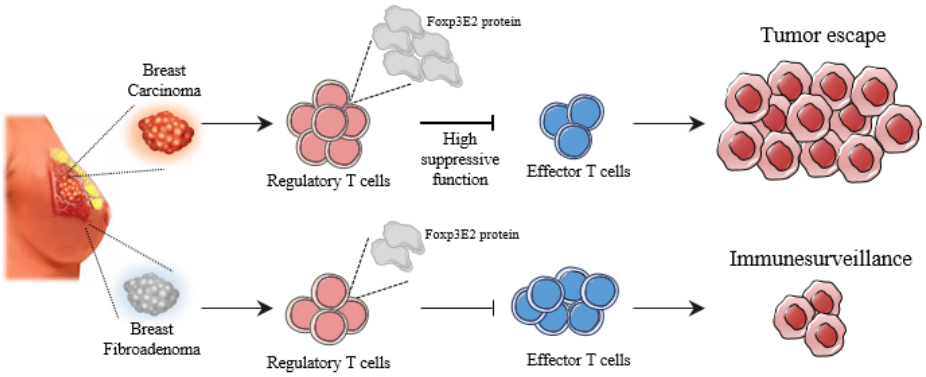
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN

MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIII CYCLE

Preferential accumulation of Foxp3E2^{+} regulatory T cells with highly immunosuppressive phenotype in breast cancer subjects



Tutor
Prof. Giuseppe Matarese

Candidate
Dr. Clorinda Fusco

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Abstract

Regulatory T $CD4^+Foxp3^+$ (Treg) cells are a cellular subset involved in the maintenance of immune self-tolerance and homeostasis but, as a double-edged sword, they can also suppress anti-tumor immune response and favor tumor progression. Therefore, $Foxp3^+$ Treg cells represent a primary target for cancer immunotherapy, which finally aims at restoring the ability of the immune system to detect and destroy cancer cells. The tumor microenvironment has been reported to contain a "rich milieu" of molecules able to increase the recruitment of $Foxp3^+$ Treg cells to the tumor site. Compelling experimental evidence has shown an increased percentage of $Foxp3^+$ Treg cells in the tumor microenvironment of subjects with different tumors, including breast cancer (BC). Moreover, their abundant presence in tumor infiltrates leads to reduced survival in cancer subjects and inversely correlates with clinical response of BC to therapy. The transcription factor Foxp3 plays a critical role in regulating the development and the immunosuppressive function of Treg cells and up to 8 different Foxp3 splicing variants have been described in human subjects, but their role and function still remain elusive. Recently, it has been found that among all the different Foxp3 splicing forms, those containing the exon2 (Foxp3E2) are necessary for the induction and establishment of the suppressive phenotype of Treg cells. The aim of this thesis was to evaluate the role of $Foxp3E2^+$ Treg cells in the context of tumor growth, dissecting whether increased immunosuppression observed in BC subjects, could be secondary to the preferential accumulation of $Foxp3E2^+$ Treg cells. In conclusion, the evaluation of the number of $Foxp3E2^+$ Treg cells in BC tumors could represent a prognostic assay for the assessment of tumor progression, severity and prognosis. In addition, $Foxp3E2^+$ Treg cells could be pharmacological targeted in order to inhibit their immunosuppressive activity in the tumor microenvironment, thus sustaining anti-tumor immune response and reducing tumor progression.

Background

1. Breast cancer

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of death among women (Bray F, 2018). The etiology of BC comprises multiple risk factors that mainly include age, family history, reproductive factors (timing of menarche and menopause, number and timing of pregnancies), hormonal factors and life style (diet, smoking, alcohol consumption), but it is also influenced by genetic and environmental factors (Li J, 2018). Indeed, several studies have identified many genes involved in BC development, confirming that mutations of both oncogenes and anti-oncogenes play key roles in BC initiation and progression (Sever R, 2015). The most well-known genes associated with BC are BRCA1 and BCRA2 that have a pivotal role in maintaining DNA integrity; indeed, their mutations significantly increase lifetime risk of BC developing (King MC, 2003), estimating BC penetrance of 60% for BRCA1 and 55% for BRCA2 (Mavaddat N, 2013). In addition to the BRCA1 and BRCA2 genes, several other genetic alterations have been associated with an increased risk of developing BC. Among these, many genes are involved in the maintenance of DNA fidelity, such as TP53 coding for p53, an essential checkpoint protein of cell cycle and PTEN, engaged in blockage of cell-cycle progression in G1 phase and implicated in the process of DNA repair. Other genes are involved in the signal transduction pathways controlling cell growth and differentiation, such as Myc and ERBB2 (Risom T, 2020; Perou CM, 2000). All these genes and many others may contribute to the pathogenesis of BC, for this reason new technologies, such as next-generation sequencing (NGS) or development of novel bioinformatics approaches, are becoming fundamental in order to improve clinical practice.

1.1 Breast cancer classification

BC is considered a highly heterogeneous disease characterized by several pathological features at histopathological, molecular and clinical level, which can differently impact on the clinical courses and responses to therapy (Cancer Genome Atlas Network, 2012). According to the histopathological evaluation, BC classification is based on the site from which the tumor originates: *ductal carcinoma* and *lobular carcinoma* which originate from the internal lining epithelium of the ducts or from the lobules that surround the ducts with milk, respectively. Another classification is established on the basis of abnormal proliferative activity of neoplastic cells in the breast tissue. More specifically, it is classified as carcinoma *in situ* when limited to the epithelial component of the breast tissue or *invasive* (infiltrating) carcinoma when it has invaded the stromal tissue. Invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) are most common types of BC and accounts for 72–80% and 5–15% respectively of all invasive BCs (Arps DP 2013; Guiu S, 2014). Furthermore, histopathologic features of BC also include other factors that reflect aggressiveness and progression of tumor such as the tumor differentiation grade and lymph node status. In this context, American Joint Commission of Cancer (AJCC) and the Union for International Cancer Control (UICC) have developed an established tool for classification of solid tumors known as TNM system (Sawaki M, 2019), devised by Pierre Denoix between 1943 and 1952 (Denoix P, 1946). TNM system classifies solid tumor cancers on the basis of the size of the primary tumor (T), regional lymph nodes that are involved in the lesion (N) and spreading of distant metastasis (M). A further prognostic and predictive tool, used in association with TNM system, is the Nottingham histological grading (NHG) that evaluates the level of differentiation and the proliferative grade of tumor cells (Todd JH, 1987). The NHG is expressed as grades between I-III, with I which is a well-differentiated tumor and III identified as poorly differentiated. Nowadays, TNM and NHG

index represent the fundamentals prognostic tools used in diagnostic and clinical practices for the choice of BC treatment (Rakha EA, 2014; Park YH, 2011; Frkovic-Grazio S, 2002).

1.2 Hormone-Receptor expression in breast cancer

Even though histopathological classification provides key information related to the clinical behavior of BC, in the last decades new approaches have been considered to explain the molecular basis for heterogeneity of BC. This molecular classification, introduced by Perou et al. in 2000 (Perou CM, 2000), identifies different classes of BC through the expression of hormone receptors (HR), including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), by immunohistochemistry (IHC) staining and the fluorescence in situ hybridization (FISH) technique. Therefore, BCs are stratified according to ER expression status as luminal (ER+) and non-luminal (ER-) tumors. Luminal tumors are further subdivided into luminal A that are HR positive (ER+\PR+), HER2 negative and with low levels of the proliferation marker Ki-67 expression, and luminal B which is hormone-receptor positive (ER+\PR+), HER2 positive or HER2 negative but with high levels of Ki-67. Luminal A cancers grow slowly and have the best prognosis in contrast with Luminal B cancers that generally grow faster and have a worsen prognosis. Molecular subtyping is essential for BC management and treatment, since different molecular subtypes lead to different prognosis and therapeutic options. Actually, molecular classification has been already approved for clinic use and it has been shown to have prognostic value and to be predictive of the response to chemotherapy.

2. Dual role of immune system in cancer: “concomitant immunity”

The role of immune system during tumor development was initially studied through murine models showing that progressive growth of a primary tumor suppressed the development of a newly implanted tumor, through a mechanism involving the immune system, a phenomenon also known as concomitant immunity (CI) (Ruggiero RA, 1989; North RJ, 1977). Specifically, North and colleagues, in animal tumor models, found that during the growth of immunogenic tumors, two different subset of suppressor T lymphocytes are sequentially generated. The first class, corresponding to $CD8^+$ T lymphocytes, appears during the early stages of tumor growth and is characterized by the ability, upon passive transfer, to suppress delayed-type hypersensitivity (DTH) reaction against tumors antigens in tumor-immunized recipients. On the contrary, the other T lymphocytes subset appears later on during tumor growth and has been identified, in adoptive transfer experiments, as made of $CD4^+$ T lymphocytes able to suppress “concomitant” immunity” against tumors (Di Giacomo, 1986). More recently, a clear evidence of the presence of cancer immunosurveillance comes from several studies showing the presence of tumor-infiltrating lymphocytes (TILs) in tumor of cancer patients (Savas P, 2016; Dadmarz R, 1995; Ruffell B, 2012). Specifically, several investigations have shown that high frequency of TILs in a primary tumor correlates with good prognosis for disease-free and overall survival in large cohort of cancer patients, supporting the notion that immune system cells control cancer growth and invasion (Galon J, 2006; Hanahan D, 2011). However, according to concomitant immunity theory, it has been observed that immune system has a dual role since it protects not only the host against tumor development but it also has the ability to favor tumors that are either poorly recognized by the immune system or that have acquired mechanisms to evade immune effector functions. Therefore, in order to describe more appropriately the dual host-protective and tumor-promoting roles of the immune system, the new "cancer

immunoediting" hypothesis has been introduced, which explains the complex interaction between immunity and cancer, describing how tumor microenvironment can induce tumor cells to deceive immunosurveillance (Dunn GP, 2002). The cancer immunoediting process involves three sequential phases: 1) the elimination phase in which the immune system tries to destroy the developing-tumor; 2) the equilibrium phase in which the immune system promotes the generation of tumor cell variants with increased capacity to escape immune system attack and in which a continuous sculpting of tumor cells produces cells resistant to immune effector cells; 3) the escape phase in which the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host (**Figure 1**).

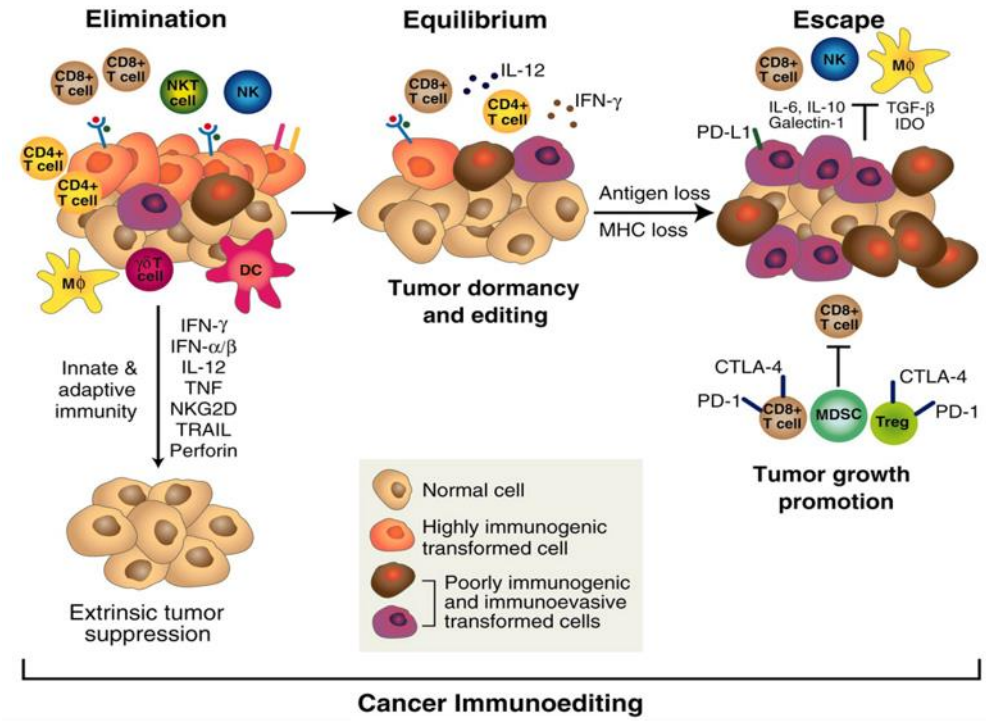

Image adapted from Schreiber, 2011

Figure 1. The Three Phases of the Cancer Immunoediting Process: elimination, equilibrium and escape. The innate and adaptive immune cells try to eliminate the developing-tumor in the elimination phase. Whether this process is not successful, tumor cells enter in the equilibrium phase in which the tumor cells and the host immune system are in balance: the immune system inhibits proliferation of immune sensitive tumor cells variants and remaining resistant tumor cells acquire mutations that protect them from immune detection, creating an immunosuppressive tumor microenvironment. These variants may eventually evade the immune system by a variety of mechanisms and become clinically detectable in the escape phase

2.1 Elimination phase

Elimination phase corresponds to the initial theory of tumor immunosurveillance. It is the phase in which immune system recognizes and eradicates the developing tumor cells. This phase occurs when the cell intrinsic tumor suppressor mechanisms failed and it needs the combined action of the innate and adaptive immune response. In recognition step, innate immune cells such as Natural Killer cells (NK), Natural killer T (NKT) cells as well as

macrophages and dendritic cells (DCs) are the first cells recruited and/or activated by pro-inflammatory cytokines produced by tumor cells. The activation of innate immune cells, such as NK and NKT, promotes the release of interferon-gamma (IFN-γ) which in turn induces tumor cells death (Shankaran V, 2001) and the production of chemokines such as CXCL10, CXCL9 and CXCL11, which block the formation of new blood vessels and tumor cell proliferation. Thus, tumor apoptotic bodies are engulfed by dendritic cells that migrate to tumor-draining lymph nodes, resulting in antigen presentation to naive T $CD4^+$ cells, which in turn facilitates the development of cytotoxic $CD8^+$ T cells. Tumor antigen-specific $CD4^+$ and $CD8^+$ T cells reach the primary tumor site, where the clonal expansion of T cells (CTLs) eliminate the remaining tumor antigens-expressing tumor cells, also selecting tumor cells with a reduced immunogenicity.

2.2 Equilibrium phase

Equilibrium is the longest phase of immunoediting and it can last even many years. It is characterized by a delicate balance between the elimination of tumor cells by the immune system and the production of new tumor variants cells that are resistant to immune response. In this context, the lymphocytes and IFN-γ put an immune selection pressure on tumor cells: they kill tumor variants from original tumor but promote proliferation of new variants of cancer cells with non-immunogenic phenotype, sustaining their survival and growth in tumor microenvironment. Recent studies have shown that tumor environment in equilibrium phase displays high amount of pro-inflammatory cells and low proportions of anti-inflammatory cells (Wu X, 2013), thus suggesting that the equilibrium phase might represent a condition of tumor "dormancy" in which outgrowth of occult tumors is specifically controlled by immune system.

2.3 Escape phase

Escape phase is a crucial point of tumorigenesis, in which surviving tumor cells variants, that have acquired the capability to escape to immunologic detection and elimination, proliferate in an uncontrolled manner. These cells are not only resistant to immune-mediated killing but they are able to induce an immunosuppressive microenvironment tolerating or even fostering tumor growth. Several mechanisms exist that lead to escape of cancer cells to immune system control, including: the reduced immune recognition through downregulation/loss of classical MHC class I expression; the exposure of self-antigens toward which the immune system is already tolerant and the increased survival, mediated by the expression of several anti-apoptotic molecules and/or development of an immunosuppressive tumor microenvironment. Indeed, the tumor microenvironment has been reported to contain a variety of tumor-derived soluble factors which contribute to create a immunosuppressive milieu, including different cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), IL-10 and immuno-regulatory molecules such as indoleamine 2,3-dioxygenase (IDO), galectin and LAG-3. In this immunosuppressive *scenario,* a key role is represented by the recruitment at the tumor site of a T $CD4^+$ cell subset called Regulatory T cells (Tregs), which express the transcription factor forkhead box (Foxp3). Specifically, these cells are able to suppress anti-tumor immune responses and favor tumor progression. Instead, the immunosuppressive molecules in the tumor milieu increas the conversion of conventional $CD4^+$ T cells into $Foxp3^+$ Treg cells, the recruitment of peripheral Treg cells to the tumor site and the expansion of tumor-resident Treg cells. Supporting the evidence of an immunosuppressive role of Treg cells in tumors is the finding showing that Treg cell removal from tumor tissues results in an enhanced anti-tumor immune response (Shimizu J, 1999). For these reasons, $Foxp3^+$ Treg cells represent one of a primary target cells for cancer immunotherapy, which finally aims at restoring the ability of

immune system to detect and destroy cancer cells, by overcoming the mechanisms by which tumors can evade the immune response.

3. Regulatory T cells (Treg cells)

Several regulatory mechanisms are essential to maintain immune homeostasis, to minimize deleterious inflammation caused by pathogens and immune responses against self- and environmental antigens. These mechanisms are regulated by different cellular subsets including Regulatory T (Treg) cells that are key players in the maintenance of peripheral self-tolerance and immune homeostasis. They represent 5-10% of $CD4^+$ T cells that constitutively express the IL-2 receptor alpha chain (CD25). Besides CD25, they also express several surface molecules: chemokine receptors like CCR4, CCR7 and CCR8 involved in lymph node homing of naïve Treg cells, selectins such as CD62L and integrin such as CD103, negative co-stimulatory molecules, such as CTLA4 and PD1. In addition, several groups have shown that Treg cells are also characterized by low levels of CD127 (IL-7 receptor α-chain) (Marinić I, 2006; Liu W, 2006; Seddiki N,2006). Moreover, they are characterized by the expression of the transcription factor Forkhead-box-P3 (Foxp3), that acts as a master gene for cell-lineage commitment and developmental differentiation of Treg cells (Fontenot JD, 2003; Miyara M, 2009; Walker MR, 2003). Foxp3 is not only a lineage-specific transcription factor required for their differentiation but is also crucial for their suppressive activity toward a wide range of cell populations such as $CD4^+$ and $CD8^+$ T cells, B cells, NK, NKT, dendritic cells (DCs), monocytes, and macrophages both *in vivo* and *in vitro* (Sakaguchi S, 2008; Ohkura N, 2013). As concerns the origin of Treg cells, they can originate either in the thymus from immature $CD4^+$ precursors by the stimulation of self-antigens and they are known as thymus-derived Treg (tTreg) cells or natural Treg (nTreg) cells. Alternatively, they can be differentiated from naïve $CD4^+$ T cells upon antigen stimulation under certain conditions in peripheral tissues and

they are known as peripheral Treg cells (pTregs). In addition, "induced" Treg (iTreg) cells is the term used to describe Treg cells generated *in vitro* by the culture of naïve $CD4^+$ T cells upon T cell receptor (TCR) activation in the presence of cytokines such as TGF-β and IL-2. During thymic development, Treg cells are positively selected through their TCR affinity with self-peptides presented on MHC class II molecules by thymic stromal cells (Bensinger SJ, 2001; Aschenbrenner K, 2007). Thus, $CD4^+$ thymocytes bearing upregulated CD25 become more sensitive to IL-2 signaling, which in turn facilitates induction of Foxp3 and drives Treg cell fate and commitment (Lio CW, 2008). Their thymic origin has been demonstrated in mice through neonatal thymectomy experiments within 3 days after birth leading to autoimmune damage of different organs and the presence of tissue-specific autoantibodies in circulation (Sakaguchi S, 2008). In the periphery, Treg cells can develop from naïve $CD4^+Foxp3^-$ T cells (Tconv) upon chronic exposure to antigens or/and in the presence of immune-regulatory cytokines including IL-10, TGFβ and IL-2 (Barrat F, 2002; Fu S, 2004; Zheng SG, 2007). More specifically, IL-2 activates the transcription of transcriptional factor STAT5, which binds several sites on the Foxp3 promoter to enhance Foxp3 expression, establishing the genetic program driving Treg cell commitment. On the other hand, Foxp3 cooperates with other transcription factors and coactivators/corepressors in order to bind the IL-2 promoter and halt its transcription, rendering Treg cells hyporesponsive to classical TCR stimulation and highly dependent on exogenous IL-2 for their maintenance and function. Therefore, due to the constitutive expression of high-affinity IL-2R in Treg cells, low concentration of IL-2 can promote their activation and proliferation. Activated Treg cells can inhibit the differentiation and development of effector T cells (Dowling MR, 2018), including autoreactive Th1 and Th17 cells, by reducing costimulatory signals, depleting IL-2 and secreting IL-10, so as to regulate the immune balance between effector T cells and regulatory T cells. Therefore, IL-2 pathway is a milestone for Treg cells development,

homeostasis and survival, as demonstrated by several studies in which IL-2-deficiency has been associated with autoimmune diseases (Yamanouchi J, 2007). Therefore, constitutively high expression of CD25, dependency on exogenous IL-2 and TCR stimulation have all pivotal roles in controlling Treg cell function and in the conversion of Tconv cells into Treg cells both *in vivo* and *in vitro* (Yamaguchi T, 2013).

3.1 Foxp3 gene and its splicing variants

Foxp3 gene expression is necessary to confer Treg cell suppressive activity and it is critical for Treg cell differentiation. Indeed, Foxp3 alterations result in increased susceptibility to several autoimmune diseases, such as IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), or immunopathology and allergy (Sakaguchi S, 2008; Bennett CL, 2001). The Foxp3 gene, located on X chromosome at Xp11.23, is well conserved in mammalians and contains 11 coding exons and 3 non-translated exons. In humans, several splicing isoforms of Foxp3 have been described (Miyara M, 2009; Ito T, 2008; Allan SE, 2005; Smith E, 2006) which identify different phenotype and function of Treg cells (Roncador JD, 2003; Joly AL; 2018; Krejsgaard T, 2008), in contrast with mouse Treg cells in which Foxp3 is only expressed as a full length protein (Fontenot JD, 2005). Indeed, human Treg cells express four Foxp3 isoforms: the full-length isoform (Foxp3all) and shortened isoforms as a result of exclusion of either exon 2 (FoxpΔ2), exon 7 (Foxp3Δ7), or both exons 2 and 7 (Foxp3Δ2Δ7) from the Foxp3 transcripts (**Figure 2**). Compared to full length Foxp3 which contains the sequences involved in the interaction with retinoic acid-related orphan receptor α and γt (RORα and RORγt), Foxp3Δ2 is unable to interact with RORα (Du J, 2008) and RORγt (Zhou L, 2008) to inhibit Th17 cell function and development. A third isoform has also been described, lacking both exon 2 and exon 7 (Foxp3Δ2Δ7); this isoform facilitates Th17 differentiation cells, differently

from the other two isoforms (Bennett CL, 2001). Recent study has demonstrated that the different Foxp3 splicing variants are influenced by different specific metabolic programs such as glycolysis, fatty acid oxidation and mitochondrial oxidative phosphorylation in order to provide energy and precursors to support Treg cells activity during immune responses (De Rosa V, 2015). In particular, it has been recently shown that the induction and suppressive function of human iTreg cells tightly depended on glycolysis, which controls the expression of Foxp3 splicing variants containing exon 2 (Foxp3E2), through the glycolytic enzyme enolase-1 (De Rosa V, 2015).

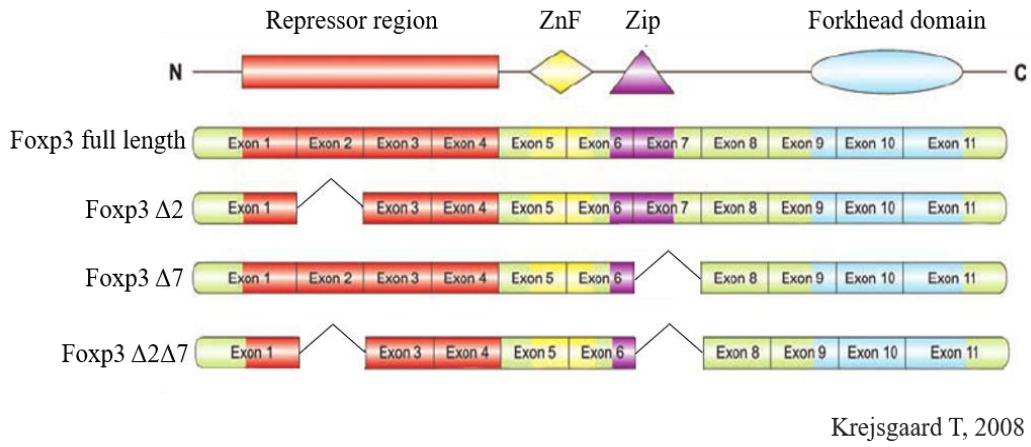

Figure 2. Schematic representation of Foxp3 mRNA splicing variants.

3.2 Treg cell mechanism of suppression

Several studies have demonstrated that Treg cells suppress the activation and expansion of several subsets of immune cells. Treg cell suppressive mechanisms can be classified into different categories, according to their modes of "action": (i) suppression through cell-to-cell contact that includes modulation of antigen-presenting cell (APC) maturation/function (ii)

suppression by killing of target cells, or (iii) suppression by contact-independent mechanisms, mediated by the release of inhibitor cytokines (**Figure 3**). The contact-dependent mechanism of suppression is mediated by several accessory molecules expressed on Treg cell surface, including Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), programmed cell death 1 (PD-1), Glucocorticoid-induced TNF receptor-related protein (GITR) and lymphocyte activation gene 3 (LAG3). The key role of such molecules in the control of Treg cell function has been demonstrated by the findings showing that CTLA-4 deficiency abrogates Treg cells-suppressive function and causes severe autoimmune diseases, similarly to what observed during Foxp3 deficiency (Sakaguchi S, 2008; Huang CT, 2004; Sharpe AH, 2007). Specifically, CTLA4 acts by downregulating or preventing the upregulation of CD80 and CD86 (the main costimulatory molecules) on APC, or inhibiting T cell activation, since it competes for CD28 in binding to CD80/86, interfering with the ability of T cells to form stable conjugates with APC cells (Schildberg FA, 2016). Similarly, to CTLA4, LAG-3 interacts with MHC class II on APC cells, causing an inhibitory signal that suppresses dendritic cells maturation and immunostimulatory capacity (Huang CT, 2004). While CTLA4 dampers T cells function during priming phase of their activation, PD1 expression is induced after T cells activation and its main role is to limit the activity of T cells in peripheral tissues during an inflammatory response, trough inhibition of molecules that are involved in T cell activation, proliferation and migration (Freeman GJ, 2000; Chemnitz JM, 2002). Other cell-surface molecules implicated in cell-to-cell suppressive functions of Treg cells are CD39 and CD73, two ectoenzymes that hydrolyze extracellular ATP to AMP. CD39 thus regulates immune T cell suppression by the downstream production of adenosine acting via the A2A receptor, which inhibits T cell proliferation. (Deaglio S, 2007; Borsellino G, 2007). In addition, Treg cells have been shown to act also by cytolysis against B cells, NK cells, monocytes and T cells through the release of perforin and granzyme A, similarly to NK cells and

$CD8^+$ cytotoxic T lymphocytes (Grossman WJ, 2004). One contact-independent mechanism by which Treg cells suppress T cell-mediated immune responses is through the secretion of immunosuppressive cytokines, such as TGF-β, IL-10, and IL-35. These cytokines are involved in inhibition of differentiation, proliferation, and activation of T effector cells, as they suppress pro-inflammatory cytokine production by T effector cells and promote the conversion of activated Tconv to cells into regulatory T cells, by inducing the expression of Foxp3. More precisely, TGF-β plays a critical role in maintaining peripheral tolerance and it is fundamental to the generation and maintenance of Treg cells (Freudenberg K, 2018), while IL-10 downregulates the expression of MHC-II and co-stimulatory molecules (CD80/CD86 and CD28) (Mittal SK, 2015; Saraiva M, 2010). Additionally, IL-10 reduces the release of pro-inflammatory cytokines by mast cells and macrophages and suppress their function and activation (Mosser DM, 2008).

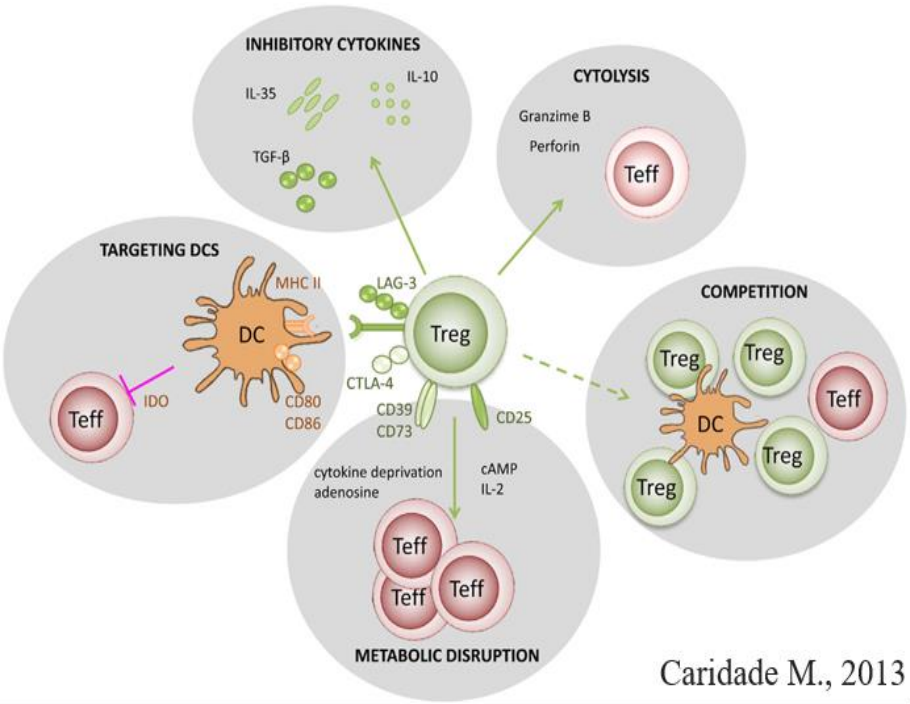

Figure 3. Schematic representation of Treg cells suppression mechanisms. Targeting of dendritic cells (DC) through downregulation of costimulatory molecules on their surface mediated by Treg cells, leading to abrogated signals to effector T cells; Metabolic disruption includes cytokine deprivation or cyclic AMP-mediated inhibition; Competition for critical cytokines or direct disruption of effector cell engagement with APCs; Cytolysis that acts by direct cytotoxic effect through the production of Granzyme B and Perforin and consequent apoptosis of effector T cells or APCs; Production of inhibitory cytokines, including IL-10, IL-35, and TGF-β.

3.3 Treg cells in breast cancer

The fundamental functions of Treg cells, engaged in suppressing abnormal or excessive immune responses against self/environmental antigens, is demonstrated by a plethora of diseases and pathological conditions characterized by Treg cell alterations. In particular, it is well known that alterations of Treg cells play a key role in the development of multifactorial autoimmune diseases, such as type 1 diabetes (T1D), rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis which afflict 10% of the

population worldwide (Cooper GS, 2009). Treg cell defects found in these autoimmune diseases concern both their suppressive function and number. In multiple sclerosis, Treg cells display an impaired *in vivo* and *in vitro* proliferative capacity that correlates with the clinical state of the disease, whit increasing disease severity associated with a decline in Treg cell expansion (Carbone F, 2014). On the contrary, an increased Treg cell number has instead been found in several types of cancers, including lung, ovarian, gastric, breast and pancreatic cancers (Salama P, 2009). In this context, tumors recruit, expand, differentiate, and activate Treg cells by multiple mechanisms, which result in the suppression of the tumor immunosurveillance. Indeed, several studies have demonstrated that the frequency of Treg cells is increased in the peripheral blood, tumor microenvironment and among tumor-infiltrating lymphocytes (TILs) in subjects with different types of cancer, including breast cancer (Liyanage UK, 2002; Perez SA, 2007). More specifically, the increased presence of Treg cells in breast tumor biopsies is associated with an invasive cancer phenotype and diminished relapse-free as well as overall survival (Bates GJ, 2006; Ohara M, 2009), due to Treg-mediated suppression of anti-tumor immunity. Treg cells play a central role not only in tumor progression, through the suppression of antitumor immunity, but also in the development of tumor resistance to immunotherapies, such as immune checkpoint inhibitors (ICIs), contributing to tumor growth and expansion. Indeed, most immunotherapies for BC treatment involve the manipulation of key immune checkpoints that regulate the adaptive immune system and are highly expressed by Treg cells, including CTLA4, PD1, and PD-L1. On the other hand, several studies have demonstrated that beneficial effects of different anti-cancer therapies are associated with the downregulation or ablation of Treg cells in BC. While the silencing of immune checkpoint molecules has no significant effect on tumor progression, its combination with Treg cells depletion by anti-CD25 antibodies suppress tumor growth in mouse breast cancer models (Liu Y, 2011). The suppressive function and higher recruitment of Treg cells in the tumor in BC

subjects depends on environmental factors, tumor-derived products and locally produced cytokines that can influence Treg cells biology, identity and function in order to inhibit anti-tumor immune response, thus favoring tumor growth.

Aim of the study

Compelling experimental evidence has shown an increased percentage of tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment of subjects with different tumors, including BC. Moreover, the Treg cells abundance in tumor infiltrates leads to reduced survival in cancer subjects and inversely correlates with clinical response of BC to therapy (Liyanage UK, 2002; Perez S, 2007). In this context, the transcription factor Foxp3 plays a critical role in regulating the development and the immunosuppressive function of Treg cells. Until now, 8 different Foxp3 splicing variants have been described in human subjects, but their specific role and function still remain uncleared. Recently, it has been found that the Foxp3 splicing forms containing the exon2 (Foxp3E2) are necessary for the induction and establishment of the suppressive phenotype of Tregs (De Rosa V, 2015). In particular, Foxp3E2 inhibition has been linked to the generation of "defective" Treg cells, which are not able to efficiently suppress immune T cell responses. The aim of this thesis is to evaluate the role of Foxp3E2^{+} Treg cells in the context of tumor growth, dissecting whether increased immunosuppression, observed in BC subjects, could be secondary to the preferential accumulation of Foxp3E2^{+} Treg.

Materials and Methods

1. Subjects and study design

The study was approved by the Institutional Review Board of the Università degli Studi di Napoli "Federico II". Between June 2016 and January 2021, Breast Cancer (BC) subjects were enrolled by our clinician collaborator at Instituto Nazionale Tumori – IRCCS "Fondazione G. Pascale". Peripheral blood was obtained from BC and healthy subjects (HS) after they signed a written informed consent approved by institutional review boards. All blood samples were collected at $9{:}00_{AM}$ into heparinized Vacutainers (BD Biosciences) and were processed within the following 4 hours. Demographic and clinical characteristics of study cohorts are shown in **Table 1**. All subjects were naïve-to-treatment and with definite clinicopathological parameters evaluated for each subject included age, tumor size, tumor-node-metastasis (TNM) stage, Ki-67 index, estrogen receptor (ER) and progesterone receptor (PR) status and human epidermal growth factor receptor 2 (HER2) status. For each subject, a detailed past medical history was recorded to exclude intake of steroids and/or antihistamine drugs in the 2 months preceding the enrolment and previous diagnosis of chronic inflammatory, immune or other neoplastic diseases. All tissue and blood samples from ER^+PR^+ BC subjects were collected prior to chemotherapy, radiotherapy, endocrine therapy or any other treatment that could affect the immune state. All patients underwent to breast cancer surgery or core needle biopsies, collected with ultra-sound guidance. BC subjects were classified in immunohistochemically defined surrogate molecular subtypes, according to the recommendations of St. Gallen International Breast Cancer Conference (Rageth CJ, 2019). HS were matched for age, body mass index, and sex and had no history of inflammation, endocrine, or autoimmune disease. The ethnic distribution among the groups was comparable, with all participants being Caucasian.

Table 1. Clinical characteristic of 35 BC subjects.

Variables	N. of patients (%)
BC subjects	35
Age at diagnosis, mean ± SD, year	54 (±6)
Tumor staging	
T1	20 (54)
T2	12 (34)
T3	2 (6)
T4	1 (3)
Lhymph Nodes metastasis	
0	23 (66)
1-3	9 (26)
≥4	3 (8)
Histological grading	
I	2 (6)
II	25 (71)
III	8 (23)
Immunohistochemical markers	
ER positive	35 (100)
PR positive	35 (100)
HER2 overexpression	0
Ki-67 positive	35 (100)
Molecular subtype	
Luminal A	25 (71)
Luminal B	10 (29)

2. Immunohistochemistry

Immunohistochemical staining was performed on slides from formalin-fixed, paraffin embedded tissues to evaluate the expression of CD3, CD8 and Foxp3 markers in Fibroadenoma and Breast cancer tissues. Paraffin slides were de-paraffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed with slides heated in 0.01 M citrate buffer (pH 6.0) in a bath for 20 minutes at 97°C. After antigen retrieval, the slides allow to cool. The endogenous peroxidase was inactivated with 3% hydrogen peroxide was inactivated with 3% hydrogen peroxide. After protein block (BSA 5% in PBS 1x), every slide was incubated with specific primary antibodies: CD3 (anti-CD3 rabbit monoclonal Ab, clone 2GV6, Verdana), CD8 (anti-CD8 rabbit monoclonal Ab, clone CAL66, Verdana) and Foxp3 (anti-Foxp3 rabbit monoclonal Ab, clone D2W8E, Cell Signaling). The sections were incubated for 1 hour with Novocastra Biotinylated Secondary Antibody (HRP-conjugated) and visualized with DAB chromogen. Finally, the sections were counterstained with hematoxylin and mounted. CD3, CD8 and Foxp3 positive nuclei were counted evaluating at least five fields at 400x magnification. All sections were evaluated in a blinded fashion by 2 investigators.

3. Tissue samples preparation

Dissected tumor fragments from fresh surgical from BC subjects were directly transferred in GentleMACS C tubes (Miltenyi Biotec) containing calcium- and magnesium-supplemented HBSS with 0.5 mg/mL Collagenase IV (Sigma), 50 ng/mL DNAse I (Worthington), 2% FBS and 10% BSA. Tissue dissociation was made on a GentleMACS Octo Dissociator with Heaters (Miltenyi Biotec), by performing the program “h_tumor 01_03” twice. After an incubation of 10 minutes at 37°C, another round of dissociation by “h_tumor 01_03” program it was made. Single cell suspensions were obtained by disrupting the fragments

with a syringe plunger over a cell strainer, washing with cold HBSS. Then, cells were pelleted through a 40% isotonic Percoll solution, and finally centrifuged over a Lympholyte density gradient.

4. Cell purification

Peripheral blood mononuclear cells (PBMCs) from HS and BC subjects were isolated after Ficoll–Hypaque gradient centrifugation (GE Healthcare). Peripheral Treg ($CD4^+CD25^+$) and Tconv ($CD4^+CD25^-$) cells were purified (90–95% pure) from PBMCs of HS and BC subjects by using the The $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec).

5. Flow cytometry, proliferation and CFSE staining

Freshly isolated PBMCs from HS and BC subjects were surface stained with the following mAbs: APC-H7–conjugated anti-human CD4 (RPA-T4), PerCP-Cy5.5–conjugated anti-human CD197/CCR7 (150503), BB515–conjugated anti-human CD45RA (HI100), BV421–conjugated anti-human CD279/PD-1 (EH12.1), BV510 conjugated anti-human CD62L (DREG-56) and PE-Cy7–conjugated anti-human CD8 (RPA-T8) all from BD Biosciences. Thereafter, cells were washed, fixed, and permeabilized (Anti-Human FOXP3 staining Set PE; eBioscience) and stained with following mAbs: PE-conjugated anti-human FOXP3 from eBioscience (PCH101) that recognizes all splicing variants of FOXP3 (through an epitope of the amino terminus of FOXP3), and PE-conjugated anti-human FOXP3 from eBioscience (150D/E4) that recognizes FOXP3E2 through an epitope present in the exon 2 only, APC–conjugated anti-human CD152/CTLA-4 (BNI3) (from BD Biosciences) and Alexa Fluor 647–conjugated antibody to ribosomal protein S6 phosphorylated at Ser235 and Ser236 (D57.2.2E) (from Cell Signaling). Cells were analysed with FACSCanto II (BD Biosciences) and FlowJo software (Tree Star).

For T cell proliferation and suppression assays, $CD4^+CD25^-$ T cells (90-95% pure) (2×10^4 cells per well) from HS and BC subjects were purified by magnetic cell separation $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec). The fluorescent dye CFSE was used at a concentration of 1 μg/ml (Invitrogen). Flow cytometry analyzing CFSE dilution was performed by gating on $CD4^+CD25^-CFSE^+$ cells from HS and BC subjects stimulated for 72 hours in round-bottomed 96-well plates (Corning Falcon) with anti-CD3/anti-CD28 mAb coated beads (0.2 bead per cell; Thermo-Fisher) alone or cultured with pTreg cells from HS and BC subjects, respectively.

6. Statistical analysis

Statistical analyses were performed using GraphPad program (Abacus Concepts). Results were expressed as mean ± s.e.m.; a P value ≤ 0.05 was indicative of statistical significance. The nonparametric Mann-Whitney U-test and the Wilcoxon matched-pairs signed-rank test were used. We used two-tailed test for all analyses. To evaluate the relationship between the percentage of $Foxp3E2^+$ Treg cells in BC subjects and the percentage of proliferation marker Ki-67 in tumor cells, we computed the nonparametric Spearman rank correlation.

Results

1. Accumulation of $CD4^+$ T cells in tumor-infiltrating leukocytes within breast cancer tissue

Immune surveillance and immune tolerance are essential mechanisms in the maintenance of immune homeostasis. However, in tumor tissues this balance is often disrupted and defective, allowing tumour cells to evade from attack by the immune system resulting in their continuous proliferation and growth. Indeed, during tumor development, cancer cells promote the recruitment in the tumour tissues of different immune cells, predominantly $CD8^+$ T lymphocytes and $FoxP3^+$ Tregs, which are the main mediators of immune surveillance and immune tolerance, respectively. In order to define the composition of immune infiltrate in BC tissues, we performed immunohistochemistry (IHC) analysis on paraffined breast cancer tissue. Specifically, we evaluated the percentage of the main subsets of tumor-infiltrating lymphocytes (TILs) in breast cancer tissue as compared to breast fibroadenoma (BF), known as a benign lesion in breast tissue. We performed in parallel immunostaining for CD3, CD8 and for Foxp3 (**Figure 4A**) and we found that in BF tissue CD8 subset was the predominant type of T cells (**Figure 4B**) among $CD3^+$ T cells. On the contrary, in BC tissue, we observed a reduced percentage of $CD8^+$ cells and a significant recruitment of $Foxp3^+$ cells (**Figure 4C**). In addition, since $CD8^+$ exhibit antitumor effects and Treg cells negatively regulate immune responses, we calculated the ratio of $CD8^+$: $Foxp3^+$ cells (rather than the absolute number) within the tissues in order to define the balance between pro-tumour and anti-tumour immune response. As expected, the CD8 : Foxp3 ratio was lower in BC tissue as compared to BF (**Figure 4D**), thus suggesting that Treg cells from BC, differently from those derived from BF, are directly involved in promoting tumor progression by suppressing anti-tumor immune response; whereas in

fibroadenoma the immune system is "surveilling" the lesion mainly thought CD8 T cell cytotoxic activity.

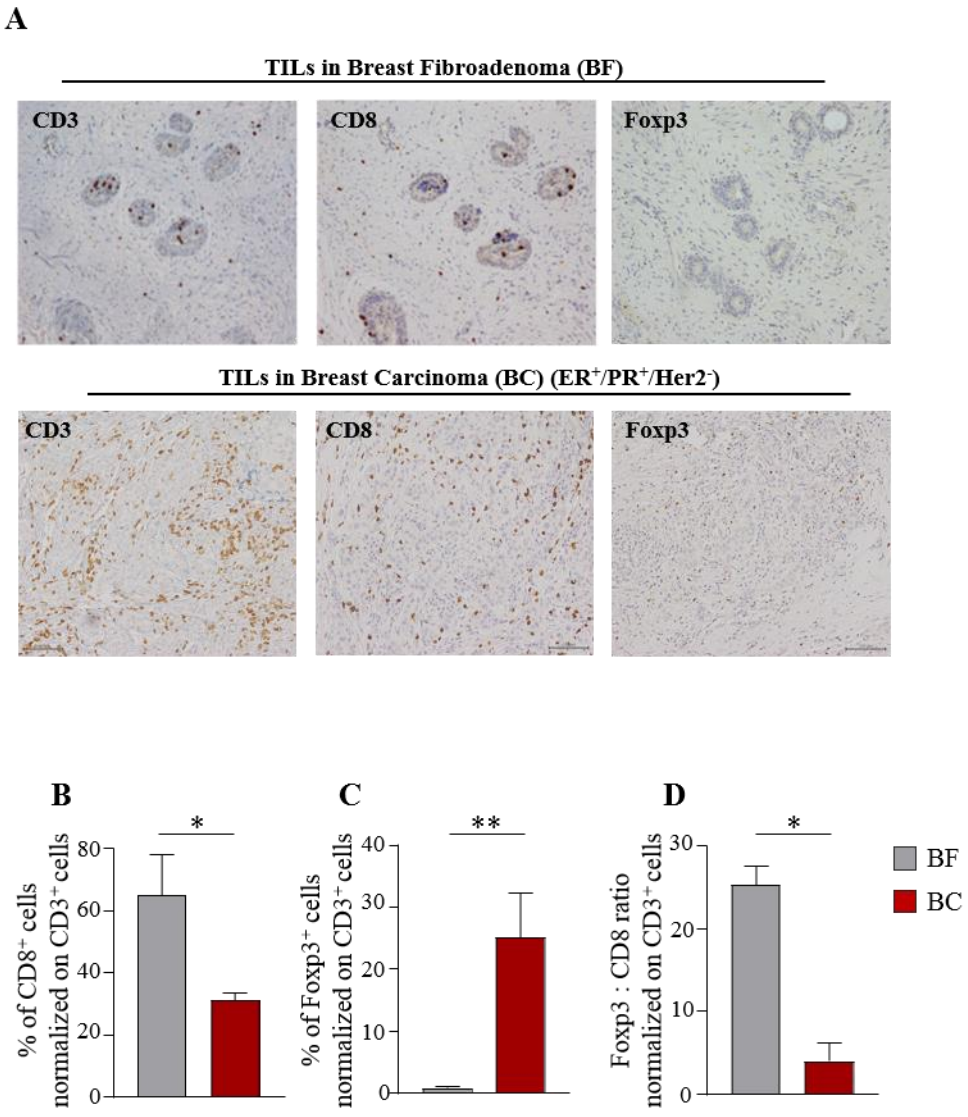

Figure 4. Increased CD4$^+$Foxp3$^+$ T cells in infiltrated breast tumors. (**A**) Immunohistochemical staining showing the stromal distribution of CD3$^+$ (left panel), CD8$^+$ (central panel) and Foxp3$^+$ (right panel) cells in tumor infiltrating-lymphocytes (TILs) from a single section of paraffined BF tissue (upper panels) and BC tissue (bottom panels). Magnification, 400x. Scale bar, 200 µm. Cumulative data showing percentage (%) of (**B**) CD8$^+$, (**C**) Foxp3$^+$ cells and (**D**) Foxp3 : CD8 ratio values, normalized on CD3$^+$ cells from 15 different sections of BF (n=15) and BC (n=15) tissues. (**B-D**) Statistical analysis was performed by using Mann-Whitney U-test (two tails) (mean ± s.e.m.); *$P \leq 0.05$, **$P \leq 0.01$.

2. Increased percentage of $CD4^+Foxp3E2^+$ Treg cells in Breast Cancer

Treg cells variably to infiltrate human BC and their frequency in the tumor microenvironment (TME) have clinical significance for BC prognosis and survival. To characterize the composition of T cell subsets resident in primary human breast tumors, we evaluated the frequency of Tumor infiltrating lymphocytes (TILs) in freshly-resected BC tissue from untreated BC subjects, also by multi-color flow cytometry analysis (FACs) (**Figure 5A**). We also found that among $CD45^+$ cells, BC tissues were characterized by an increased percentage of $CD4^+$ T cells as compared to BF tissues (**Figure 5B** and **5C**), where we observed a higher percentage of $CD8^+$ T cells (**Figure 5B** and **5D**). Moreover, $CD4^+Foxp3^+$ Treg cell frequency was significantly increased in BC, compared to BF (**Figure 5B** and **5E**). In addition, recent data have shown that a novel functionally distinct subpopulation of Treg cells expressing the Foxp3 Exon 2 (Foxp3E2) splicing variant, exhibit the strongest suppressive function (De Rosa, 2015). Therefore, we asked whether the immunosuppressive milieu characterizing tumors could be secondary to a predominant occurrence of Treg cells expressing $Foxp3E2^+$ compared to $Foxp3all^+$ in the TME. To this aim, we evaluated the expression of Foxp3 full length and Foxp3 Exon2 in TILs from all the above-mentioned experimental groups, using two different antibodies that recognize all splicing variants of Foxp3 (Foxp3all) and Foxp3 containing Exon2 (Foxp3E2), respectively. FACS analysis showed a higher percentage of $Foxp3E2^+$ Treg cell population in BC tissue compared to BF tissue (**Figure 5B** and **5F**). Furthermore, in order to evaluate the predominant immune response in BC we compared the CD8 : Foxp3 ratios in BC and BF tissues and we confirmed that it was significantly lower in BC tissue compared to BF tissue (**Figure 5G**). These data suggest that while in BF probably a great number of these lesions are resolved in tissue repair or are in an equilibrium phase with the surrounding tissue, in BC subjects there is a whole inversion of the immunological infiltrate, being Treg cells populations most represented during

the tumor escape. Interestingly, Treg cells exhibit a higher level of the Foxp3E2 splicing variant which characterizes and defines a highly immune suppressive population, able to play a key role in immune suppression and promote tumor escape from immune system control.

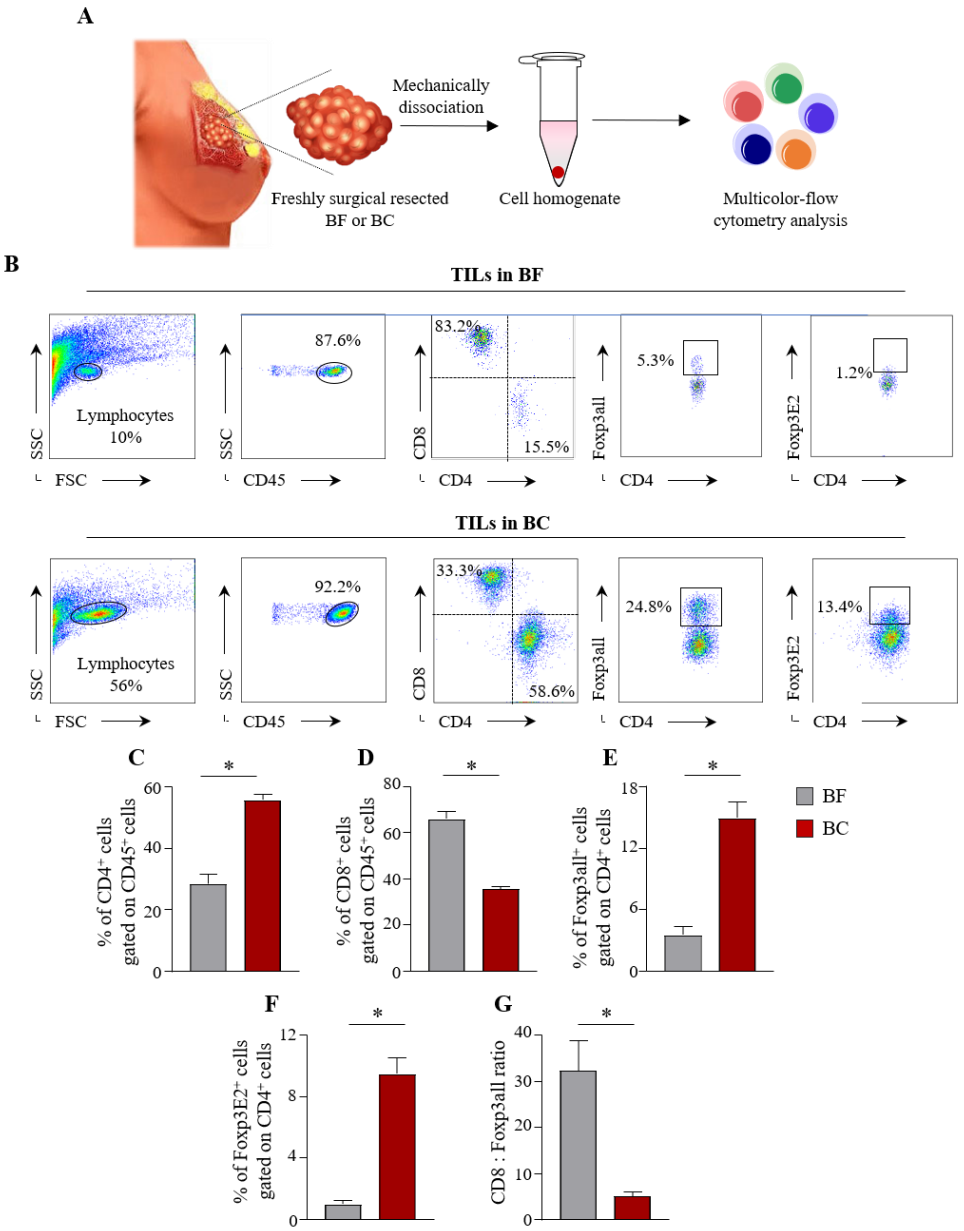

Figure 5. Increased CD4$^+$, CD4$^+$Foxp3all$^+$ and CD4$^+$Foxp3E2$^+$ frequency from TILs of BC compared to BF. (**A**) Schematic representation of the experimental procedure used to obtain cell homogenate from freshly surgical BF and BC for flow cytometry analysis. (**B**) Flow cytometry analyzing the expression of lymphocytes, CD45$^+$, CD4$^+$, CD8$^+$, CD4$^+$Foxp3all$^+$ and CD4$^+$Foxp3E2$^+$ cells in TILs from BF (upper panels) and BC (lower panels). Numbers in the plots indicate percentage (%) of positive cells; one representative experiment out of n=15 and n=20 independent experiments for BF and BC, respectively. Cumulative data showing percentage (%) of (**C**) CD4$^+$, (**D**) CD8$^+$, (**E**) CD4$^+$Foxp3all$^+$, (**F**) CD4$^+$Foxp3E2$^+$ cells and (**G**) CD8 : Foxp3all ratio values, from TILs of BF (n=15) and BC (n=20). (**C-G**) Statistical analysis was performed by using Mann-Whitney U-test (two tails) (mean ± s.e.m.); *$P \leq 0.05$.

3. Treg cells expressing Foxp3E2 exhibit a stronger suppressive phenotype than Foxp3all Treg cells

Next, to assess whether Foxp3E2^{+} Treg cell subset in BC subjects display a different immunophenotype than Foxp3all Treg cells, we compared the expression pattern of several Treg cell-associated markers in these two distinct Treg cell subsets. (**Figure 6A** and **6C**). We found that the level of suppressive markers such as CTLA4 and PD1, of the proliferative marker Ki-67 and the phosphorylation of S6 (downstream target of the metabolic checkpoint kinase mTOR) were significantly higher in the Foxp3E2^{+} subset compared to Foxp3all^{+} subset in BC tissue (**Figure 6D**), but not in BF (**Figure 6B**). On the contrary, the expression of migratory markers such as CCR7, CCR8 and cell adhesion molecule CD62L did not increase in Foxp3E2^{+} Treg population compared to Foxp3all subset both in BC and BF tissues (**Figures 6B** and **6D**). These results indicated that tumor microenvironment is enriched of Treg cells, most of which express Foxp3E2 splicing variant compared to BF, with a higher suppressive phenotype, elevated proliferative and metabolic activity, thus contributing to immune suppression which sustains tumor growth and progression.

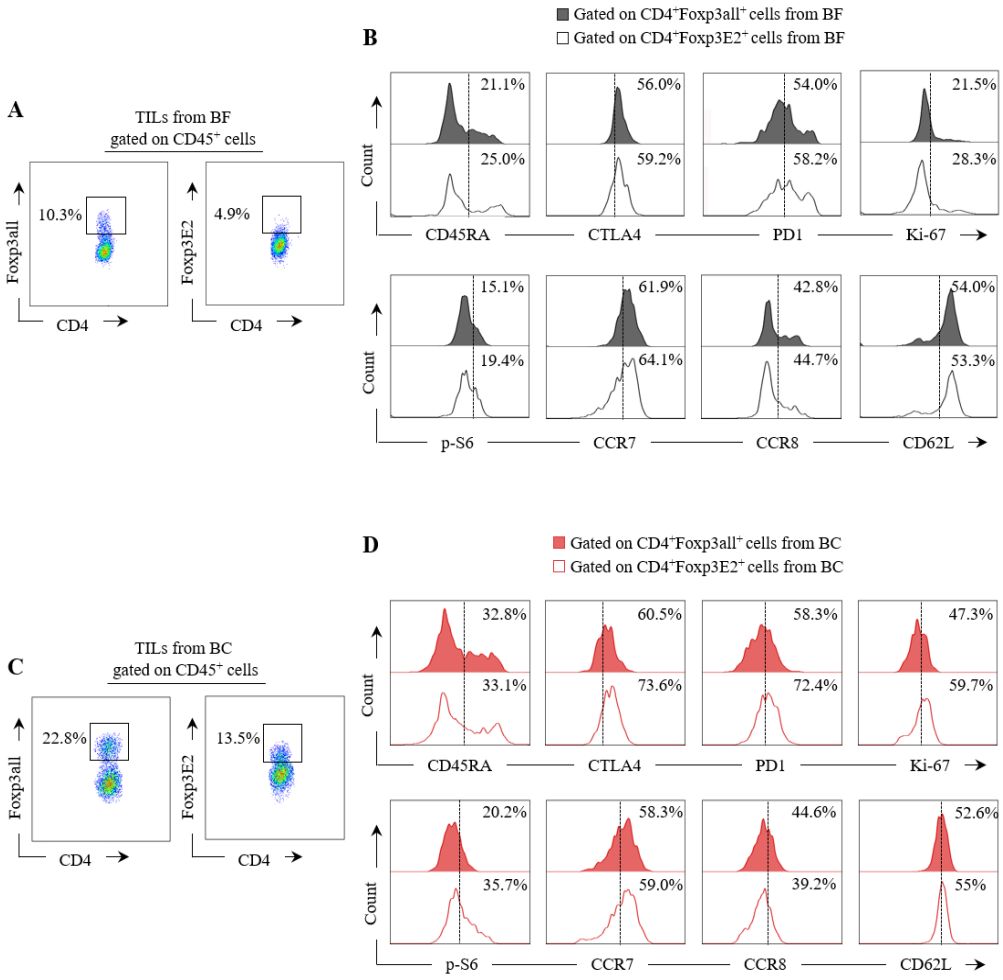

Figure 6. Increased levels of Treg cell-lineage markers in Foxp3E2$^+$ cells from TILs of BC subjects. (**A, C**) Flow cytometry analyzing the expression of CD4$^+$Foxp3all$^+$ and CD4$^+$Foxp3E2$^+$ cells in TILs from BF (upper panels) and BC (lower panels). (**B, D**) Flow cytometry analyzing the expression of specific Treg cell-lineage markers gated on CD4$^+$Foxp3all$^+$ cells (full lines) and CD4$^+$Foxp3E2$^+$ cells (empty lines) from TILs of BF (upper panels) and BC (lower panels). (**A-D**) Numbers in the plots indicate percentage (%) of positive cells; one representative experiment out of *n*=15 and *n*=20 independent experiments for BF and BC, respectively.

4. **Peripheral Treg cells from BC patients exhibit a stronger immune suppressive phenotype compared to healthy subjects**

Subsequently, we evaluated whether peripheral Foxp3all$^+$ and Foxp3E2$^+$ Treg cells from BC subjects could display different immune phenotype also in the peripheral blood, by FACS analysis. Flow-cytometric analysis of peripheral blood mononuclear cells (PBMCs) showed higher frequency of peripheral Treg cells, evaluated as both CD4$^+$Foxp3all$^+$ and CD4$^+$Foxp3E2$^+$ cells, in BC subjects when compared to HS (**Figure 7A** and **7C**). We next compared the expression of Treg cell-associated markers in both cellular subsets. We found that both CD4$^+$Foxp3all$^+$ and Foxp3E2$^+$ Treg cells from BC subjects showed an increased expression of CD45RA (a surface marker of naïve Treg cells), CTLA4, PD1, Ki-67, p-S6, CCR7, CCR8 and CD62L, when compared to HS (**Figure 7B** and **7D**). In addition, we found an increased expression of CTLA4, PD1, Ki-67 and p-S6 in Foxp3E2$^+$ Treg cells as compared to Foxp3all$^+$ Treg cell counterpart from BC subjects (**Figure 7E**), indicating a higher suppressive, proliferative and metabolic phenotype of Foxp3E2$^+$ Treg cells rather than Foxp3all$^+$ Treg cells in peripheral blood of BC. Taken together our data show that BC subjects display also in the peripheral blood an increased frequency of Treg cells, characterized by higher expression of chemokine receptors and suppressive markers, especially Treg cell subset expressing Foxp3E2, in line with an increased homing of Treg cells at the tumor site.

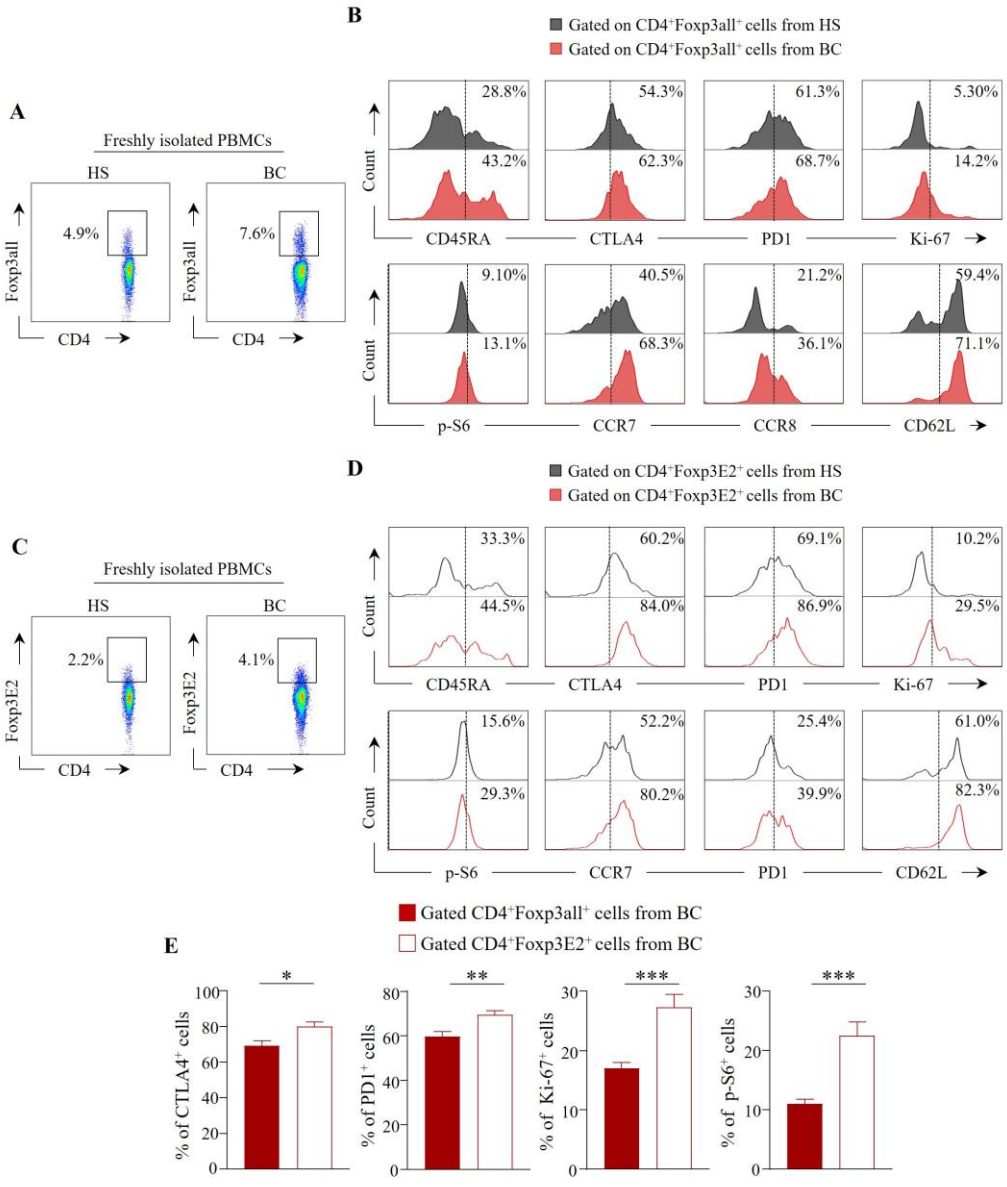

Figure 7. Increased Foxp3E2$^+$ cells and enhanced expression of Treg cell-specific markers in Foxp3E2$^+$ cells from BC subjects compared to HS. (**A, C**) Flow cytometry analyzing the expression of CD4$^+$Foxp3all$^+$ (upper panels) and CD4$^+$Foxp3E2$^+$ (lower panels) cells in freshly isolated PBMCs from BC subjects and HS. (**B, D**) Flow cytometry analyzing the expression of specific Treg cell-lineage markers gated on CD4$^+$Foxp3all$^+$ cells (full lines) and CD4$^+$Foxp3E2$^+$ cells (empty lines) in freshly isolated PBMCs from BC subjects compared to HS. (**A-D**) Numbers in the plots indicate percentage (%) of positive cells; one representative experiment out of *n*=30 independent experiments for BC subjects and HS. (**E**) Cumulative data showing percentage (%) of CTLA4$^+$, PD1$^+$, Ki-67$^+$, p-S6$^+$ cells gated on CD4$^+$Foxp3all$^+$ (full column) and CD4$^+$Foxp3E2$^+$ (empty column) in freshly isolated PBMCs from BC subjects (*n*=30). (**E**) Statistical analysis was performed by using Mann-Whitney *U*-test (two tails) (mean ± s.e.m.); *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

5. Percentage of Foxp3E2^{+} Treg cells correlates with tumor aggressiveness of tumor cells

In order to corroborate whether Foxp3E2 expression could represent a prognostic marker for BC progression, we evaluated the correlation between the percentage of Foxp3E2^{+} Treg cells with Ki-67 expression (one of the main parameters used for BC prognosis and aggressiveness) in BC cells. Correlation analysis revealed a direct and significant correlation between these parameters in TILs (**Figure 8A**) as well as in PBMCs (**Figure 8B**) from BC subjects. These results suggest that an increase of Foxp3E2 expression in Treg cells is associated with a more aggressive tumor.

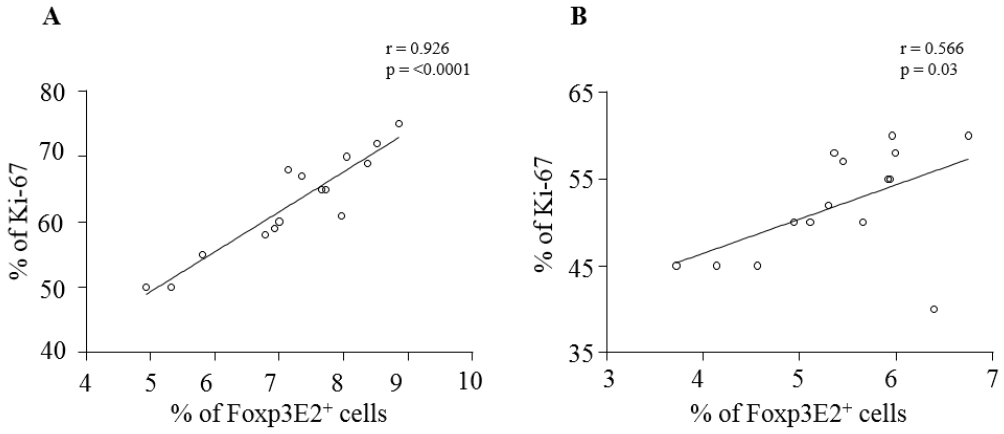

Figure 8. Correlations between Foxp3E2^{+}Treg cells and percentage of Ki-67^{+} tumor cells evaluated in TILs **(A)** and PBMCs **(B)** from BC subjects (n= 15); correlation has been computed using the nonparametric Spearman rank correlation.

6. Peripheral Treg cells from BC subjects display a stronger suppressive ability than Treg cells from healthy subjects

Next, we asked whether an increased frequency of Treg cells in peripheral blood from BC subjects was associated with their higher suppressive function *in vitro*. In particular, we assessed in *in vitro* co-culture experiments the ability of pTreg cells from BC and HS to suppress the proliferation of $CD4^+CD25^-$ T cells labelled with the division-tracking dye 5,6-carboxyfluorescein-diacetate-succinimidyl ester (CFSE). We found that pTreg cells from BC showed an increased suppressive ability on $CD4^+CD25^-$ T cells proliferation at all the different ratios analysed when compared to Treg cells obtained from HS (**Figure 9A** and **9B**). This data revealed that Treg cells from BC subjects not only show a higher immunosuppressive phenotype but also display a stronger suppressive activity, which is possibly due to their elevated expression of $Foxp3E2^+$ splicing variant.

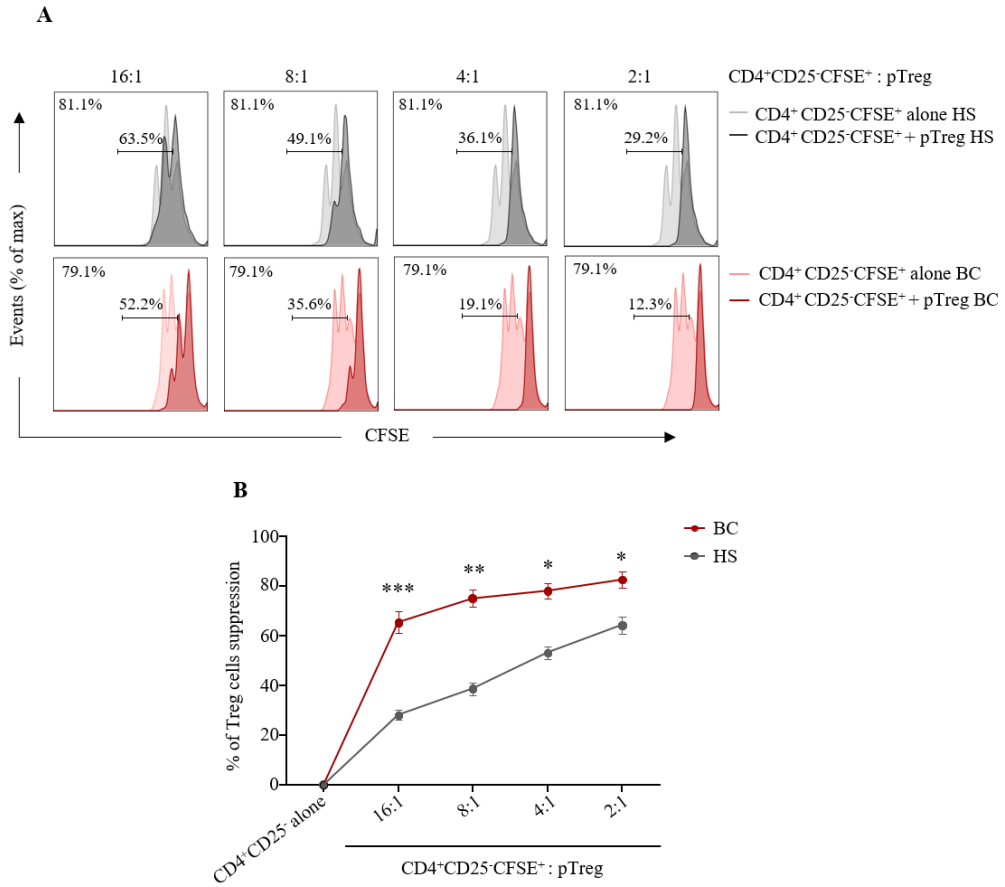

Figure 9. Increased suppressive capacity of Treg cells from BC subjects compared to HS. Proliferation of CFSE-labeled $CD4^+CD25^-$ cells from HS (*n*=30) and BC (*n*=30) stimulated for 72 hours with anti-CD3/anti-CD28 mAbs (0.2 bead per cell) alone or in the presence of pTreg cells from HS and BC subjects at a ratio of 16:1, 8:1, 4:1 and 2:1 (above plots), respectively. **(A)** Representative FACS plots of proliferation of CFSE-labeled $CD4^+CD25^-$ cells. Numbers in plots indicate percentage of CFSE dilution in $CD4^+CD25^-$ cells from HS and BC cultured alone (top left); numbers above bracketed lines indicate percentage of CFSE dilution in $CD4^+CD25^-$ cells cultured with pTreg cells from HS and BC subjects. **(B)** Percentage (%) of Treg cell suppression in HS (black line) and BC (red line) in above mentioned conditions. **(A, B)** Data are from thirty independent experiments. (**B**) Statistical analysis was performed by using Wilcoxon paired (two tails) (mean ± s.e.m.). $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Discussion

Breast cancer is a highly heterogeneous tumor and it is often induced by genetic and epigenetic changes in genes that regulate the main functions of the mammary epithelial cells. Several intrinsic tumor-suppressor mechanisms, that induce senescence or apoptosis of neoplastic cells, are involved in preventing tumor development and progression (d'Adda di Fagagna F, 2003; Pistritto G, 2016). Contextually, the immune system is recognized as an extrinsic tumor-suppressor mechanism that can eliminate transformed- epithelial breast cancer cells and limit their growth when they escape intrinsic tumor suppression process (Schreiber RD, 2011; Vesely MD, 2011). Indeed, several clinical studies have shown that a huge infiltration of TILs in several cancers correlates with a good clinical outcome and increased survival (Wang K, 2016; Nguyen N, 2016). On the other hand, since cancer originates and evolves within the context of self-tissues, the immune mechanisms of dominant and recessive tolerance to self-antigens operate concurrently to constrain the development of an anti-tumor adaptive immune response, previously described as "concomitant immunity" (North RJ, 1977; Di Giacomo A, 1986). The balance between antitumor immunity and tumor-promoting inflammation is under the control of $CD8^+$ and $CD4^+Foxp3^+$ T cell responses that significantly influence the clinical outcome, as they represent the main mediators of immune surveillance and immune tolerance, respectively. Thus, it is conceivable that a crosstalk among tumoral microenvironment, cancer cells and TILs exists, and it is able to either condition or promote tumor growth and development. Specifically, in BC, the presence or the absence of the Estrogen Receptors (ER) seems to correlate with different immune responses (West NR, 2013). Indeed, it has been shown that estrogen is able to convert $CD4^+CD25^-$ Tconv cells into $CD4^+CD25^+$ Treg cells, increasing the expression of Foxp3, thus expanding Treg cells both *in vivo* and *in vitro* (Tai P, 2008). In this study, we analysed the asset of adaptive immunity in benign and malignant lesions such as Breast Fibroadenoma (BF)

and Breast carcinoma (BC), respectively. We found that while in BF the main adaptive immune response is mediated by CD8 cytotoxic T cells, which are highly effective at surveilling transformed cells, in BC we found a prominent recruitment of Foxp3^{+} Treg cells that provide an advantage to tumor growth by inhibition of antitumor immune response in tumor-bearing hosts. Moreover, we found that in tumor microenvironment the vast majority of Treg cells express the Foxp3 splicing form containing exon2 (Foxp3E2) when compared to BF counterpart, in line with the finding showing that a E2 marks a Treg cell population with a highly efficient suppressive function (De Rosa V, 2015). Indeed, in the comparison between Foxp3E2^{+} Treg cells with Foxp3all^{+} Treg cells, we found a significant increase in the expression of the main suppressive markers only in the E2 subset from BC patients, while this increase was not evident in healthy subjects. These data suggested a selective induction of Foxp3E2-expressing Treg cells with enhanced suppressive activity only at the tumor site. In addition, Foxp3E2 Treg cells from BC display also an advanced proliferative rate and higher metabolic activity when compared to Foxp3E2 Treg cells from BF, as evidenced by their higher levels of Ki-67 and p-S6, respectively. The mechanism underlying Treg cell enrichment in the tumor milieu remains to be elucidated, but Treg cell trafficking from peripheral tissues to cancer tissue and *vice-versa* represents an important process to improve the immunosuppressive scenario characterizing tumor settings. Interestingly, we found that in addition to tumor setting, also in the peripheral blood an increased frequency of Foxp3E2^{+} Treg cells has been observed in BC subjects as compared to HS. The higher immunosuppressive phenotype of peripheral Foxp3E2 Treg cells from BC is confirmed by the increased expression of CTLA4 and PD1, as well as the higher proliferative rate and enhanced metabolic activity. Furthermore, both Foxp3all and Foxp3E2 Treg cells in peripheral blood from BC show an increased expression of migratory markers and cell adhesion molecules compared to HS. This could be probably due to the sustained production of cytokines/chemokines secreted by tumor

cells, which in turn can favour Treg cell recruitment. Interestingly, Treg cells both in tumor tissue and peripheral blood from BC subjects display a naïve phenotype, as evidenced by the high level of CD45RA expression, which has been previously shown to correlate with efficient suppressive activity of Treg cells (Seddiki N, 2006; Valmori D, 2005; Fritzsching, B., 2006). Existing evidence suggests that Treg cell depletion or interference with their suppressive mechanisms can result in enhanced anti-tumor immunity with a clear therapeutic potential (Selby MJ, 2013; Kurose K, 2015). The introduction of immunotherapy has revolutionized the treatment of several cancer types, shifting the focus from cytotoxic therapies toward treatments that can boost anti-tumor immune responses. To this end, the identification of new predictive biomarkers and cellular targets, such as Foxp3E2-expressing Treg cells, which could help to stratify patients and guide the therapeutic decision, is urgently needed. Furthermore, recent experimental studies have highlighted that even metabolic inhibitors can also have effects on Treg cells in cancer therapy. In this context, a recent study from our group has demonstrated the crucial role of glycolysis in the induction of human inducible Treg cells (iTreg), by modulating the expression of Foxp3E2 splicing variants, through the glycolytic enzyme enolase-1 (De Rosa V, 2015). In particular, it has been demonstrated that Foxp3-E2 was indispensable for the regulatory function of iTreg since iTreg cells generated in the presence of siRNA-E2 displayed less suppressive ability than iTreg cells generated in the presence of control siRNA with a scrambled sequence (siRNA-Scr). The reason why Treg cells expressing a specific Foxp3 isoform containing exon 2 (FOXPE2) have a greater suppressive capacity is still unknown and the possible molecular mechanism underlying this phenomenon is the subject of extensive scientific investigations. Thus, therapeutic strategies designed to target for example glycolytic metabolism, enolase-1 or Treg cells which express high levels of Foxp3E2 are an intense area of research, as they can directly impact on Treg cell function, thus sustaining tumor immune response. The pharmacological

action of glycolytic enzyme inhibitors, could therefore have a novel meaning in the light of their capability to “selectively” modulate Treg cell specific reprogramming in the tumor milieu.

Conclusions and future perspectives

Data produced in our laboratory has previously demonstrated a functional role of the exon 2 in the regulation of Foxp3-related suppressive function. Since increased Treg cell suppressive activity has been linked to a poor immunological response and represents a critical mechanism of immune evasion by tumors (Zhou Y, 2017; Liu C, 2019), the aim of this thesis was to evaluate whether increased immunosuppression observed in BC subjects with poor prognosis could be secondary to the induction of Treg cells with stronger suppressive activity, with specific Foxp3 splicing variants which account for stronger immunosuppressive function. In conclusion, our study suggests Foxp3E2 Treg cell population has a fundamental impact in immune suppressive context of breast cancer and could be considered a novel target for the depletion of tumor-resident Treg cells. These cells would therefore represent a promising tool for the immunotherapy. Moreover, we believe that the study of Foxp3E2^{+} Treg cells could be clinically relevant for BC management, with high numbers correlating with a poorer prognosis. This could be of great innovation for early decisions in BC therapy, correlating the frequency of Foxp3E2^{+} Tregs and clinico-pathological data including age, nodal status, tumour size, grade, and human epidermal growth factor receptor 2 (HER2) and estrogen receptor (ER) status. In the next future, we aim at understanding the precise molecular mechanisms that preferentially induce the Foxp3E2 splicing variant in the tumor context, through the analysis of the main processes that influence Treg cell development, homeostasis and trafficking.

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