44 – Histological analysis for collagen type IV and I (upper panel) and PD-1 (panel below) performed on SN25ASP tumors treated with or without ZA.
Interestingly, *in situ* immunostaining for Gr-1 shows a reduction in myeloid cells infiltrating tumors from ZA treated mice (*Figure 45A*). Performing a semi quantitative PCR analysis for PD-L1, we found a significant down-modulation of PD-L1 in G-MDSC subsets (*Figure 45B*). Overall these data show that ZA treatment could be a strategy to revert *in vivo* the immune suppressive environment of ECM3 tumors.

**Figure 45** – **A.** Quantitative IHC data for Gr-1 were obtained by counting the number of Gr-1+ cells in 5 fields from SN25A and SN25ASP treated with or without ZA. **B.** Semiquantitative real-time PCR analysis of PD-L1 was performed on the G-MDSC and M-MDSC subsets isolated from tumors of SN25A and SN25ASP (with or without ZA).

### 4.2.7 Characterization of MDSCS after ZA treatment in HGBS patients

ZA administration is a standard care for BC patients with bone lesions or with a recurrence in bone. The possibility to perform multiple liquid biopsies allows evaluating whether ZA administration is able to revert immunosuppressive status in patients. We monitored every 28-days the peripheral blood parameters. In particular, peripheral blood samples have been collected (n=4) the day before (d0), 28-days (d28) and 56-days (d56) after ZA administration. We performed FACS analysis on PBMC for different immune cells population, MDSC. We observed a reduction of the
frequency of MDSCs in HGBC patients 28 days after the first administration of ZA with an increase of the frequency after 56 days (Figure 46A). For only one patient, consistently to the available material, it was possible to isolate, through FACS sorting, MDSCs fraction and we performed a RT-PCR analysis for PD-L1 and STAT3. MDSCs isolated from this patient showed a reduction in the expression of the immunosuppressive markers STAT3 and PD-L1 after ZA treatment (Figure 46B).

Figure 46 – A. FACS analysis of MDSCs performed on PBMC of metastatic BC patients treated with ZA. B. Semiquantitative real-time PCR analysis of PD-L1 and STAT3 on MDSC isolated, through FACS sorting, from one BC patient treated with ZA.

In our institute BC patients with bone metastasis are now treated with denosumab, a fully human monoclonal antibody that specifically binds receptor activator of nuclear factor ligand (RANKL) to inhibit osteoclast activity that results in reduced bone resorption, tumor-induced bone destruction, and skeletal-related events (SREs) [281].
Indeed, ZA and denosumab are two molecules with similar application used in clinical practice to prevent SREs in patients with bone metastases or osteoporosis. It has been shown in different double-blind studies that denosumab was better than ZA for prevention of SREs, and potentially represents a novel treatment option in men with bone metastases from castration-resistant prostate cancer and from BC [281,282].
5. DISCUSSION

The ECM regulates tissue development and homeostasis and contributes to tumour growth sustaining all processes of tissue transformation. Accumulating evidences suggest that ECM is not merely a scaffold but provides critical biochemical and biomechanical cues able to directly affect tumour growth, survival, migration and differentiation, angiogenesis but also chronic inflammation and immune suppression. All these features are known as the hallmarks of cancer [211]. Although the prime driver of cancer cell fate specification lies within the oncogene activity, in our laboratory evidences were provided that under the same oncogenic pressure alteration in the ECM composition are able to influence the phenotypic outcome of a genetically-determined cancerogenesis towards more indolent or aggressive histotypes. Transgenic TRAMP mice, which develop prostate adenocarcinomas, had a prominent differentiation towards a neuroendocrine phenotype, when cross to Sparc-deficient mice or Spp1-deficient mice [283,284]. On the same line, Fas mutant lpr/lpr mice, which basically develop autoimmunity and rarely a diffuse large B-cell lymphoma, develop a B-cell chronic lymphocytic leukemia or highly aggressive DLBCL, when crossed to Sparc-deficient or Spp1-deficient mice, respectively [285,286]. In all these cases a different ECM/stroma/immune contexture engendered by the ECM proteins SPARC or Osteopontin, have been shown to play a direct role in tumour evolution and specification [287,288].

In BC, the interplay between the ECM and immune cells in tumour evolution have been demonstrated through data showing that the aggressive EMT phenotype of SPARC expressing tumours depends on the capacity of SPARC to promotes the recruitment of MDSCs. The last occurs through the activation of COX2, which in turn sustains the
production of chemokines and cytokines involved in the differentiation (G-CSF, GM-CSF, IL-6) and local recruitment (SDF1) of MDSCs [262].

All these features have been shown to define in situ ECM3 patients that molecularly are those characterized for a worse survival probability, poor response to therapy and EMT features [261].

In the era of immunotherapy the prognostic relevance of the ECM3 signature was recently strengthened by data showing that this signature is able to identified “cold” tumours, which almost completely lacks T-cell infiltration and that would not benefit from immunotherapeutic treatments.

So far, we attributed this cold microenvironment to the enrichment in MDSCs, which characterizes ECM3+ patients. Now with the data generated in this thesis we extend the influence of the ECM to T-cells, showing a direct effect of the ECM on Treg activities, an effect that is mediated by the matricellular protein SPARC, a key gene belonging to the ECM3 signature. Furthermore the activity of SPARC over Treg is so relevant such to impact on Treg composition in PB of BC patients.

In our prospective clinical study, based on the collection of blood samples of HGBC patients, we showed that the PB of ECM3+ patients is significantly enriched in PD-1<sup>neg</sup> Treg if compared to non-ECM3 cases. Modelling ECM3+ tumours in mice we showed that SPARC, the key functional genes of the ECM3 signature, directly controls PD-1 expression in the Treg population. Particularly, SPARC decreases PD-1 expression on Treg leading to the expansion of a population of highly suppressive PD-1<sup>neg</sup> Treg.

The suppressive activity of PD-1 on Treg that we observed in our models, is in line with data published by Lowther et al. who proposed PD-1 as marker of dysfunctional and exhausted Treg in malignant gliomas [185]. In a recent paper Tan et al. showed that PD-1 deficiency in Treg cells increases their suppressive function reducing
autoimmunity development in an experimental autoimmune encephalomyelitis (EAE) and diabetes [186].

Overall our data suggest that the expansion of PD-1<sup>neg</sup> Treg along with the increased recruitment of MDSCs [262,268], might account for the highly immune suppressed environment of ECM3+ tumors. A further level of complexity is added by the fact that SPARC is expressed by MDSCs where it plays a key functional role. Indeed, back to mouse model, using Sparc-deficient mice, we were able to formally demonstrate that SPARC is a new MDSC marker able to sustain MDSC suppressive functions. With MDSC we define a specific functional state of myeloid cells, in which MDSCs are able to suppress CD4 and CD8 T-cell proliferation. MDSCs can be both of monocytic or granulocytic origin, as described. In our mouse models we found a strong expansion of PMN-MDSC that truly suppressed T-cell proliferation. This data fit well with other data published in our laboratory showing that one of the immune population mostly affected by SPARC is that of neutrophils. In autoimmunity models, the absence of SPARC worsened the autoimmune phenotype promoting neutrophil recruitment, impairing their clearance by macrophages and sustaining the extrusion of NETs [289,290]. A formal demonstration on the dual role of extracellular SPARC and neutrophil/G-MDSC-derived SPARC was obtained transducing 4T1cl5 cells with the Stearoyl-CoA desaturating SCD5 enzyme, which provide catalytic conversion of saturated fatty acids (SFAs) into mono-unsaturated FAs (MUFAs). Indeed, the enforced expression of SCD5 into 4T1cl5 cells blocked the extracellular release of SPARC reducing lung metastasis (results obtained in collaboration with Istituto Superiore di Sanita’, Bellenghi et al, Oncogene submitted). The injection of SCD5-transduced cells into Sparc-deficient mice was additive and promoted a further reduction in lung metastases and tumor growth. The most evident phenotype was associated to a change in PMN-MDSCs that were not longer suppressive and displayed a more mature phenotype, evaluated in term of GR-1
expression and nuclear morphologic features through Wright-Giemsa staining [291]. The degree of maturation increased when SCD5-transduced cells were injected into *Sparc*-deficient mice (Bellenghi et al, Oncogene submitted).

To explain the reduced suppressive capacity of *Sparc*-deficient MDSC, we analysed the different transcription factors that have been involved in the acquisition of MDSC suppressive phenotype, among which the best characterized are STAT3, STAT1 and NF-kB. STAT3 works preferentially on PMN-MDSC and is largely involved in MDSC expansion. In our models, Arginase-I and NOS2, whose expression can be controlled by STAT3, were surprisingly not down-regulated in Sparc−/− MDSC, which are low suppressive. Flow cytometry analysis showed equal phosphorylation of STAT3 in WT and Sparc−/− MDSC. Searching for possible relevant differences between Sparc−/− and WT MDSC we found reduced nuclear translocation of the NF-kB p50 subunit, in the former. This may suggest that reduced level of p50 subunits may limit the formation of immunosuppressive p50:p50 homodimers in favor of the p65:p50 inflammatory heterodimers. Supporting this hypothesis, the production of TNF by Sparc−/− MDSC was significantly higher than by WT MDSC. Furthermore, recently Veglia et al. reported that the deletion of the fatty acid transport protein 2 (FATP2) abrogated the suppressive activity of PMN-MDSC leaving unaffected the expression of Arginase 1 and Nos2 [292]. This discrepancy in expression of suppressive genes and MDSC suppressive activity was explained showing reduced PGE2 production by PMN-MDSC from Fatp2-KO compared to WT mice. Differently from Veglia et al. [292] we found that PMN-MDSC isolated from the spleen of tumor-bearing WT and Sparc−/− mice showed a strongly reduced ROS expression in those from Sparc−/− mice. Overall these results suggest that the reduced ROS expression combined to the high production of TNF could account for the anti-tumor activity of Sparc−/− myeloid cells. In fact TNF if produced at high doses becomes a key factor in mediating tumor-rejection [293].
Therefore, it is foreseeable that an unbalance in TNF production could skew MDSC toward an inflammatory, anti-tumor phenotype. Unexpectedly, despite the influence of MDSC-derived SPARC on EMT markers and immune suppression, the tumor volume of SN25ASP tumors injected in WT and Sparc$^{-/-}$ mice was similar at the end, although the differed kinetics of growth that was initially faster in WT mice. Data suggest that PMN-MDSC from Sparc$^{-/-}$ mice behave as N1-like neutrophils rather than MDSC, a condition that allows them to initially control tumor growth until other immune suppressive mechanisms take over (i.e., CD8 T cells exhaustion). We propose SPARC as a new potential marker of MDSC, in both human and mouse, with the additional feature of controlling MDSC suppressive activity with the aim of preventing an excessive anti-tumor inflammatory state.

Similarly to what is occurring in myeloid cell, recent data showed an activity of SPARC when directly produced by Treg. Indeed, Shahnen F. et al [294] demonstrated that Tregs accumulates in venous blood clots to regulate thrombolysis by controlling the recruitment, differentiation and matrix metalloproteinase (MMP) activity of monocytes. In this context they provided evidences that SPARC+ Tregs were crucial for blood clot resorption. On the same line, Ni Xia et al demonstrated that SPARC is highly expressed by heart Tregs to protect the heart against myocardial infarction [295]. In the same paper they also showed a key role of environmental SPARC (from fibroblasts and monocytes) in protecting from myocardial infarction. So, when analyzing SPARC activity on immune cells a commonalities emerging in the different pathological setting consists in the fact that there is a cell- endogenous SPARC activity to be combined to an extracellular SPARC activity. The last could involve the engagement of specific receptor such as integrin/ILK pathway as described by Alachar et al in leukemic cells [296] or the different production of regulatory cytokines, mediated by SPARC.
In this thesis we show that SPARC directly regulates PD-1 expression on Tregs acting on IL-23 secretion.

The PD-1 pathway delivers inhibitory signals that control adaptive immune response and tolerance. PD-1 is inducible expressed on conventional CD4+ and CD8+ T cells as well as Treg cells during immune activation and chronic inflammation. PD-1 inhibitory signals play critical roles in regulating the threshold for T cell activation and limiting effector T cell responses, as well as controlling T cell tolerance and resolution of inflammation.

In CD8 T-cell subset PD-1 is firstly a marker of activation however its engagement by PD-L1 promotes CD8 exhaustion. Considering the effect of SPARC on PD-1 expression in Tregs we evaluated whether PD-1 expression on this subset was also affected by SPARC. Considering CD8 T-cells, we showed a similar activity of SPARC on this population, being CD8 T-cells associated to SPARC-expressing tumors characterized by a low expression of PD-1 along with reduced production of IFNγ. This suggested a less activated phenotype.

Mechanistically, in both Tregs and CD8 we showed that SPARC controls PD-1 expression through a regulation of the chromatin organizer special AT-rich sequence-binding protein-1 (SATB1). SATB1 is involved in controlling the development of mature CD4+ T and CD8+ T but also of Foxp3+ Treg cells in the thymus [198,297]. In the absence of SATB1, thymocyte development is blocked at the double-positive stage [197]. SATB1 regulates immune cell responses and is important in tumorigenesis and tumor progression in cancer [298]. Studies have reported that SATB1 expression was negatively associated with cancer progression and survival in breast cancer [299,300]. SATB1 has been shown to suppress PD-1 expression in CD8 T cells through recruitment of the nucleosome remodeling deacetylase (NuRD) repressive complex.
The expression of SATB1 expression is reduced in presence of TGFb, a condition that remove the repression of the Pdcd1 locus promoting PD-1 expression [192]. Among positive regulators of SATB1 expression IL-23, which is required for Th17 development and for the differentiation of Treg cells into IL-17A-producing Th17-like Treg cells [301], has been reported to be responsible for SATB1 activation.

For this reason, we evaluated the expression of IL-23 on SPARC -high and SPARC -low available BC cells. Interestingly, in SPARC high BC cell lines we observed an increase in Il-23 expression. Back to humans we showed a significantly increased expression of IL-23 on tumour biopsies from ECM3+ compared to non-ECM3- patients. Mechanistically we show that SPARC induces the expression of IL-23, which is ultimately responsible for SATB1 induction and PD-1 down-modulation on Treg cells.

Considering the peculiar immune suppressive environment of ECM3 tumors, we evaluated whether ZA could be adopted as a strategy to revert the immunosuppression, particularly acting on MDSCs. Indeed our lab previously showed the capacity of ZA to interfere with MDSCs suppressive activities [262].

We observed that the treatment with ZA reduced collagen deposition in mice injected with SPARC high BC cells along with the reduction of Gr-1+ cells and with an increase of PD-1+ cells. According to data generated in mouse models, in a small number of available HGBC patients with bone metastasis and treated with ZA we observed a reduction of the frequency of MDSCs and we showed that FACS-sorted CD33+ cells had decreased expression of PD-L1 and STAT3 at day 28 and 56 after the administration of ZA.

Taking together, our results clearly demonstrate the immune suppressive activity of the ECM and suggest that ECM3 patients might have a very low chance of responding to therapy with immune checkpoint inhibitors.
For these reasons, new therapeutic strategies aiming at reverting this immune suppression represent a clinical challenge. In this context, we believe that hydrophobic statins such as fluvastatin and simvastatin that have been shown to down-regulate SATB1 at the post-translational level and in a time- and dose-dependent manner in human colon cancer cells [200] could be potentially exploited in combination with conventional therapy.
6. REFERENCES


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