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ROLE OF MIR-19A RELEASED BY INFLAMMATORY BREAST CANCER CELLS IN THE REGULATION OF DENDRITIC CELL FUNCTIONS: IN VITRO MODEL OF CROSSTALK IN THE TUMOR MICROENVIRONMENT OF INFLAMMATORY BREAST CANCER

Simone Anfossi

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By

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DEDICATION

… to mom, dad and the grandparents.

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ROLE OF MIR-19A RELEASED BY INFLAMMATORY BREAST CANCER CELLS IN THE REGULATION OF DENDRITIC CELL FUNCTIONS: *IN VITRO* MODEL OF CROSS-TALK IN THE TUMOR MICROENVIRONMENT OF INFLAMMATORY BREAST CANCER

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Inflammatory breast cancer (IBC) is a rare but very aggressive form of locally advanced breast cancer (1-6% of total breast cancer patients in United States), with a 5-year overall survival rate of only 40.5%, compared with 85% of the non-IBC patients. So far, a unique molecular signature for IBC able to explain the dramatic differences in the tumor biology between IBC and non-IBC has not been identified. As immune cells in the tumor microenvironment plays an important role in regulating tumor progression, we hypothesized that tumor-associated dendritic cells (TADC) may be responsible for regulating the development of the aggressive characteristics of IBC. MiRNAs can be released into the extracellular space and mediate the intercellular communication by regulating target gene expression beyond their cells of origin. We hypothesized that miRNAs released by IBC cells can induce an increased activation status, secretion of pro-inflammatory cytokines and migration ability of TADC. In an *in vitro* model of IBC tumor microenvironment, we found that the co-cultured of the IBC cell line SUM-149 with immature dendritic cells ($iDC^{SUM-149}$) induced a higher degree of activation and maturation of $iDC^{SUM-149}$ upon stimulation with

lipopolysaccharide (LPS) compared with iDCs co-cultured with the non-IBC cell line SUM-159 ($iDC^{SUM-159}$), resulting in: increased expression of the costimulatory and activation markers; higher production of pro-inflammatory cytokines (TNF-α, IL-6); and 3) higher migratory ability. These differences were due to the exosome-mediated transfer of miR-19a and miR-146a from SUM-149 and SUM-159, respectively, to iDCs, causing the downregulation of the miR-19a target genes PTEN, SOCS-1 and the miR-146a target genes IRAK1, TRAF6. PTEN, SOCS-1 and IRAK1, TRAF6 are important negative and positive regulator of cytokine- and TLR-mediated activation/maturation signaling pathway in DCs. Increased levels of IL-6 induced the upregulation of miR-19a synthesis in SUM-149 cells that was associated with the induction of $CD44^{+}CD24^{-}ALDH1^{+}$ cancer stem cells (CSCs) with epithelial-to-mesenchymal transition (EMT) characteristics.

In conclusion, in IBC tumor microenvironment IL-6/miR-19a axis can represent a self-sustaining loop able to maintain a pro-inflammatory status of DCs, leading to the development of tumor cells with high metastatic potential (EMT CSCs) responsible of the poor prognosis in IBC patients.

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INTRODUCTION

BACKGROUND ON BREAST CANCER

Over the past decades, the rates of death for breast cancer have seen a steady decrease, mainly in younger women. This is mostly due to the improvement in screening methods, the development of more effective and less toxic treatment and the progress in the earlier detection. Nevertheless, breast cancer still remains the second leading cause of death in women after lung cancer, with 232,340 estimated new cases (accounting for 29% of all new cancer cases among women) and 39,620 estimated deaths in 2013 in US (1, 2).

Breast cancer is a heterogeneous disease characterized by a wide spectrum of clinical and molecular factors that identify distinct biological subtypes. Based on the immunohistochemical (IHC) markers (the estrogen receptors: ER^+; progesterone receptor: PR^+; the amplification of the human epidermal growth factor receptor 2: $HER2^+$) and gene expression studies using the cDNA microarray, five major breast cancer subtypes have been identified: luminal A (ER^+ and/or PR^+, $HER2^-$, Ki-67<14%); luminal B (ER^+ and/or PR^+, $HER2^-$, Ki-67≥14% or ER^+ and/or PR^+, $HER2^+$); $HER2^+$ (ER^-, PR^-, $HER2^+$); basal-like (ER^-, PR^-, $HER2^-$ "triple-negative" breast cancer (TNBC) with cytokeratin (CK) 5/6 and/or 17 positive and/or epidermal growth factor receptor (EGFR) positive and normal breast-like tumors (3-5). As the majority of the TNBC (approximately 80%) breast cancer express basal-like genes, triple-negative and basal-like breast cancer are considered synonymous (6). The distinct subtypes are characterized by different biological, clinical and pathological features that account for different responsiveness to treatment and outcome. Particularly, the hormone receptors

positive breast cancers (ER^+ and/or PR^+, $HER2^-$) are the most common type of breast cancer, representing approximately 70% of invasive breast cancers (7) and have been associated with the most favorable prognosis. The $HER2^+$ breast cancers represent approximately 15-20% of invasive breast cancers and the overexpression of HER2 is associated with an increased aggressiveness and shorter survival (8). The TNBC represents the 12-17% of invasive breast cancers and is one of the most aggressive and lethal subtypes (3, 4, 9-11) characterized by high proliferative rate, genetic instability, high histological grade, and poor differentiation.

TRIPLE NEGATIVE BREAST CANCER

Compared with the other subtype, TNBC is a very heterogeneous disease and presents a higher degree of complexity, as it exhibits a wide spectrum of clonal genotypes (12) and encompasses additional molecular subtypes: the claudin-low subgroup, which are enriched in cells with stem cell ($CD44^+CD24^-$ and $ALDH1^+$) and epithelial-to-mesenchymal transition features; the interferon-rich subgroup characterized by better prognosis compared with other TNBC; and the normal-breast-like subgroup which may represent an artifact due to contamination of cell from normal epithelium (stromal and normal cells) (13). More than 75% of breast cancers positive for BRCA1 mutation have TN phenotype, while up to 70% of TNBC had overexpression of EGFR and more than 50% had alteration of PTEN expression (loss of heterozygosis, chromosomal instability, epigenetic silencing)(13-16). As TNBC do not express ER, PR and HER2 receptors, they cannot benefit form target therapy, like the tamoxifen and

trastuzumab for ER^+/PR^+ and $HER2^+$ breast cancer patients. TNBC are treated exclusively with conventional chemotherapy and there is no preferred standard for of chemotherapy. Although a minority of patients may achieve complete pathological response and excellent outcome, the majority of patients fail to achieve complete pathological response and their prognosis is poorer than patients of the other subtypes (17). As little is know on the pathogenesis of TNBC/basal-like, the elucidation of the molecular and biological mechanisms that drive this subtype of breast cancer will have an important impact in the development of new druggable targets.

BACKGROUND ON INFLAMMATORY BREAST CANCER

The inflammatory breast cancer (IBC) is a rare disease (~ 5% of all diagnosed breast cancer), but it is the most aggressive form of locally advanced breast cancer (LABC)(18). Contrary to the steady decreasing incidence of non-IBC breast cancer, IBC incidence continues to increase (19). IBC is characterized by fast progression, high invasiveness, highly angiogenic ability and lymphovascular invasion (LVI) (20) that accordingly confer to IBC an extremely high metastatic potential. Indeed, at diagnosis the majority of the patients present with advanced disease, with the involvement of axillary lymph nodes and distant metastases for ~35% of patients. IBC often shows high histological grade and negativity for hormone receptors (HR) and triple negative IBC are associated with poor prognosis (21). Despite the advances in the multidisciplinary treatments, IBC remains the most lethal type of LABC and the prognosis remain much less favorable with a 5-year overall survival rate of only 40.5%, compared with 85% of

the non-IBC patients (22). The diagnosis is mainly based on clinical and pathological criteria. Symptoms have characteristic features at presentation and include diffuse skin erythema (at least one-third of the breast) and oedema (more than two-thirds of the breast), peau d'orange, swelling, tenderness, induration and warmth in the breast. The pathological hallmark of IBC is represented by dermal lymphatic involvement with the presence of tumor emboli in the dermal lymphatic vessels, as a result of the high migratory and invasive capability. This is required by pathologist to confirm the clinical diagnosis (T4d according to the TNM staging criteria). However, in some instances, the clinical and pathologic findings may not concord for the diagnosis of IBC. Indeed, the dermal lymphatic invasion is identified in only 50-75% of IBC cases (23) and the changes of the skin may derive from acute mastitis or non-IBC advanced breast cancer. Therefore, the distinction from non-IBC may result difficult and this may lead to a misdiagnosis of IBC.

So far, there are no molecular diagnostic and prognostic biomarkers available for IBC and little is know about the molecular mechanism and genetic alterations that could explain the higher aggressiveness and poor prognosis of IBC compared with non-IBC. Their identification would help to improve the accuracy of diagnosis and develop new target therapy. Despite the effort to establish a molecular signature able to distinguish IBC from non-IBC, the results generated by several studies are poorly comparable and overlapping. Recently, a comprehensive analysis of three large datasets generated by the World IBC Consortium showed that a very small number of genes have a uniquely IBC-specific gene expression (24). This small difference in the gene expression profile might

not explain the difference in the behavior of tumor cells. Therefore, it is conceivable that the complex network of interactions among cells in the microenvironment may play a relevant role in determining the relevant differences in the degree of aggressiveness and clinical outcome of IBC compared non-IBC. In the tumor microenvironment, the cross-talk between cancer cells and tumor associated cells is particularly relevant in promoting tumor progression and metastasis formation.

Metastasis is the final step of cancer progression and represents the major cause of death by cancer. About 90% of the deaths of cancer patients are determined by development of metastases rather than primary disease (25). Important step in the process of metastasis formation is the acquisition of invasive and migratory ability by tumor cells that allows tumor cells to invade the surrounding tissue, gain access to the neovasculature and disseminate to distant organ. These two biological characteristics are particularly important in IBC, as they are responsible of the fast progression (26, 27). It is a traditional notion that tumor cells disseminate at later stage during the cancer development. However, recently, gene expression profiling studies have challenged this concept (28). Indeed, tumor cells capable to disseminate and give rise to metastases are already present at early stage of the disease, are genetically predetermined, and different from the other cells in the primary tumor (29, 30). Indeed, in a epidemiological analysis involving more than 12,000 breast cancer patients, it was reported that metastasis might have started already 5-7 years before the primary tumor diagnosis (31). In addition, the presence of disseminated tumor cells (DTCs) in the bone marrow of early stage cancer

patients without evident metastases supports the concept of early dissemination of tumor cells (32).

TUMOR MICROENVIRONMENT

Besides the genetic changes, also the paracrine cross-talk between cancer and bystander cells in the tumor microenvironment has been shown to play an important role at early stage in the regulation of tumor dissemination and promotion of metastasis formation (33-38). In fact, tumor is a heterogeneous population of cells consisting not only of cancer cells, but also endothelial cells, mesenchymal cells, fibroblasts and immune cells (33). All of these cell types are involved in the regulation tumor microenvironment homeostasis that is harnessed by tumor cancer cells to favor their own survival. Over the past years, more insights have been gained on the complex relationship between immune and tumor cells and on the role of immune system in the regulation of tumor development and progression (36, 39-41). In the tumor microenvironment immune cells paradoxically play two opposite roles in the regulation of tumor cell dissemination during the early stage of tumor progression. Indeed, they can have an anti-tumor activity by killing the developing cancer cells that start accumulating mutations and therefore they can prevent tumor cell dissemination (cancer immunosurveillance)(42-44). On the other hand, immune cells can contribute to tumor formation and progression by their chronic activation status and secretion of pro-inflammatory cytokines, like TNF-α, IL-1, IL-6, IL-8, TGF-β, VEGF, IFN-γ (chronic inflammation) (33, 36, 39, 45, 46). DCs play a central role in the regulation of tumor

microenvironment immune responses by orchestrating both the innate and adaptive arms of the immune systems. DCs reside in the tissue as immature dendritic cells (iDCs), characterized by low levels expression of the costimulatory (CD80, CD86, CD40) and activation/maturation (CD83, HLA-DR) surface markers and low secretion of cytokines. In this status, iDCs maintain the immunological homeostasis in the tissue. At early stage, when tumor cells start to develop and proliferate, two major scenarios can occur. First, tumor cells can induce activation of tumor-associated iDCs that subsequently upregulate the expression of CD80, CD86, CD40, CD83, HLA-DR and cytokine secretion leading to effective immune responses. Indeed, according to the pattern of cytokines that activated DCs secrete three different T cell-mediated responses can be induced: 1) IL-1, IL-6, TGF-β, IL-21 and IL-23 induce the differentiation of Th-17 cell that mediate pro-inflammatory responses (IL-17, IL-21, IL-22); 2) IL-12 and IL-18 induce the differentiation of Th1 cells that activate cell-mediated anti-tumor responses that are able to eradicate cancer cells (antigen-specific $CD8^+$ T cytotoxic lymphocytes: $CD8^+$ CTL; and natural killer cells: NK) by the secretion of IFN-γ and IL-2; and 3) IL-4 and IL-10 induce the differentiation of Th2 cells that inhibit the anti-tumor responses Th1-mediated by the secretion of IL-4, IL-5 and IL-13 (47, 48). In the second scenario, tumor cells may not induce activation of tumor-associated iDCs that remain in an immature status and therefore are not able to induce effective immune responses (Th-17, Th1 or Th2). On the contrary iDCs induce the differentiation of the immunosuppressive T regulatory (Treg) cells by TGF-β secretion leading to a general suppression of the immune cell function and therefore determining the tumor immune escape.

DCs can also drive a tumor promoting response directly by sustaining chronic inflammation by secretion of high levels of TNF-α, IL-1, IL-6 and recruiting polymorphonuclear leucocytes (PMNLs) by IL-8 (39, 40, 49). Furthermore, DCs can also contribute to tumor progression by promoting tumor angiogenesis (36, 50), supporting the development and expansion of breast cancer stem cells (BCSC) (33, 46, 51-54) and by activating T cells and macrophages (55) to secrete the EMT-inducing cytokines TGF-β, IL-6, TNF-α (56). EMT is a biological process in which epithelial cells lose their epithelial characteristics and acquire a mesenchymal phenotype along with increased migratory and invasive behavior. Particularly, epithelial cells lose the epithelial morphology, the intercellular adhesion molecule (E-cadherin), the epithelial marker (cytokeratin) and acquire the mesenchymal markers (fibronectin, N-cadherin, and vimentin), spindle-like morphology and increased motility, invasiveness and metastatic ability. It is know that the EMT process in involved not only in the induction of increased tumor migration and invasion during the metastatic process, but also in the generation of cancer cells with stem cell-like characteristics that acquire resistance to chemo- and radiotherapy (57).

On the other hand, tumor cells can negatively regulate the effector function of immune cells in the tumor microenvironment by developing multiple strategies able to elude the anti-tumor immune responses (42). These strategies include mechanisms able either to prevent and hinder the activation of an effective anti-tumor immune response (tumor escape) by the secretion of immunosuppressive cytokines (TGF-β and IL-10) or impair tumor recognition by immune effector cells through the downregulation of tumor

cell surface markers (tumor specific antigen, MHC class I molecules, immune-cell activatory/costimulatory ligands) and death-receptor signaling pathways (Fas, TNF receptors). Genetic and epigenetic alterations that occur at early stage of tumor development may drive the acquisition of resistance to immune cell-mediated detection and elimination of tumor cells resulting in tumor growth (immunoediting). Therefore, at early stage, tumor cells with high aggressive characteristics, like increased migratory, invasive ability and EMT/CSC-like phenotype may be induced by a pro-inflammatory tumor microenvironment, can be positively selected by immunoediting, can disseminate to distant organ by the acquisition of migratory, invasive ability and finally generate micrometastasis even before primary tumor can become clinically detectable.

We can conclude that at early stage, immune cells play an important role in the determining the fate of tumor cells in terms of tumor-promoting or anti-tumor immune responses.

CIRCULATING MIRNAs

In the tumor microenvironment there is a continuous crosstalk between tumor and tumor associated immune cells. This communication is mediated mainly by cytokines, growth factor and cell-to-cell interactions. However, recently it was reported that a new intercellular messenger can regulate the crosstalk: circulating microRNAs (miRNAs).

MiRNAs are small noncoding RNA molecules typically of 18-24 nucleotides in size with regulatory functions. They modulate the translation of specific target mRNAs by binding either to the complementary sequences in the 3' untranslated regions (3' UTR)

of mRNA or to the amino acid coding sequence (CDS) of the mRNA (58). This binding determines the inhibition of gene expression through either the degradation or the inhibition of translation by ribosomes or the localization to P-bodies of the target mRNA (59). It is estimated that in human cancers more than 50% of miRNAs are located in chromosomal regions subjected to genetic alterations (deletions, amplification, translocation and mutation) (60) and this determines an aberrant expression pattern in the tumor cells (61).

Recently, it was reported that miRNAs can be detected outside the cellular environment and in biological fluids (serum, plasma, urine, milk, pleural fluid and saliva), as circulating miRNAs (62, 63). They can be secreted by cells into the extracellular space as a highly stable form of cell-free nucleic acids in three different fashion: 1) encapsulated in exosomes; 2) associated with Argonaute2 (Ago2) protein forming extracellular Ago2-miRNA complexes; or 3) contained inside apoptotic bodies (64-69). Therefore, miRNAs released through these ways can resist degradation by RNase in the extracellular space and when taken up they can regulate target gene expression beyond their cell of origin (64, 70-72). Immune cells can use circulating miRNAs to communicate and mediated their functions. Indeed, T and B lymphocytes can exchange functional miRNAs bidirectionally (73); DCs can take up EBV-encoded miRNAs secreted by EBV-infected B cells that are able to downregulate the expression of the immunostimulatory gene CXCL11 in DCs (74); DCs communicate with neighboring DCs through the transfer of functional microRNAs (75); and finally, miRNAs can be transferred from the IL-4 alternatively activated (M2) tumor-associated

macrophages (TAMs) into breast cancer cells and promote invasion ability in recipient breast cancer cells (76).

Besides tumor cells, altered levels of miRNAs can be found also in the serum of cancer patients and their aberrant pattern may correlate with the dysregulated pattern of parental tumor (62, 63). However, as most of the circulating miRNAs originate from blood cells (77) it is conceivable to assume that serum miRNA pattern may reflect the overall pathological changes associated with the development of the disease with the contribution of both cancer and blood cells. Therefore, circulating miRNAs have a relevant potential use as clinical diagnostic and prognostic biomarkers (62, 63).

MIR-19A AND MIR-146A EXPRESSION IN BREAST CANCER AND DCs

MiR-146a and miR-19a are overexpressed in breast cancer and regulate the tumor progression. The expression of miR-146a is upregulated in the highly invasive MDA-231 and SUM-159 breast cancer cell lines, compared with the less-invasive MCF-7 cells (78). Furthermore, miR-146a was found to be highly expressed in triple negative and basal-like breast cancer cells and be responsible of BRCA1 downregulation (miR-146a target), explaining the low levels of BRCA1 in about 30% of sporadic breast cancer (79). MiR-146a is also upregulated in breast cancer cells that developed cisplatin-resistance (80).

MiR-19a belongs to the miR-17-92 cluster, composed by seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92-1 and was found to be overexpressed in breast (81, 82) and is the major player in the induction of

tumorigenesis (83). MiR-19a regulates tumor angiogenesis (84), induces c-Myc-mediated lymphomagenesis and decrease apoptosis by the suppression of PTEN (85, 86) and it is overexpressed in a mouse model of human breast cancer bone metastasis (82).

Besides tumor cells, miR-146a and miR-19a have regulatory functions also in DCs. Indeed, they can regulate the activation of iDCs by modulating the signaling pathways of TLR4 and CD40 upon binding with their ligands: lipopolysaccharide (LPS) and CD40L respectively. LPS in the major component of the gram-negative bacterial wall and is a strong activator of iDC to mount immune responses against bacterial infection. The binding of TLR4 with LPS induces the nuclear translocation of NF-kB and the subsequent activation of transcription of pro-inflammatory cytokines, like IL-1β, IL-6, and TNF-α (87). Also fatty acids can bind TLR4 inducing pro-inflammatory responses in the adipose tissue of obese mice and humans. This pathway is responsible of chronic inflammation in obese people leading to the development of insulin resistance (88, 89). This is particularly important in IBC patients where obesity were associated with shorter survival (90). CD40-CD40L interaction is the major activator/maturation signaling that occur in the lymph node between iDCs and $CD4^+$ T cells to develop adaptive immune responses (antigen-specific). This is the central pathway of iDC activation for the induction of the antigen-specific cell-mediated anti-tumor responses.

MiR-146a regulates iDC activation/maturation by regulating TLR4 and CD40 signaling. Particularly miR-146a can inhibit TLR4 signaling by targeting IRAK1 and TRAF6 (91). IRAK1 and TRAF6 are two adaptor proteins activated during TLR4 signaling. TRAF6 is also a component of the signaling cascade in DC40 pathways (92)

The downregulation of IRAK1 and TRAF6 impairs DC maturation induced by TLR4 and CD40-mediated signaling and important functions, like cytokine production and T cell activation (91, 92). MiR-19a can regulate PI3K/Akt-mediate signaling upon the binding of LPS and CD40L to TLR4 and CD40 respectively, by the downregulation of the phosphatase and tensin homologue (PTEN) a potent inhibitor of PI3K/Akt signaling (93, 94). PI3K/Akt signaling plays an important role in promoting survival, migration and maturation of DCs (95, 96). Furthermore, miR-19a downregulates the expression of SOCS-1 an important negative regulator of IL-6, IL-8 and TLR4 signaling (93, 97, 98).

RESEARCH OBJECTIVE

OVERALL GOAL

The overall goal of this research project is understand the role of DCs in the tumor microenvironment of IBC. Particularly, we wanted to assess how the cross-talk between IBC tumor cells and DCs can induce a pro-inflammatory microenvironment responsible of the development of tumor cell with aggressive behavior that determine the worse clinical outcome of IBC patients.

CRITICAL OBSERVATION AND CRITICAL QUESTION

A comprehensive analysis of three large gene expression profile datasets (World IBC Consortium) could not establish a unique and definitive molecular signature able to distinguish IBC from non-IBC and explain the higher aggressiveness and poor prognosis of IBC. Therefore, we asked if the immune cells present in the IBC tumor microenvironment can play a role in determining such differences.

CENTRAL HYPOTHESIS

- As circulating miRNAs can mediate intercellular communication regulating the gene expression of recipient cells
- As DCs are the master regulator of immune responses in the tumor microenvironment and can secrete pro-inflammatory cytokines able to induce the development of tumor cells with aggressive characteristics (EMT/CSC-like phenotype), we hypothesized that:

in the IBC tumor microenvironment miRNAs released by IBC cells can be taken up by tumor-associated iDCs and can induce higher levels of activation/maturation of iDCs upon stimulation with LPS compared with non-IBC cells leading to increased synthesis of pro-inflammatory cytokines.

SPECIFIC AIMS

Three specific aims are proposed:

1. show that miRNAs released by breast cancer cells can be taken up by iDCs
2. show that the taken up miRNAs can regulate DC status and functions:
    a. upregulation of the expression costimulation/activation (CD80, CD86, CD40) and maturation (CD83, HLA-DR) surface markers
    b. increase in migration ability
    c. increased synthesis of pro-inflammatory cytokines (IL-6, TNF-α).
3. show that pro-inflammatory secreted by DCs induce tumor cell aggressive phenotype:
    a. cancer stem cell-like (CSCs) phenotype
    b. epithelial-to-mesenchymal transition phenotype

Particularly, in an *in* vitro model of tumor microenvironment, we wanted to assess if miR-19a and miR-146a are differentially expressed and released by breast cancer tumor cells (IBC vs non-IBC), and if they play a different roles in the regulation of DC activation/maturation and functions (cytokine synthesis and migratory ability).

Chapter 1:

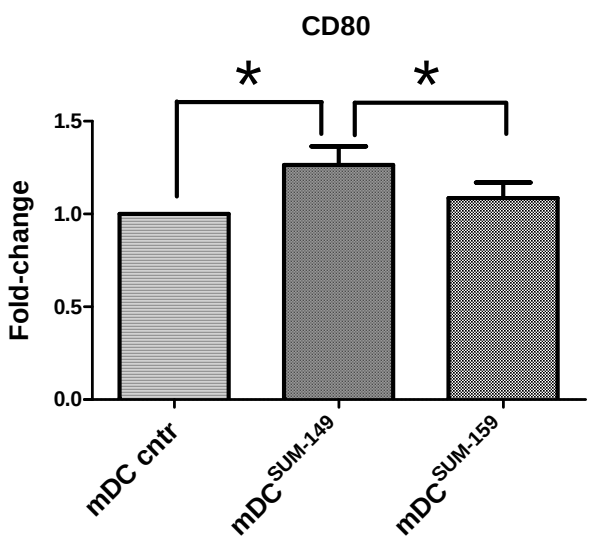
Effect of the co-culture with SUM-149 and SUM-159 breast cancer cell lines on activation and maturation of DCs upon LPS stimulation

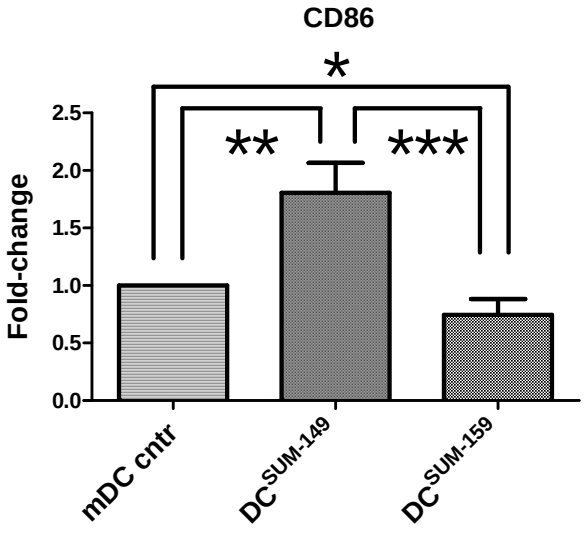
SUM-149 and SUM-159 have different effects on DC functions: modulation of the expression levels of costimulatory/activation and maturation markers upon DC maturation with LPS.

To test this hypothesis an *in vitro* model of tumor microenvironment was set up. In order to evaluate the effect of soluble factors on DC function, transwell plate with a membrane of 1.0 μm pore size was used. The size of the pores were big enough to allow soluble factors to flow between the two chambers (upper and lower chamber), but small enough to prevents tumor cells to migrate across the membrane and to establish a cell-to-cell contact with DCs. Breast cancer cell lines were plated in the upper chamber and the immature dendritic cells (iDCs) in the lower chamber.

The expression levels of the costimulatory/activation markers (CD80, CD86, CD40) and maturation markers (CD83, HLA-DR) are upregulated upon stimulation with LPS during the maturation process from immature (iDCs) to mature DCs (mDCs). The evaluation of the expression levels of these surface markers is generally used to evaluate the degree of activation and maturation of mDCs.

After 24 h of co-culture with either SUM-149 or SUM-159, iDCs were activated with LPS 100 ng/ml for 18 h to induce maturation. When iDCs were previously co-cultured with SUM-149 ($iDC^{SUM\text{-}149}$), there was a significant increased upregulation, after maturation with LPS, in the expression levels of all the costimulatory/activation and maturation markers of $mDC^{SUM\text{-}149}$ compared with those of control mDCs cultured in RPMI 10% only (Fig.1).


CD80
Fold-change
1.5
1.0
0.5
0.0
*
*
mDC cntr
mDCSUM-149
mDCSUM-159


CD86
Fold-change
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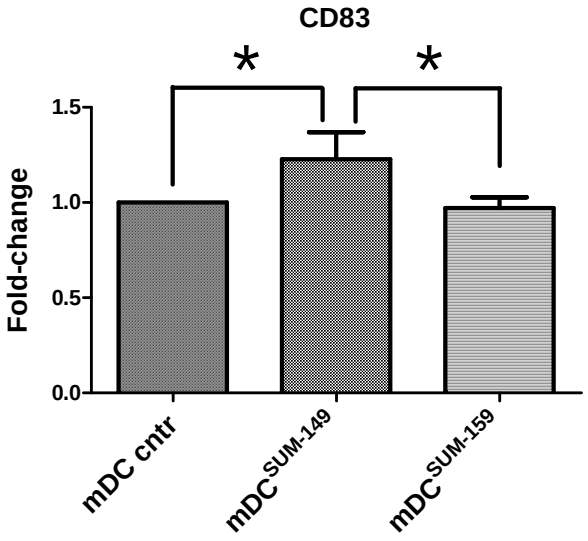
2.0
1.5
1.0
0.5
0.0
*
**
***
mDC cntr
DCSUM-149
DCSUM-159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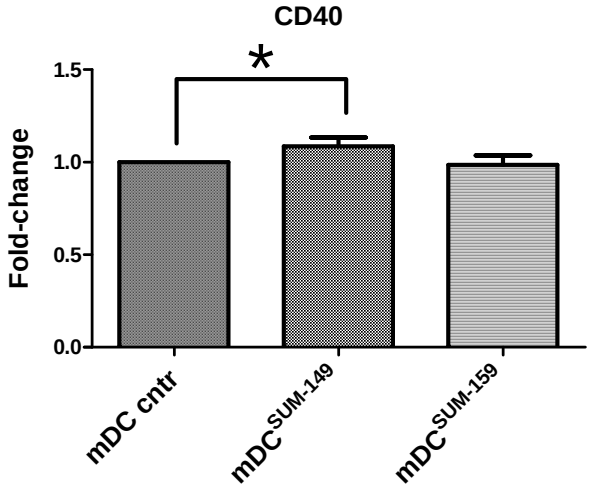
1.0
0.5
0.0
*
*
mDC cntr
mDCSUM-149
mDCSUM-159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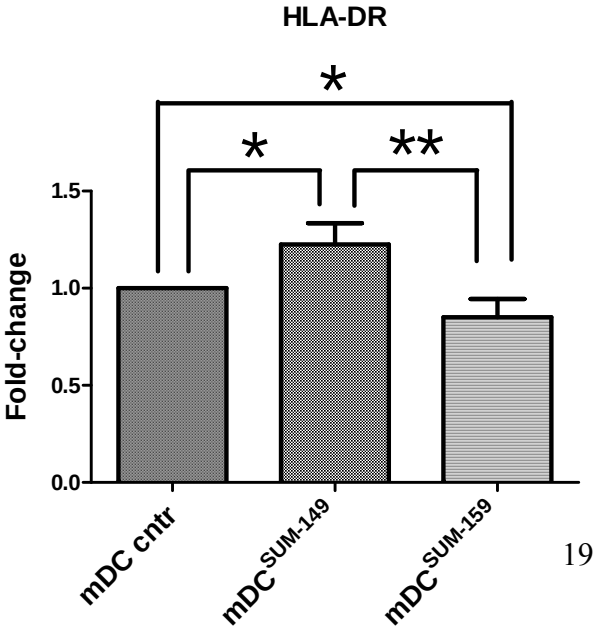
1.0
0.5
0.0
*
mDC cntr
mDCSUM-149
mDCSUM-159


HLA-DR
Fold-change
1.5
1.0
0.5
0.0
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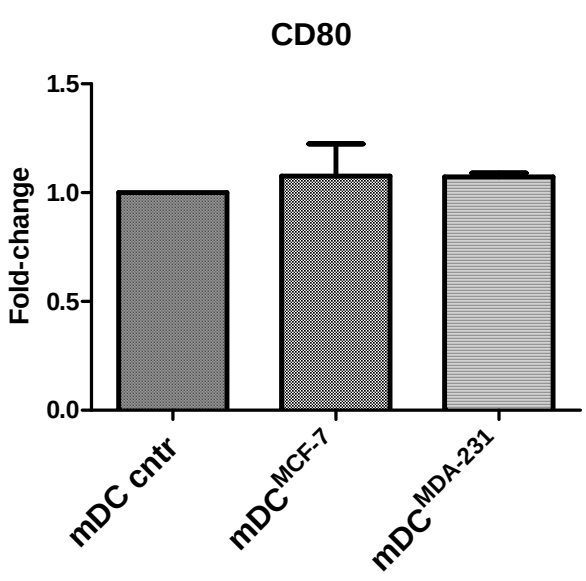
mDC cntr
mDCSUM-149
mDCSUM-159

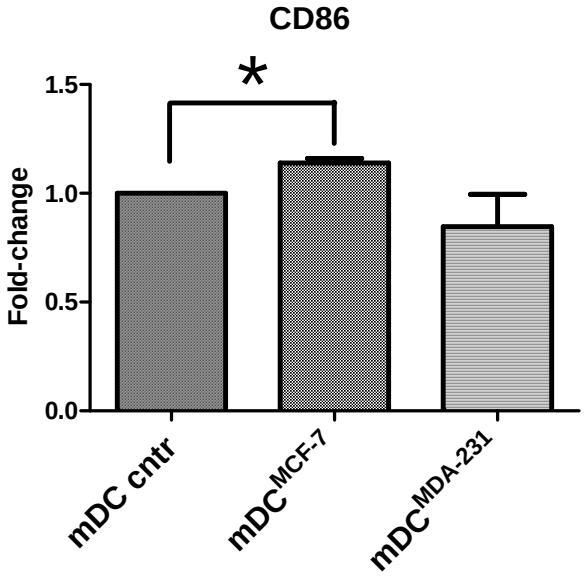
Fig. 1. Effect of co-culture with SUM-149 and SUM-159 cells on the expression levels of costimulatory/activation and maturation markers of DCs upon stimulation with LPS

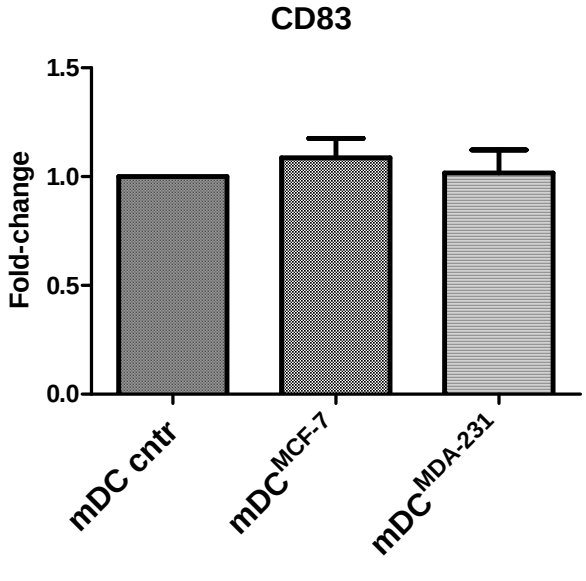
Analysis of the upregulation (fold-changes) of the costimulatory/activation (CD80, CD86, CD40) and maturation (CD83 and HLA-DR) marker expression levels of mDCs (mature DCs) previously co-cultured with SUM-149 ($mDC^{SUM-149}$) and SUM-159 ($mDC^{SUM-159}$) compared with control mDCs (cultured in RPIM 10% FBS) upon LPS maturation. The results represent the mean ± standard deviation of 4 independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

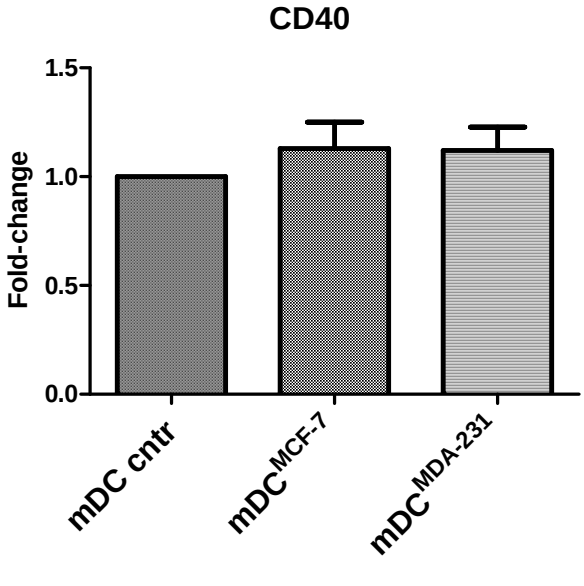
On the contrary, when iDCs were previously co-cultured with SUM-159 (iDC $^{SUM-159}$), mDC$^{SUM-159}$ showed a reduced upregulation, after maturation with LPS, in the expression levels of both CD86 and HLA-DR compared with control mDCs cultured in RPMI 10% only (Fig.1); while there was not any significant effect on the other surface markers (CD80, CD83, CD40). Furthermore, there were also significant differences in the upregulation of CD80, CD86, CD83, and HLA-DR of mDC$^{SUM-149}$ compared with mDC$^{SUM-159}$. This may imply that soluble factors released by SUM-149, during the co-culture, affected the ability of iDCs to be activated by LPS, inducing a higher degree of activation and maturation. This effect has a relevant consequence on the ability of mDC to activate T cells, in particularly because the upregulation of CD80, CD86 and HLA-DR is essential for effective activation of $CD4^+$ and $CD8^+$ T lymphocytes by mature DCs (mDCs) during immune responses. Indeed, the lack of CD80, CD86 and HLA-DR upregulation can hamper the full activation of $CD4^+$ and $CD8^+$ T lymphocytes and induce a suppressed status called anergy. This is one of the major ways by which the immune responses are negatively regulated by tumor cells in order to escape by the attack of the anti-tumor responses.

Other breast cancer cell lines were tested for also. No significant differences were measured in the degree of upregulation of surface costimulatory/activation markers compared with control mDCs, when iDCs were previously co-cultured with the ER/PR^+ MCF-7 cell line (iDC^{MCF-7}) and the TN MDA-231 cell line (iDC$^{MDA-231}$), except for the CD86 of mDC^{MCF-7} (Fig.2).


CD80
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^MCF-7
mDC^MDA-231


CD86
*
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^MCF-7
mDC^MDA-231


CD83
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^MCF-7
mDC^MDA-231


CD40
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^MCF-7
mDC^MDA-231

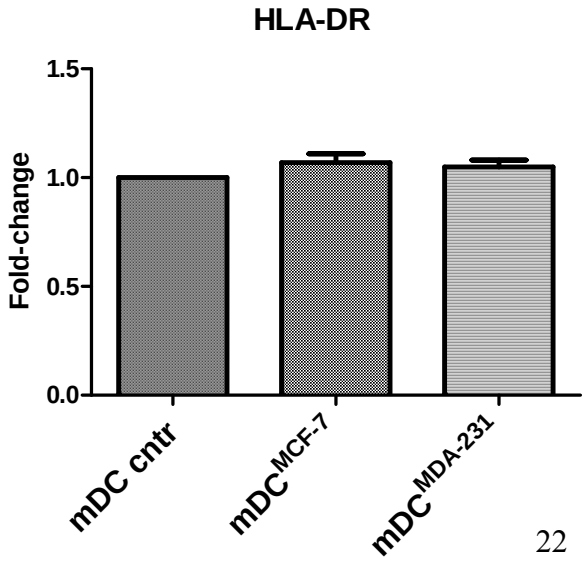

HLA-DR
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^MCF-7
mDC^MDA-231

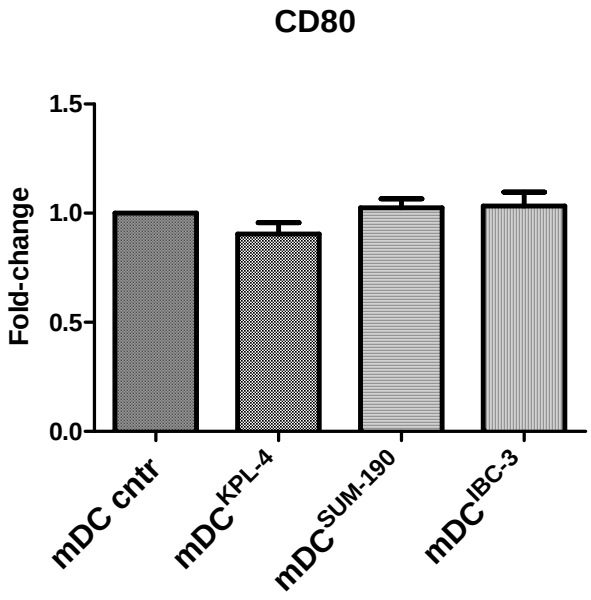
Fig. 2. Effect of co-culture with MCF-7 and MDA-231 cells on the expression levels of costimulatory/activation and maturation markers of DCs upon stimulation with LPS

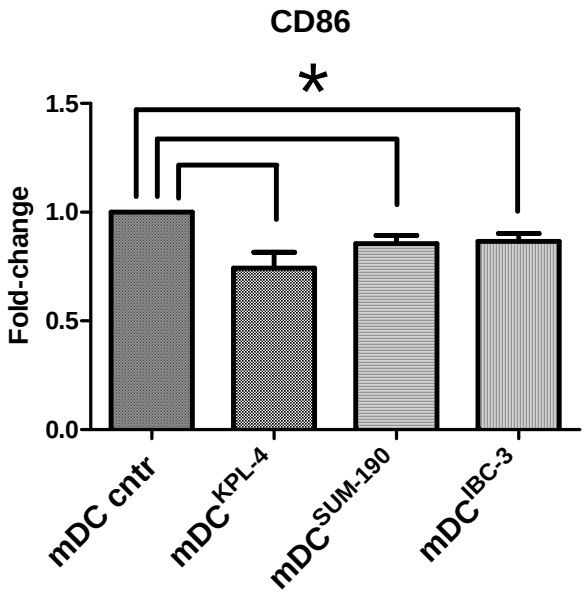
Analysis of the upregulation (fold-changes) of the costimulatory/activation (CD80, CD86, CD40) and maturation (CD83 and HLA-DR) marker expression levels of mDCs (mature DCs) previously co-cultured with MCF-7 (mDC^{MC-7}) and MDA-231 ($mDC^{MDA-231}$) compared with control mDCs (cultured in RPIM 10% FBS) upon LPS maturation. The results represent the mean ± standard deviation of 3 independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

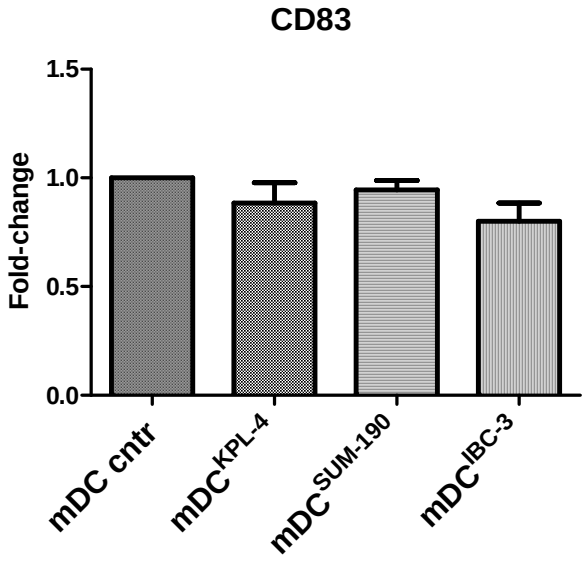
As SUM-149 is a TN IBC cell line, while SUM-159 is a TN non-IBC cell line we evaluated if the effect on DC activation and maturation was associated to the IBC type. Therefore, additional IBC cell lines were tested: KPL-4 ($HER2^+$ IBC), SUM-190 ($HER2^+$ IBC) and IBC-3 ($HER2^+$ IBC). There were not significant differences in the degree of upregulation of CD80, CD83 and HLA-DR after LPS maturation, when iDCs were previously co-cultured with KPL-4 (iDC^{KPL-4}), SUM-190 ($iDC^{SUM-190}$) and IBC-3 (iDC^{IBC-3}) (Fig.3). However, mDC^{KPL-4}, $mDC^{SUM-190}$, and mDC^{IBC-3} showed a reduced degree in the upregulation of CD86 compared with control mDCs and mDC^{IBC-3} showed a reduced degree in the upregulation of CD40 compared with control mDCs. As KPL-4, SUM-190, and IBC-3 are $HER2^+$ IBC cell lines and cannot affect the degree of iDC activation and maturation by LPS stimulation, it can be hypothesized that the ability to induce an increased activation and maturation of iDCs by LPS might be related to the triple negative receptor phenotype of SUM-149. Indeed, it seems that the $HER2^+$ IBC phenotype did not have the same effect on iDCs; rather $HER2^+$ IBC phenotype had a slightly immunosuppressive effect (Fig.3). It could also be hypothesized that the different effect might be related to specific characteristics of SUM-149, like cytokine and miRNA expression pattern.

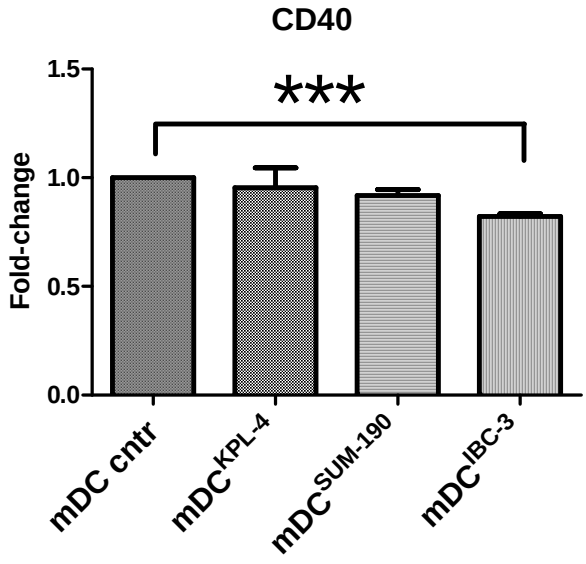
As the SUM-159 SUM149 cell lines had the most marked differences in affecting the degree of activation and maturation of iDCs and they might represent two good cellular models to understand the differences between IBC and non-IBC tumor microenvironment. Therefore, they were considered for further analyses. However, as SUM-149 and SUM-159 are triple negative cell lines, it should be pointed out that they

are not representative of all IBC and non-IBC respectively, rather they can be considered a model for the study of a subset of tumor microenvironment.


CD80
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^KPL-4
mDC^SUM-190
mDC^IBC-3


CD86
*
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^KPL-4
mDC^SUM-190
mDC^IBC-3


CD83
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^KPL-4
mDC^SUM-190
mDC^IBC-3


CD40
***
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^KPL-4
mDC^SUM-190
mDC^IBC-3

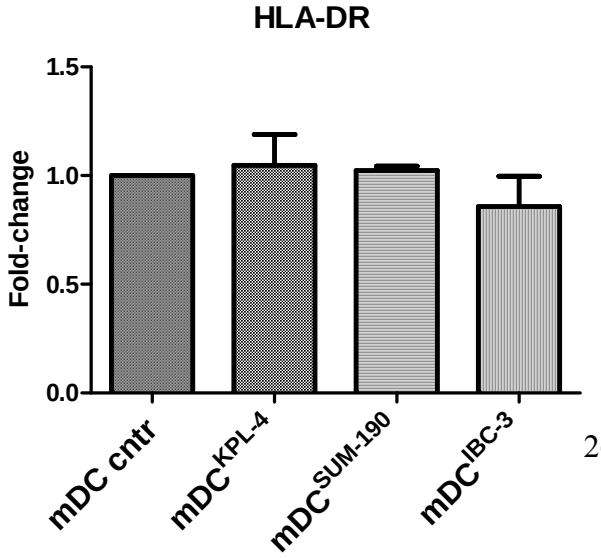

HLA-DR
Fold-change
1.5
1.0
0.5
0.0
mDC cntr
mDC^KPL-4
mDC^SUM-190
mDC^IBC-3

Fig. 3. Effect of co-culture with KPL-4, SUM-190 and IBC-3 cells on the expression levels of costimulatory/activation and maturation markers of DCs upon stimulation with LPS

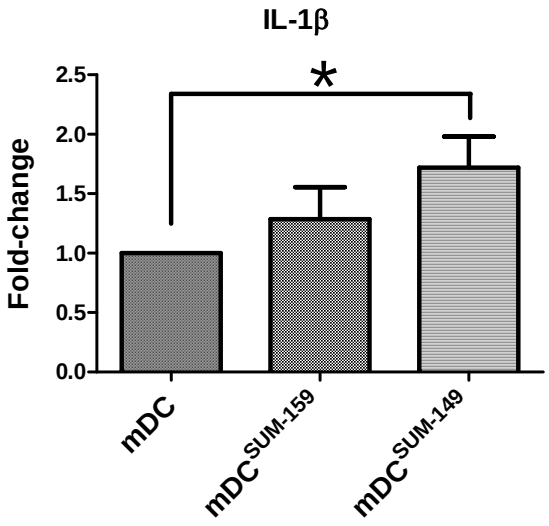
Analysis of the upregulation (fold-changes) of the costimulatory/activation (CD80, CD86, CD40) and maturation (CD83 and HLA-DR) marker expression levels of mDCs (mature DCs) previously co-cultured with KPL-4 (mDC^{KPL-4}), SUM-190 ($mDC^{SUM-190}$), and IBC-3 (mDC^{IBC-3}) compared with control mDCs (cultured in RPIM 10% FBS) upon LPS maturation. The results represent the mean ± standard deviation of 4 independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

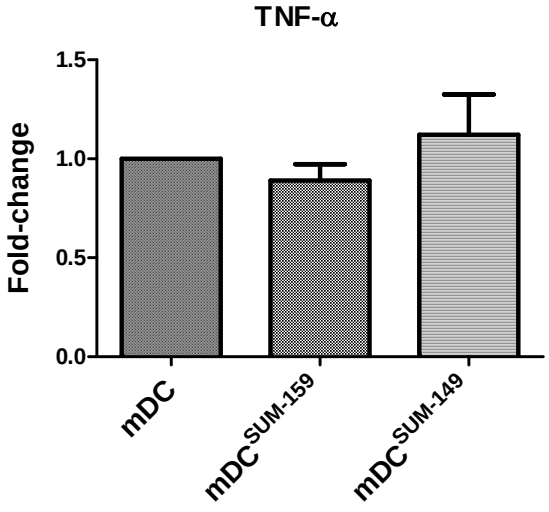
Chapter 2:

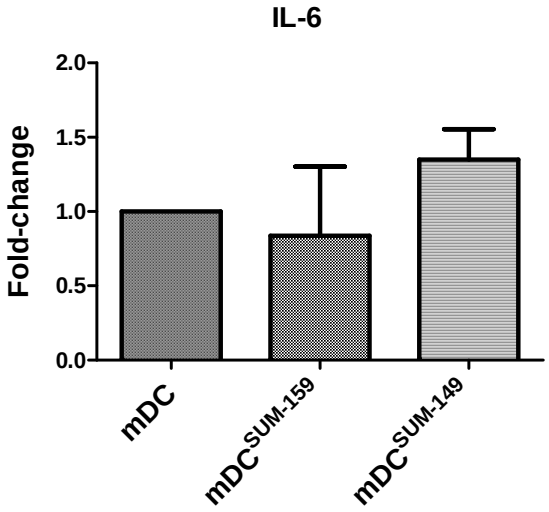
Effect of the co-culture with SUM-149 and SUM-159 breast cancer cell lines on cytokine secretion by DCs

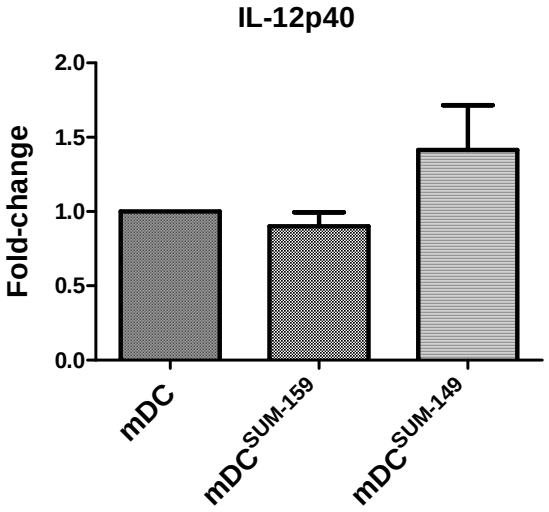
SUM-149 and SUM-159 have different effects on on DC functions: cytokine secretion by mDC$^{SUM-149}$ and mDC$^{SUM-159}$

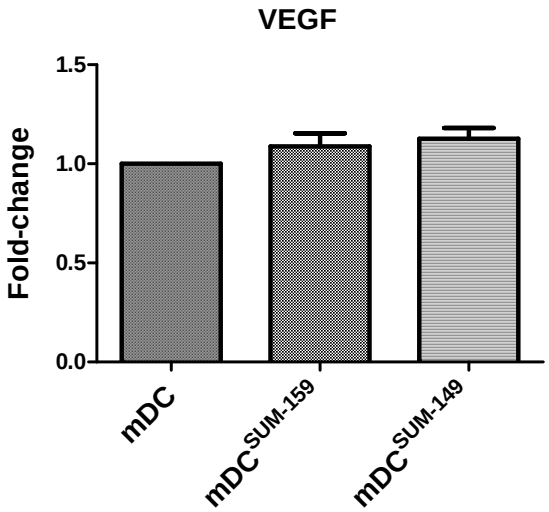
Besides the regulation of the expression levels of the surface costimulatory/activation and maturation markers of mDC$^{SUM-149}$ and mDC$^{SUM-159}$, the effect of breast cancer cell-iDC co-culture was also evaluated on another important function of mDCs: the production of cytokines. After 24 h of co-culture with SUM-149 and SUM-159, iDCs were stimulated with LPS 100 ng/ml to induce the synthesis and secretion of cytokines. Accordingly with the higher degree of maturation, mDC$^{SUM-149}$ produced higher levels of cytokines. Particularly, mDC$^{SUM-149}$ had a more marked pro-inflammatory profile of cytokine production than mDC$^{SUM-159}$. Indeed, mDC$^{SUM-149}$ secreted higher levels of IL-1β, TNF-α, IL-6 and IL-12p40 than mDC$^{SUM-159}$. Furthermore, mDC$^{SUM-149}$ secreted slightly higher levels of cytokine involved in angiogenesis (VEGF and IL-8) than mDC$^{SUM-159}$ (Fig.4).


IL-1β
*
Fold-change
2.5
2.0
1.5
1.0
0.5
0.0
mDC
mDCSUM-159
mDCSUM-149


TNF-α
Fold-change
1.5
1.0
0.5
0.0
mDC
mDCSUM-159
mDCSUM-149


IL-6
Fold-change
2.0
1.5
1.0
0.5
0.0
mDC
mDCSUM-159
mDCSUM-149


IL-12p40
Fold-change
2.0
1.5
1.0
0.5
0.0
mDC
mDCSUM-159
mDCSUM-149


VEGF
Fold-change
1.5
1.0
0.5
0.0
mDC
mDCSUM-159
mDCSUM-149

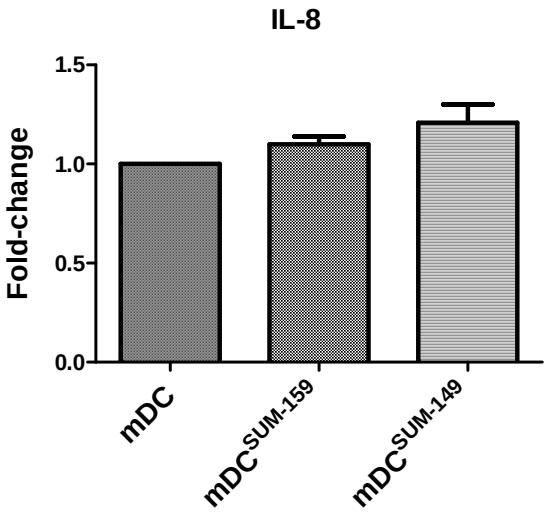

IL-8
Fold-change
1.5
1.0
0.5
0.0
mDC
mDCSUM-159
mDCSUM-149

Fig. 4. Effect of co-culture with SUM-149 and SUM-159 cells on cytokine secretion by $mDC^{SUM-149}$ and $mDC^{SUM-159}$

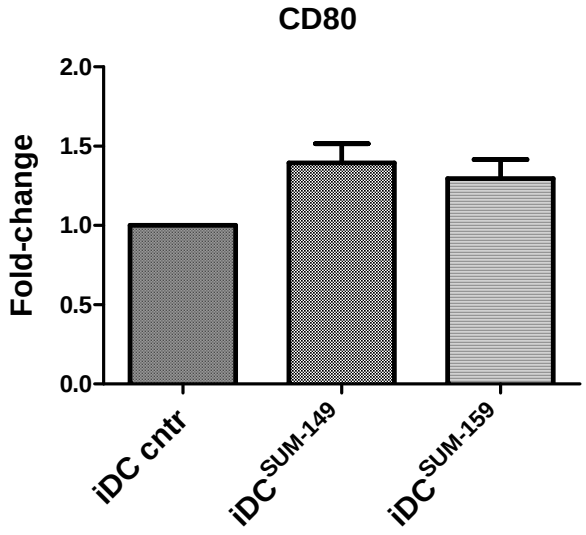
Analysis of cytokine levels in the supernatant of mDCs (mature DCs) previously co-cultured with SUM-149 ($mDC^{SUM-149}$) and SUM-159 ($mDC^{SUM-159}$) compared with control mDCs (cultured in RPIM 10% FBS). The results represent the mean ± standard deviation of 3 independent experiments except for IL-6 where the data shown are from tow experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

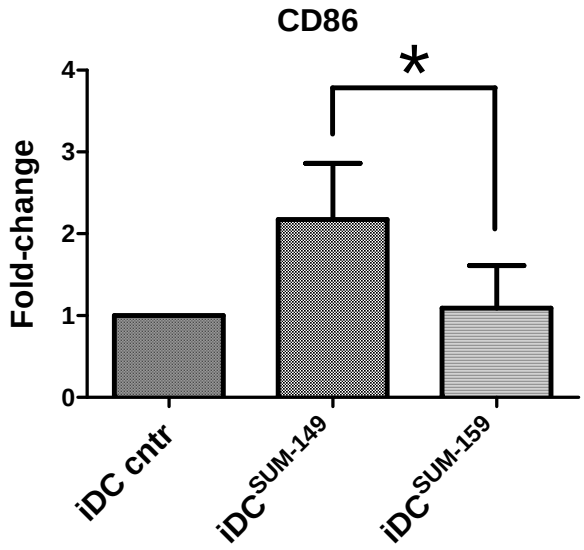
Chapter 3:

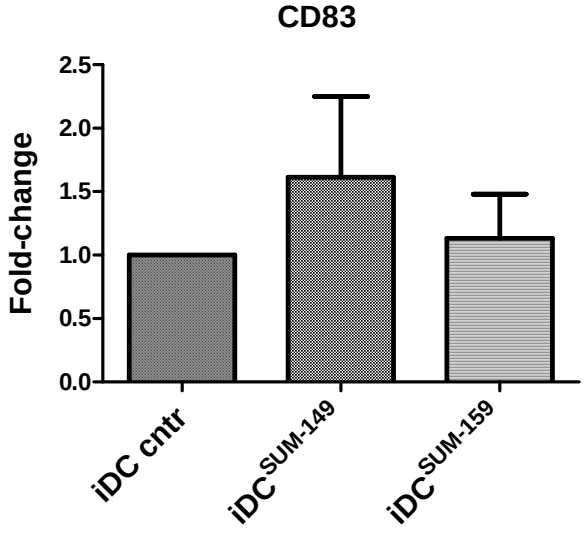
Effect of the co-culture with SUM-149 and SUM-159 breast cancer cell lines on the expression levels of costimulatory/activation and maturation markers of iDCs

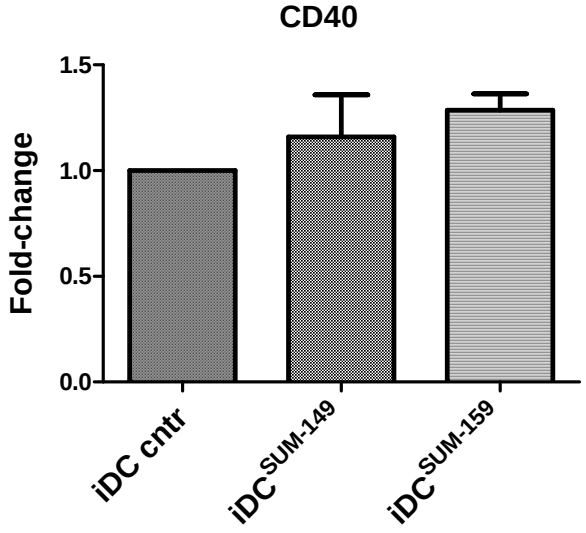
SUM-149 and SUM-159 have different effects on DC functions: modulation of the expression levels of costimulatory/activation and maturation status of iDCs

In order to assess if just the co-culture with SUM-149 and SUM-19 could induce changes in the degree of costimulatory/activation and maturation status of iDCs, we evaluated the expression pattern of the costimulatory/activation and maturation surface markers after 24 of co-culture of iDCs without the stimulation with LPS. We found that just the co-culture with SUM-149 determined a general increase of the expression levels of the surface markers in $iDC^{SUM-149}$ compared with control iDCs. Particularly, $iDC^{SUM-149}$ had higher expression levels of CD80, CD86, CD83 and HLA-DR compared with control iDCs and $iDC^{SUM-159}$, while $iDC^{SUM-159}$ had higher expression levels of CD80 and CD40 and lower expression levels of HLA-DR compared with control iDCs (Fig.5). This may mean that even without maturation with LPS, SUM-149 cells were able to induce a higher degree of activation of iDCs compared with $iDC^{SUM-159\ and}$ the control iDCs.


CD80
Fold-change
2.0
1.5
1.0
0.5
0.0
iDC cntr
iDCSUM-149
iDCSUM-159


CD86
*
Fold-change
4
3
2
1
0
iDC cntr
iDCSUM-149
iDCSUM-159


CD83
Fold-change
2.5
2.0
1.5
1.0
0.5
0.0
iDC cntr
iDCSUM-149
iDCSUM-159


CD40
Fold-change
1.5
1.0
0.5
0.0
iDC cntr
iDCSUM-149
iDCSUM-159

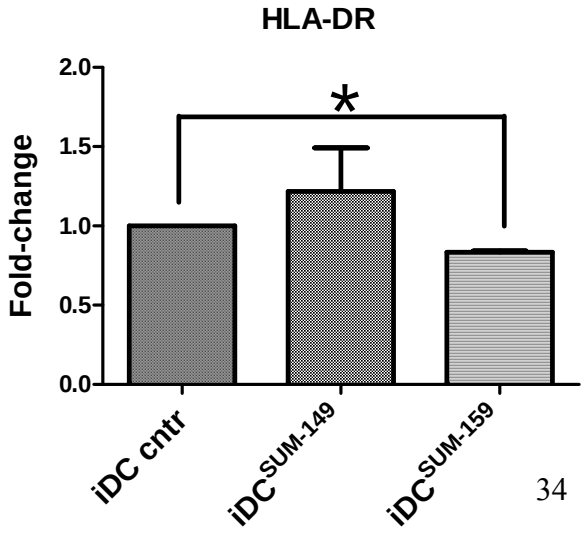

HLA-DR
*
Fold-change
2.0
1.5
1.0
0.5
0.0
iDC cntr
iDCSUM-149
iDCSUM-159

Fig. 5. Effect of co-culture with SUM-149 and SUM-159 cells on the expression levels of costimulatory/activation and maturation markers of iDCs

Analysis of the upregulation (fold-changes) of the costimulatory/activation (CD80, CD86, CD40) and maturation (CD83 and HLA-DR) marker expression levels of iDCs co-cultured with SUM-149 ($mDC^{SUM-149}$) and SUM-159 ($mDC^{SUM-159}$) compared with control iDCs (cultured in RPIM 10% FBS) without LPS maturation. The results represent the mean ± standard deviation of 2 independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

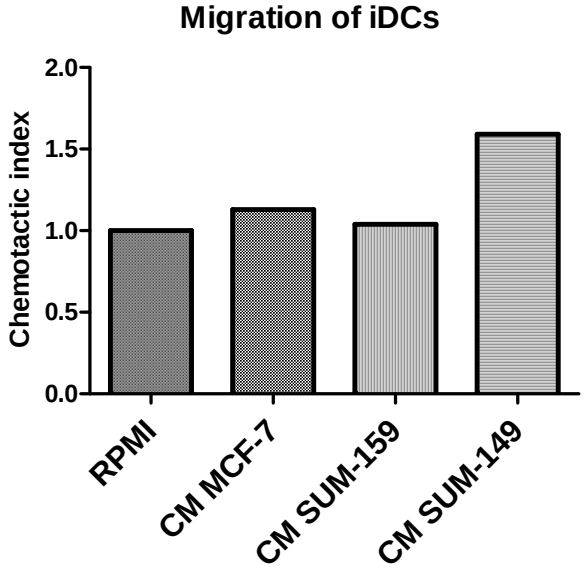
Chapter 4:

Effect of the co-culture with SUM-149 and SUM-159 breast cancer cell lines on migratory ability of DCs

SUM-149 and SUM-159 have different effect on DC functions: migratory ability

An important function of DCs in controlling the immune responses is their ability to migrate in the tissue. To evaluate their migratory ability, a transwell plate with a pore size of 8.0 μm was used. This pore size allows evaluating active migration by DCs. iDCs were plated in the upper chamber of the transwell and conditioned media from MCF-7 (CM MCF-7), SUM-149 (CM SUM-149) and SUM-159 (CM SUM-159) were used as migration stimuli and loaded in the lower chamber. The CM SUM-149 induced higher rate of migration of iDCs, compared with CM MCF-7, CM SUM-159 medium and RMPI 10% alone (Fig.6a). Then, the effect of co-culturing iDCs with MCF-7, SUM-149 and SUM-159 on migratory ability was evaluated. After 48 h of co-culture, iDC^{MCF-7} and $iDC^{SUM-159}$ showed lower migratory ability compared with control, while $iDC^{SUM-149}$ showed a higher migratory ability compared with control iDCs. Furthermore, $iDC^{SUM-149}$ showed a higher migratory ability compared with $iDC^{SUM-159}$ (Fig.6b). Therefore, SUM-149 cells secreted chemotactic factors able to stimulate higher migration of iDCs compared with MCF-7 and SUM-159 and the co-culture of iDCs with breast cancer cell line further increased these differences. These results confirmed that SUM-149 cells can increase the degree of activation of iDCs and their functions. It can be hypothesized that, in the tumor microenvironment, TN IBC cells can attract iDCs to the tumor site and induce an upregulation of their functions.

a


Migration of iDCs
Chemotactic index
2.0
1.5
1.0
0.5
0.0
RPMI
CM MCF-7
CM SUM-159
CM SUM-149

b

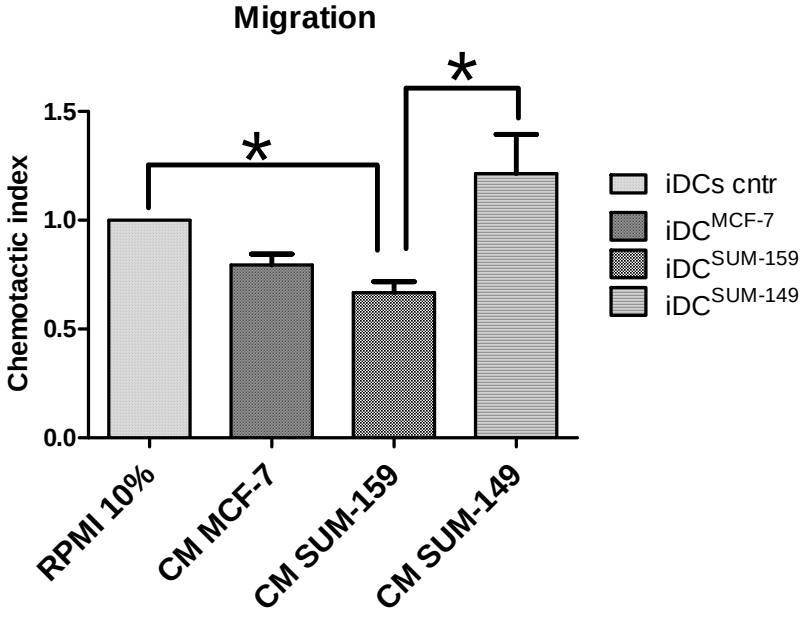

Migration
Chemotactic index
1.5
1.0
0.5
0.0
*
*
RPMI 10%
CM MCF-7
CM SUM-159
CM SUM-149
iDCs cntr
iDCMCF-7
iDCSUM-159
iDCSUM-149

Fig. 6. Effect of co-culture with SUM-149 and SUM-159 cells on migratory ability of DCs

Migration ability of iDCs (a) and, $iDC^{MCF\text{-}7}$, $iDC^{SUM\text{-}159}$ and $iDC^{SUM\text{-}149}$ (b) using conditioned media from MCF-7 (CM MCF-7), SUM-149 (CM SUM-159) and SUM-149 (CM SUM-149). In (a) it is shown the most representative experiment; in (b) the results represent the mean ± standard deviation of one experiment performed in triplicate (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Chapter 5:

Transfer of miRNAs from SUM-149 and SUM-159 breast cancer cells to iDCs

MiRNAs can be transferred from SUM-149 and SUM-159 to iDCs

As miRNAs can be secreted into extracellular space as circulating miRNAs in a very stable form, are functional and can mediate intercellular communication, we hypothesized that circulating miRNAs can also traffic between breast cancer cells and iDCs and in this way they can regulate the genes expression and activation of iDCs.

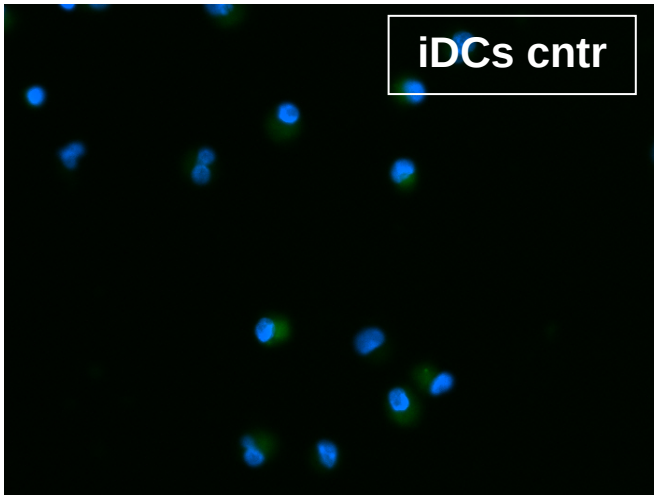
MiRNAs can be released into extracellular space either inside microvesicles (exosomes and apoptotic bodies) or in a cell-membrane-free form as Ago2-miRNA complex, thus we use two approach to track exosomes and miRNA: stain breast cancer cell with the green fluorescent dye PKH67 and transfect breast cancer cell with Dy547-labeled miRNA. PKH67 is a green fluorescent dye used for cell membrane labeling characterized by long aliphatic carbon tail that allow the dye to be trapped inside the cell membrane lipid bilayer preventing the leaking or cell-to-cell transfer, while Dy547-labeled miRNA is a non-targeting red fluorescent miRNA used as mimic negative control for miRNA transfection. The same transwell setting of co-culture was utilized (1.0 μm pore size) to assess the transfer of exosomes and miRNAs from SUM-149 and SUM-159 to iDCs. First, we labeled SUM-149 and SUM-159 with green fluorescent dye PKH67 and plated in the upper chamber of transwell. As PKH67 can be incorporated in all cell membranes, it can also stain exosomes.

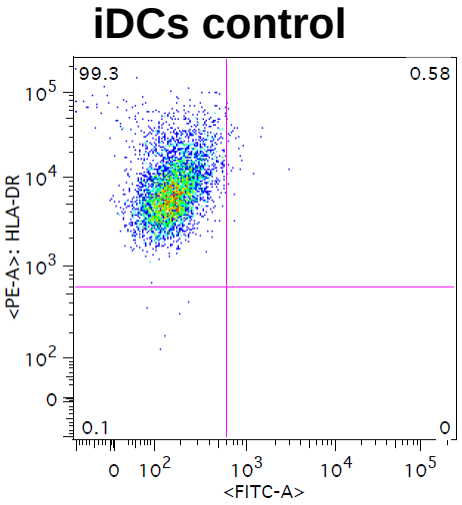
After 24 h of co-culture in transwell, the uptake of PKH67-labeled exosomes by iDCs could be detected by fluorescence microscopy, confocal microscopy and flow cytometry, as shown in fig.7-8. To confirm that miRNAs are transferred to iDCs, SUM-149 and SUM-159 were transfected with Dy547-labeled miRNA. After 24 h of culture,

cell viability was checked (>90%) before plating the transfected SUM-149 and SUM-159 into the upper chamber of the transwell. Also in this case, fluorescent staining could be detected in iDCs after 48 h of co-culture (fig.9).

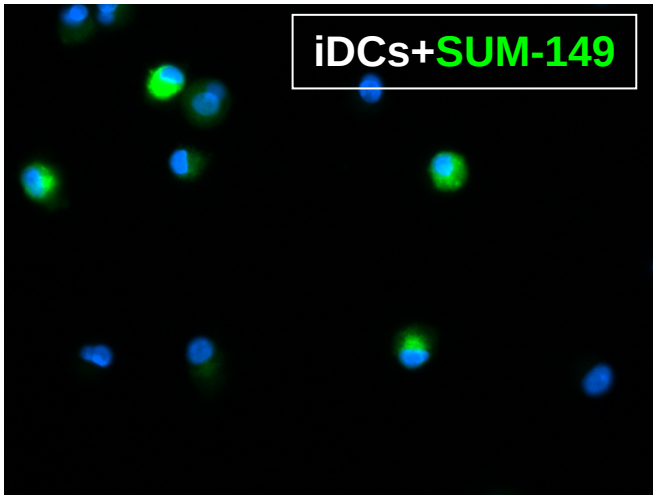
To further confirm the transfer to iDCs, exosomes were purified from the supernatants of both PKH67-labeled SUM-149 and SUM-159. To prevent the contamination of cellular debris, the supernatants were centrifuged at high speed (3000 X g for 15 minutes) before the precipitation at 4^{o}C according to the manufacturer's instruction. A further step to remove possible contaminating debris was added by filtering the centrifuged supernatant through a 0.2 μm filter. The purified PKH67-labeled exosomes were added to iDCs for 24h of culture. The confocal microscopy analysis confirmed the uptake of exosome by iDCs (fig.10).

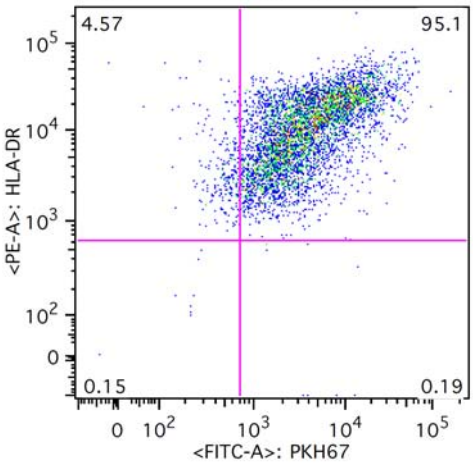
a


iDCs cntr

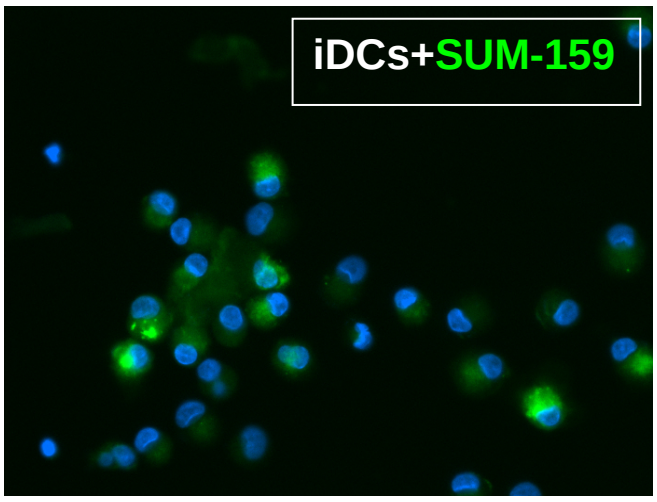

iDCs control
99.3
0.58
0.1
0
<PE-A>: HLA-DR
<FITC-A>

b


iDCs+SUM-149


iDCs + SUM-149
4.57
95.1
0.15
0.19
<PE-A>: HLA-DR
<FITC-A>: PKH67

c


iDCs+SUM-159

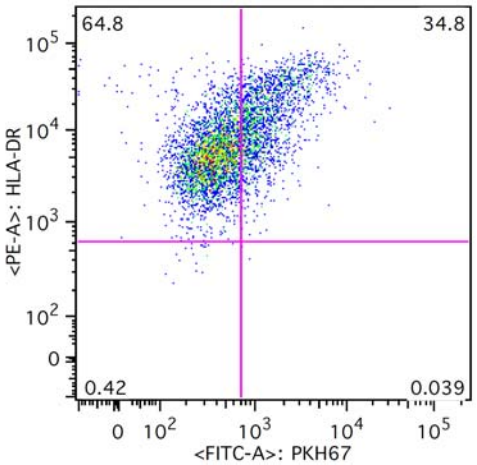

iDCs + SUM-159
64.8
34.8
0.42
0.039
<PE-A>: HLA-DR
<FITC-A>: PKH67

Fig. 7. Exosome transfer from PKH67-labeled SUM-149 and SUM-159 cells analyzed by fluorescence microcopy

Fluorescence microcopy and FACS analysis of: a) control iDCs; b) iDCs co-cultured 24 h with SUM-149; c) iDCs co-cultured 24 h with SUM-159. SUM-149 and SUM-159 were previously stained with the green fluorescent dye PKH67 and then plated in the upper chamber of 1.0 μm pore size transwell.

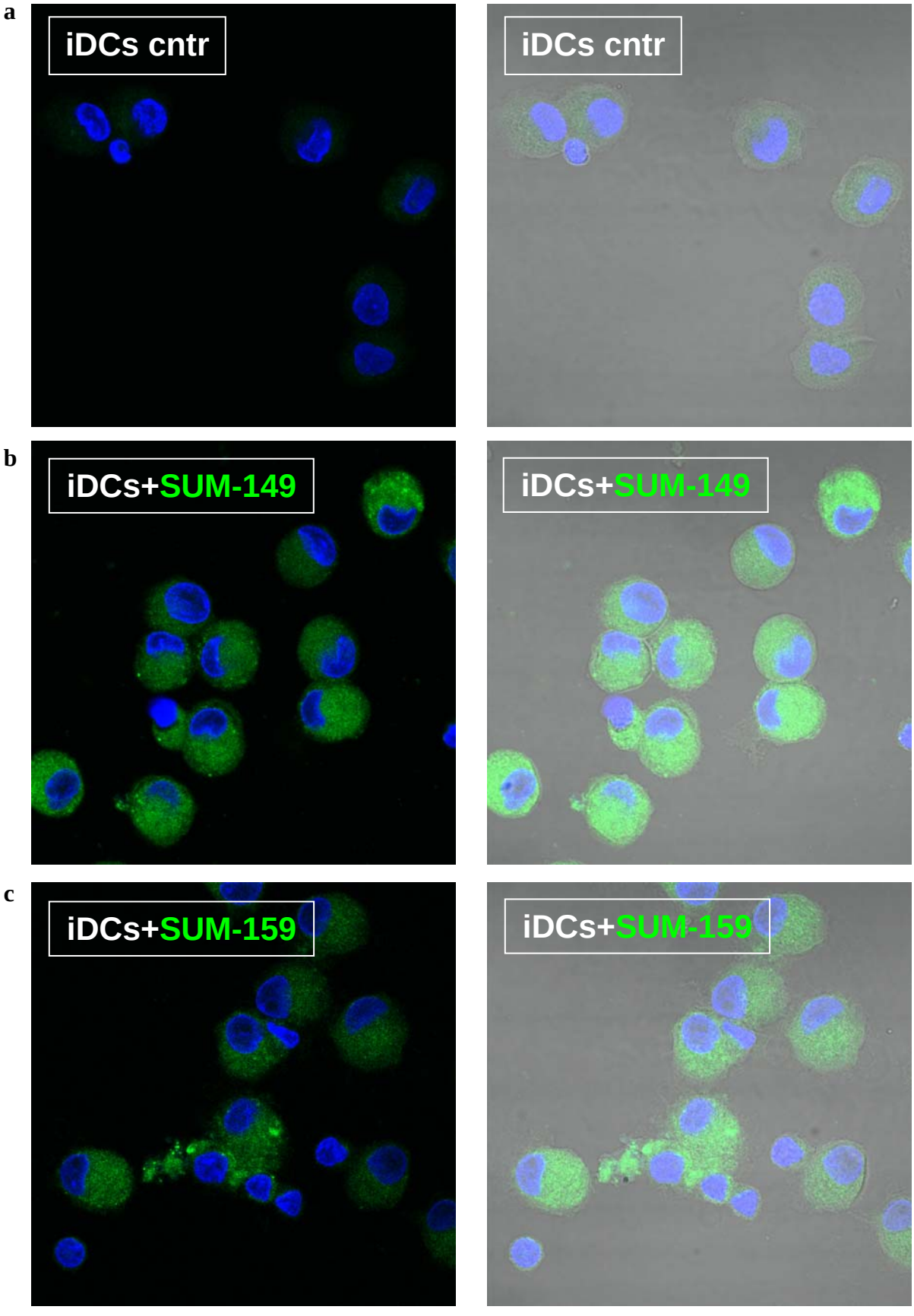

a
iDCs cntr
iDCs cntr
b
iDCs+SUM-149
iDCs+SUM-149
c
iDCs+SUM-159
iDCs+SUM-159

Fig. 8. Exosome transfer from PKH67-labeled SUM-149 and SUM-159 cells analyzed by confocal fluorescence microcopy

Confocal fluorescence microcopy analysis of: a) control iDCs; b) iDCs co-cultured for 24 h with SUM-149; and c) iDCs co-cultured 24 h with SUM-159. SUM-149 and SUM-159 were previously stained with the green fluorescent dye PKH67 and then plated in the upper chamber of 1.0 μm pore size transwell. On the right side: DAPI and FITC fluorescence overlapping with bright field images of iDCs.

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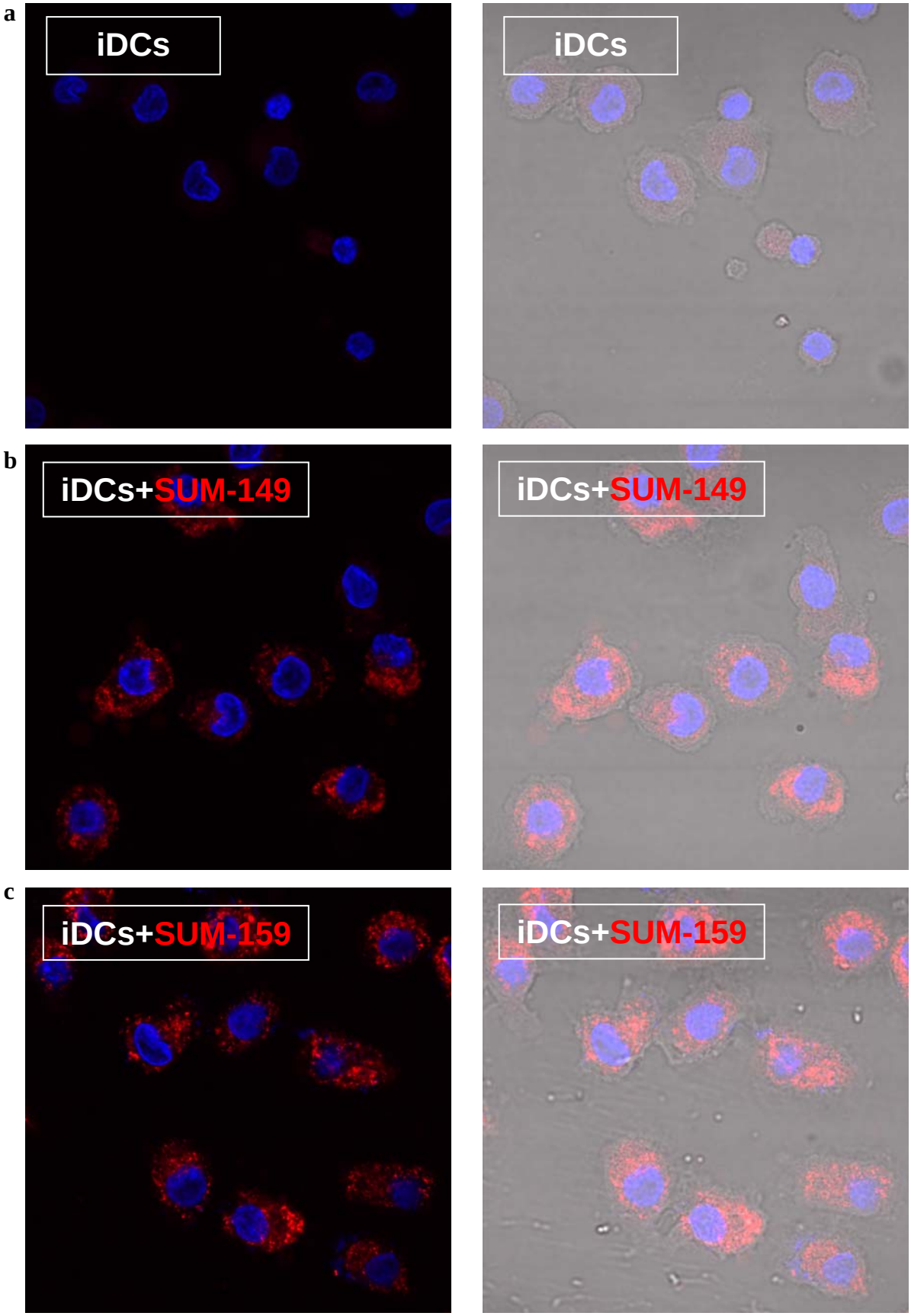
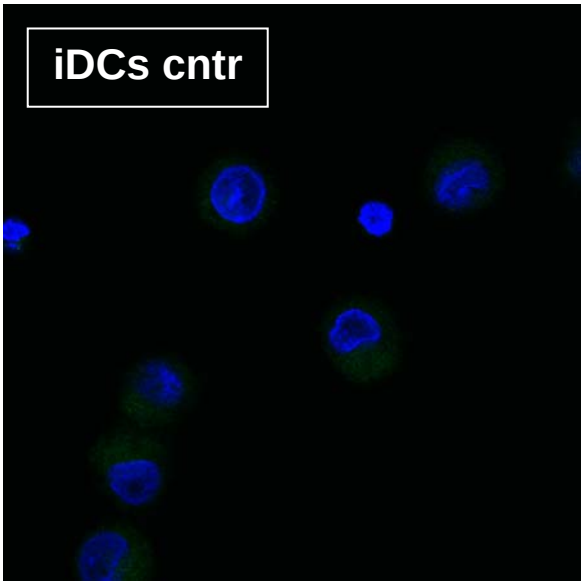

a
iDCs
iDCs
b
iDCs+SUM-149
iDCs+SUM-149
c
iDCs+SUM-159
iDCs+SUM-159

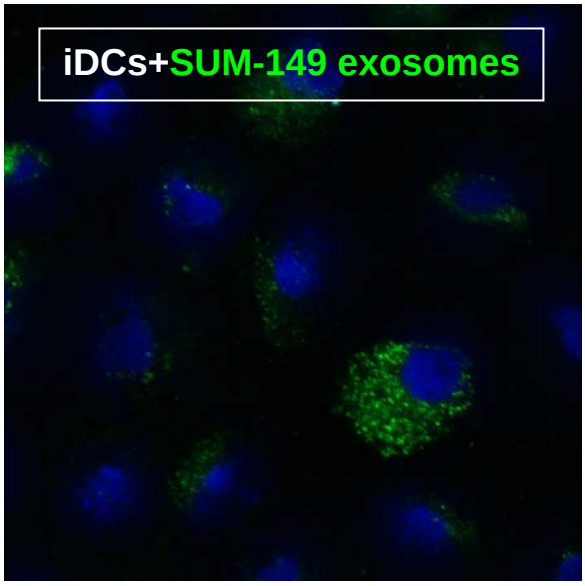
Fig. 9. Exosome transfer from SUM-149 and SUM-159 cells transfected with Dy547-labeled miRNAs analyzed by confocal fluorescence microcopy

Confocal fluorescence microcopy analysis of: a) control iDCs; b) iDCs co-cultured for 48 h with SUM-149; c) iDCs co-cultured for 48 h with SUM-159. SUM-149 and SUM-159 were previously transfected with red fluorescent Dy547-labeled miRNA and than plated in the upper chamber of 1.0 μm pore size transwell. On the right side: DAPI and TRITC fluorescence overlapping with bright field images of iDCs.

a



b



c

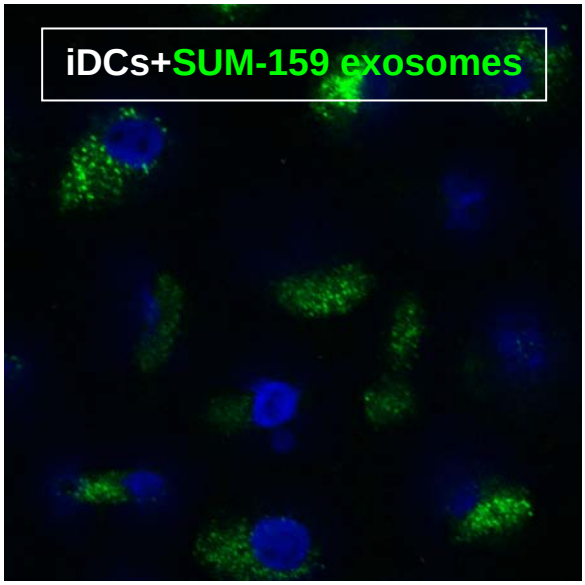


Fig. 10. Transfer of exosomes isolate from PKH67-labeled SUM-149 and SUM-159 cells analyzed by confocal fluorescence microcopy

Confocal fluorescence microcopy analysis of; a) control iDCs; b) iDCs co-cultured 24h with exosome isolated from PKH67-labeled SUM-149; c) iDCs co-cultured 24h with exosome isolated from PKH67-labeled SUM-159.

Chapter 6:

Expression levels of miR-19a and miR-146a in breast cancer cell lines and their cell culture supernatants

MiR-19a and miR-146a are differentially expressed by SUM-149 and SUM-159 and they can be detected in the cell culture media and exosomes isolated from the supernatants.

The expression of miR-19a and miR-146a was evaluated in SUM-149, SUM-159 cells line and their supernatants. For further comparison, the expression levels of these two miRNAs were also evaluated in additional cell lines: HMLE; MCF-7; MDA-231; KPL-4; SUM-190; and IBC-3.

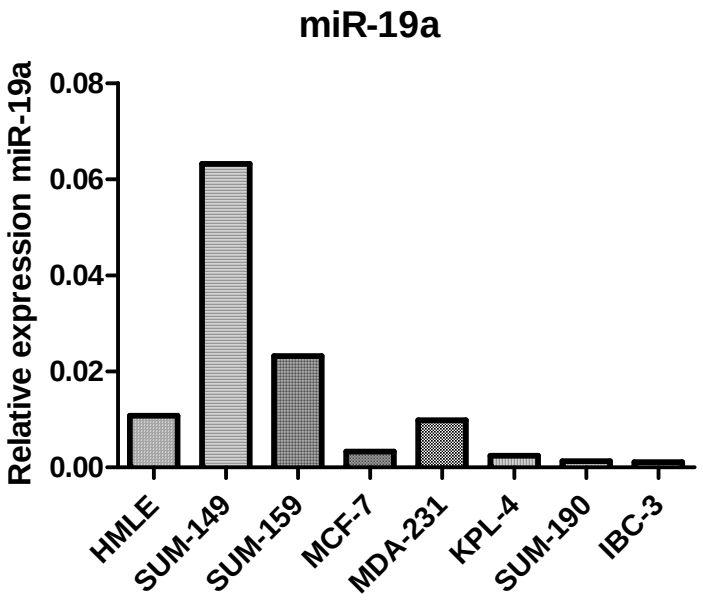
SUM-149 cells expressed higher levels of miR-19a compared with SUM-159 (~2.7-fold higher). HMLE, MCF-7, MDA-231, KPL-4, SUM-190 and IBC-3 cells expressed lower levels of miR-19a compared with SUM-149 (Fig. 11a). On the contrary, SUM-159 expressed very high levels of miR-146a compared with SUM-149 (~240-fold higher). Only MDA-231 expressed moderately high levels of miR-146a compared with SUM-159, while miR-146a levels were much lower in HMLE, MCF-7, MDA-231, KPL-4, SUM-190 and IBC-3 compared with SUM-159 (Fig. 11b). To verify that miR-19a and miR-146a can be released by the breast cancer cells, we evaluated the levels of miR-19a and miR-146a in the supernatants of SUM-149 and SUM-159. Only the supernatants of cell line with viability > 90% were used. The supernatant of SUM-149 had higher level of miR-19a compared with the supernatant of SUM-159 (~1.9-fold higher). MCF-7, MDA-232, KPL-4, SUM-190 and IBC-3, released very low levels of miR-19a compared with SUM-149 (Fig. 12a). The supernatant of SUM-159 had very high levels of miR-146a compared with the supernatant of SUM-149 (~53-fold higher). Only the supernatant of MDA-231 had fairly high amount of miR-146a, while the supernatant of

MCF-7, MDA-232, KPL-4, SUM-190 and IBC-3 had very low levels of miR-146a compared with SUM-159 (Fig. 12b).

Finally, we evaluated if the exosomes isolated from the supernatants of SUM-149 and SUM-159 contained miR-19a and miR-146a. Exosomes from both cell lines contained miR-19a and miR-146a. Exosomes from SUM-149 had higher levels of miR-19a than miR-146a (~22-fold higher), while exosomes from SUM-159 had comparable levels of miR-146a and miR-19a (Fig. 13a-b).

We can conclude that miR-19a and miR-146a are selectively expressed and released in the supernatant by SUM-149 and SUM-159.

a


miR-19a
Relative expression miR-19a
0.08
0.06
0.04
0.02
0.00
HMLE
SUM-149
SUM-159
MCF-7
MDA-231
KPL-4
SUM-190
IBC-3

b

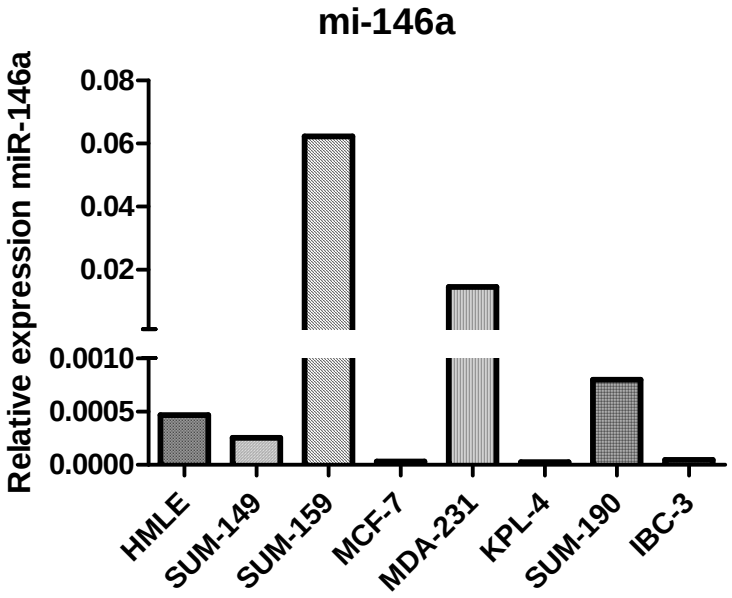
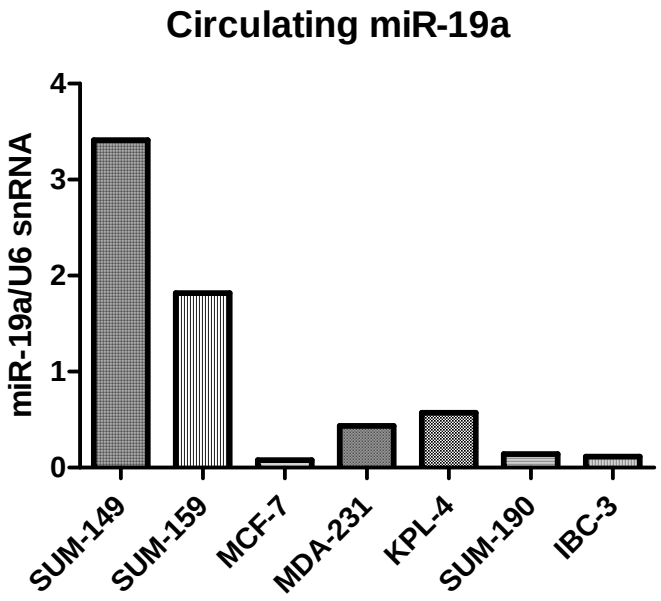

mi-146a
Relative expression miR-146a
0.08
0.06
0.04
0.02
0.0010
0.0005
0.0000
HMLE
SUM-149
SUM-159
MCF-7
MDA-231
KPL-4
SUM-190
IBC-3

Fig. 11. Expression levels of miR-19a and miR-146a in breast cancer cell lines

Expression levels of miR-19a (a) and miR-146a (b) in HMLE, SUM-149, SUM-159 MCF-7, MDA-231, KPL-4 SUM-190 and IBC-3 cell lines. The expression levels of miR-19a and miR-146a are from the most representative experiment.

a


Circulating miR-19a
miR-19a/U6 snRNA
4
3
2
1
0
SUM-149
SUM-159
MCF-7
MDA-231
KPL-4
SUM-190
IBC-3

b

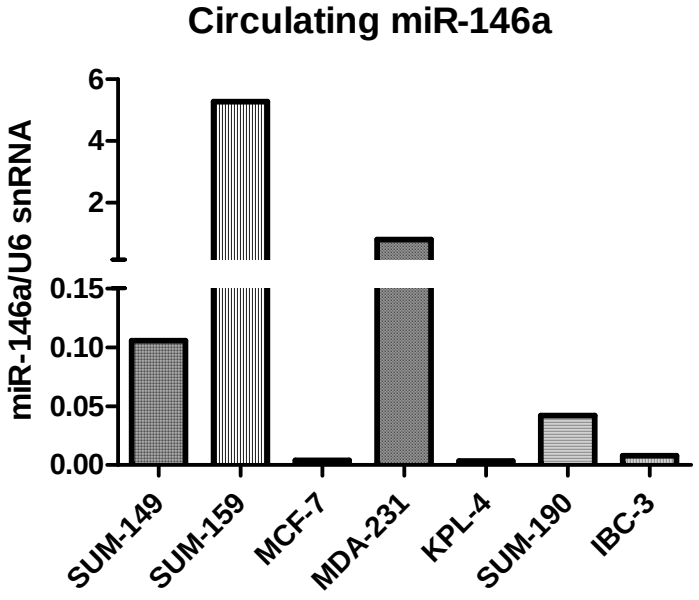
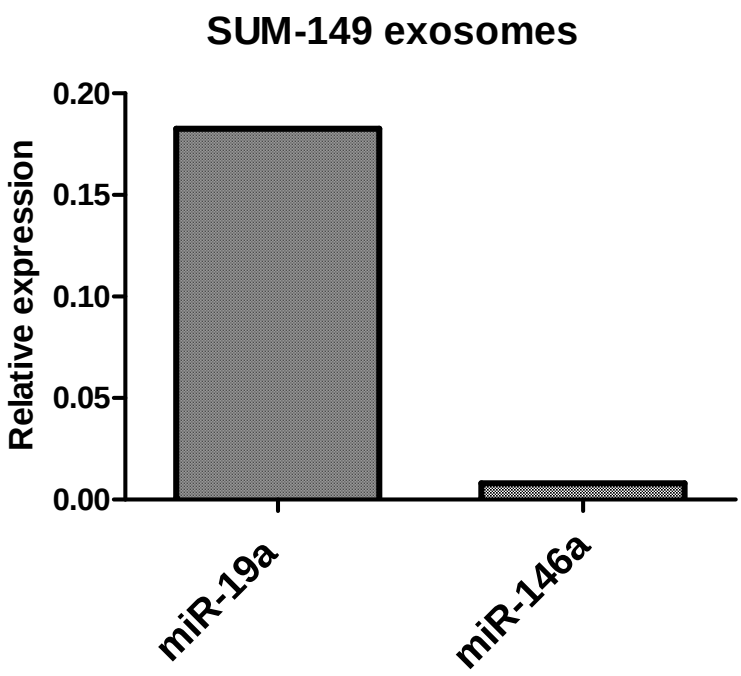

Circulating miR-146a
miR-146a/U6 snRNA
6
4
2
0.15
0.10
0.05
0.00
SUM-149
SUM-159
MCF-7
MDA-231
KPL-4
SUM-190
IBC-3

Fig. 12. Expression levels of miR-19a and miR-146a in the supernatants of breast cancer cell lines

Expression levels of miR-19a (a) and miR-146a (b) in the supernatants of: HMLE, SUM-149, SUM-159 MCF-7, MDA-231, KPL-4 SUM-190 and IBC-3 cell lines. The expression levels of miR-19a and miR-146a are from the most representative experiment.

a


SUM-149 exosomes
Relative expression
0.20
0.15
0.10
0.05
0.00
miR-19a
miR-146a

b

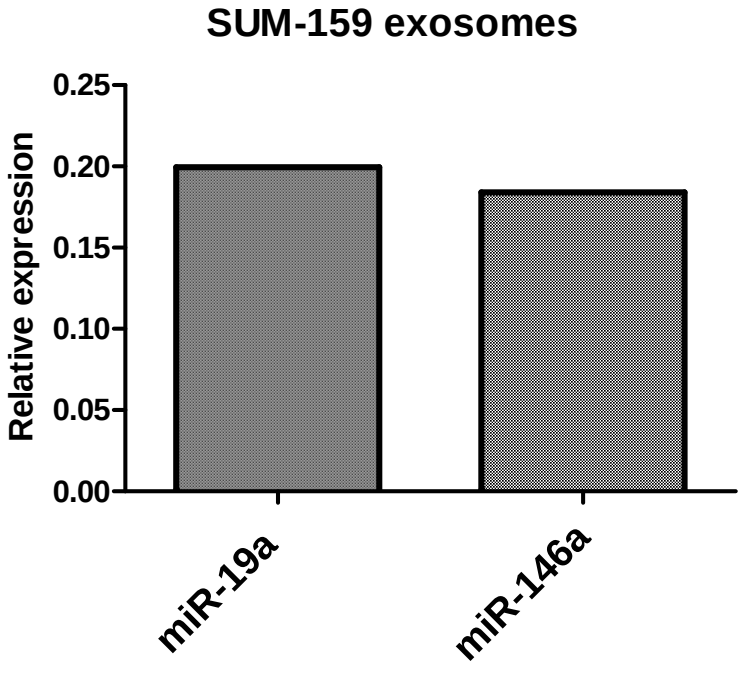

SUM-159 exosomes
Relative expression
0.25
0.20
0.15
0.10
0.05
0.00
miR-19a
miR-146a

Fig. 13. Exosomes released by SUM-149 and SUM-159 cells contain miR-19a and miR-146a

Detection of miR-19a and miR-146a in the exosomes isolated from SUM-149 (a) and SUM-159 (b) supernatants. The expression levels of miR-19a and miR-146a are from one experiment.

Chapter 7:

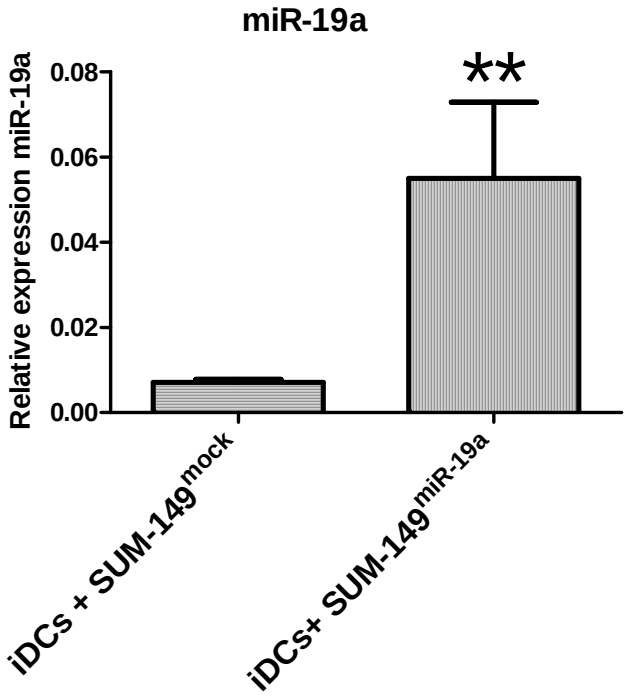
Transfer of miR-19a and miR-146a from breast cancer cell lines to iDCs

MiR-19a and miR-146a can be transferred from breast cancer cells to recipient iDCs

In order to assess if miR-19a and miR-146a can be transferred to iDCs, SUM-149 and SUM-159 were transfected respectively with 100 nM mimic miR-19a (SUM-149$^{miR-19a}$) and miR-146a (SUM-159$^{miR-146a}$) and co-cultured with iDCs in transwell 1.0 μm pores size. After 24 h of co-culture, there was a significant increase in the levels of miR-19a and miR-146a in iDCs co-cultured with SUM-149$^{miR-19a}$ (Fig. 14a) and SUM-159$^{miR-146a}$ (Fig. 14b) respectively. We also assessed if the co-culture of non-transfected SUM-149 and SUM-159 could influence the levels of miR-19a and miR-146a. Indeed, the co-culture with SUM-149 and SUM-159 induced an increase of miR-19a (Fig. 15a) and miR-146a (Fig. 15b) in iDCs, respectively.

We can conclude that miR-19a and miR-146a can be transferred from SUM-149 and SUM-159 to iDCs, respectively.

a


miR-19a
Relative expression miR-19a
0.08
0.06
0.04
0.02
0.00
**
iDCs + SUM-149mock
iDCs+ SUM-149miR-19a

b

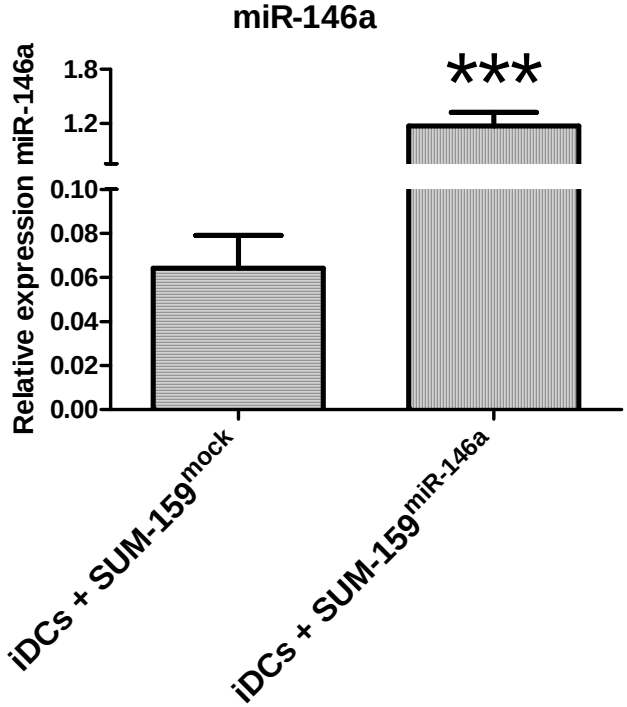
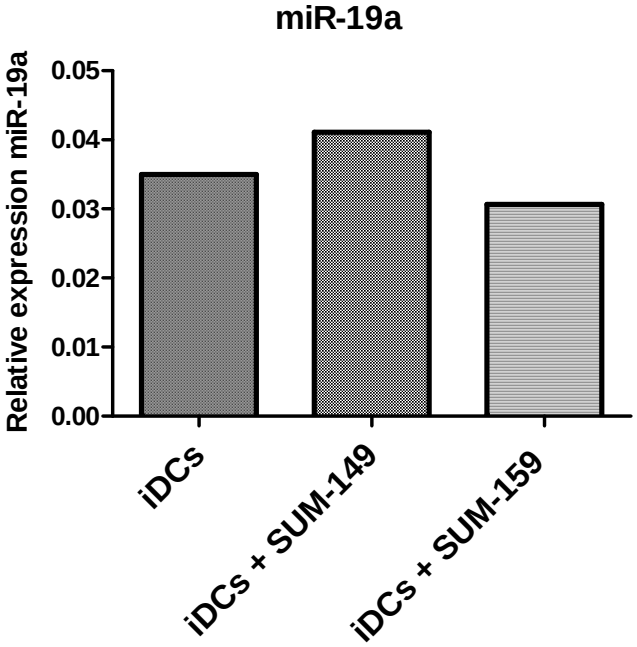

miR-146a
Relative expression miR-146a
1.8
1.2
0.10
0.08
0.06
0.04
0.02
0.00
***
iDCs + SUM-159mock
iDCs + SUM-159miR-146a

Fig. 14. Transfer of miR-19a and miR-146a from transfected SUM-149 and SUM-159 cells to iDCs

Expression levels of miR-19a and miR-146a in iDCs co-cultured with: a) SUM-149 transfected with miRIDIAN mimic miR-19a (SUM-149$^{miR\text{-}19a}$); b) SUM-159 transfected with miRIDIAN mimic miR-146a (SUM-159$^{miR\text{-}146a}$), than plated in the upper chamber of 1.0 μm pore size transwell. The results represent the mean ± standard deviation of one experiment performed in triplicate (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

a


miR-19a
Relative expression miR-19a
0.05
0.04
0.03
0.02
0.01
0.00
iDCs
iDCs + SUM-149
iDCs + SUM-159

b

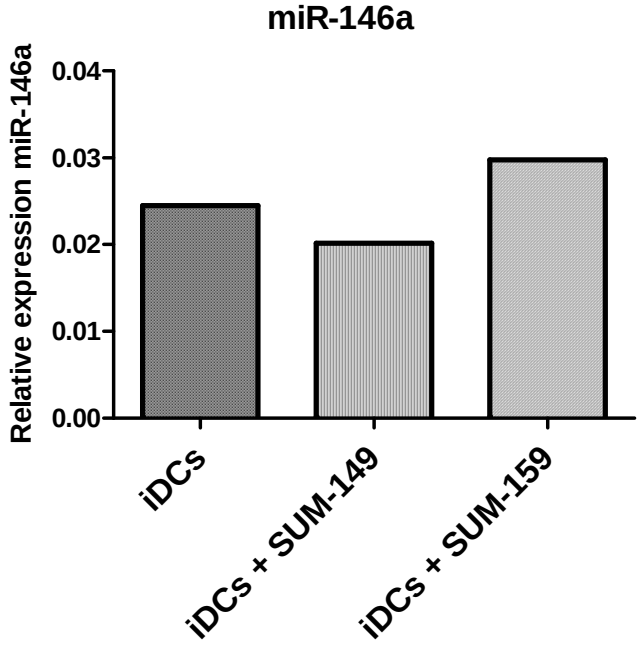

miR-146a
Relative expression miR-146a
0.04
0.03
0.02
0.01
0.00
iDCs
iDCs + SUM-149
iDCs + SUM-159

Fig. 15. Transfer of miR-19a and miR-146a from SUM-149 and SUM-159 cells to iDCs

Expression levels of miR-19a and miR-146a in iDCs co-cultured with SUM-149 (a) and SUM-159 (b) for 24 h in 1.0 μm pore size transwell. The expression levels of miR-19a and miR-146a are from the most representative experiment.

Chapter 8:

Effect of miR-19a and miR-146a on the mRNA levels of their target genes PTEN, SOCS-1, IRAK1, TAF6 and cytokine in DCs

The miR-19a and miR-146a affect the levels of cytokine mRNA synthesis in mDCs

To assess the effect of miR-19a and miR-146a on cytokine production, iDCs were transfected with mimic miR-19a 50 nM and miR-146a 100 nM. The transfection of iDCs with miR-19a ($iDC^{miR\text{-}19a}$) induced a decrease in the expression levels of its target genes PTEN and SOCS-1 compared with iDCs transfected with mimic control (iDC^{mock}) (Fig. 16a). After stimulation with LPS 100 ng/ml for 3 h, $iDC^{miR\text{-}19a}$ produced higher mRNA levels of IL-6 and TNF-α compared with iDC^{mock} (Fig. 16b). On the contrary, the transfection of iDCs with miR-146a ($iDC^{miR\text{-}146a}$) induced a decrease in the expression levels of its target genes IRAK1 and TRAF6 compared with iDCs transfected with mimic control (iDC^{mock}) (Fig. 17a). After stimulation with LPS 100 ng/ml for 3 h, $iDC^{miR\text{-}146a}$ produced lower mRNA levels of IL-6 and TNF-α compared with iDC^{mock} (Fig. 17b). We can conclude that increased levels of miR-19a in iDCs were able to enhance the ability to synthesize the pro-inflammatory cytokines IL-6 and TNF-α upon LPS activation. Therefore, miR-19a can induce a more pro-inflammatory phenotype in $iDC^{miR\text{-}19a}$. On the other hand, increased levels of miR-146a in iDCs reduced the ability to synthesize IL-6 and TNF-α upon LPS activation. Therefore, miR-146a can induce an anti-inflammatory phenotype in $iDC^{miR\text{-}146a}$.

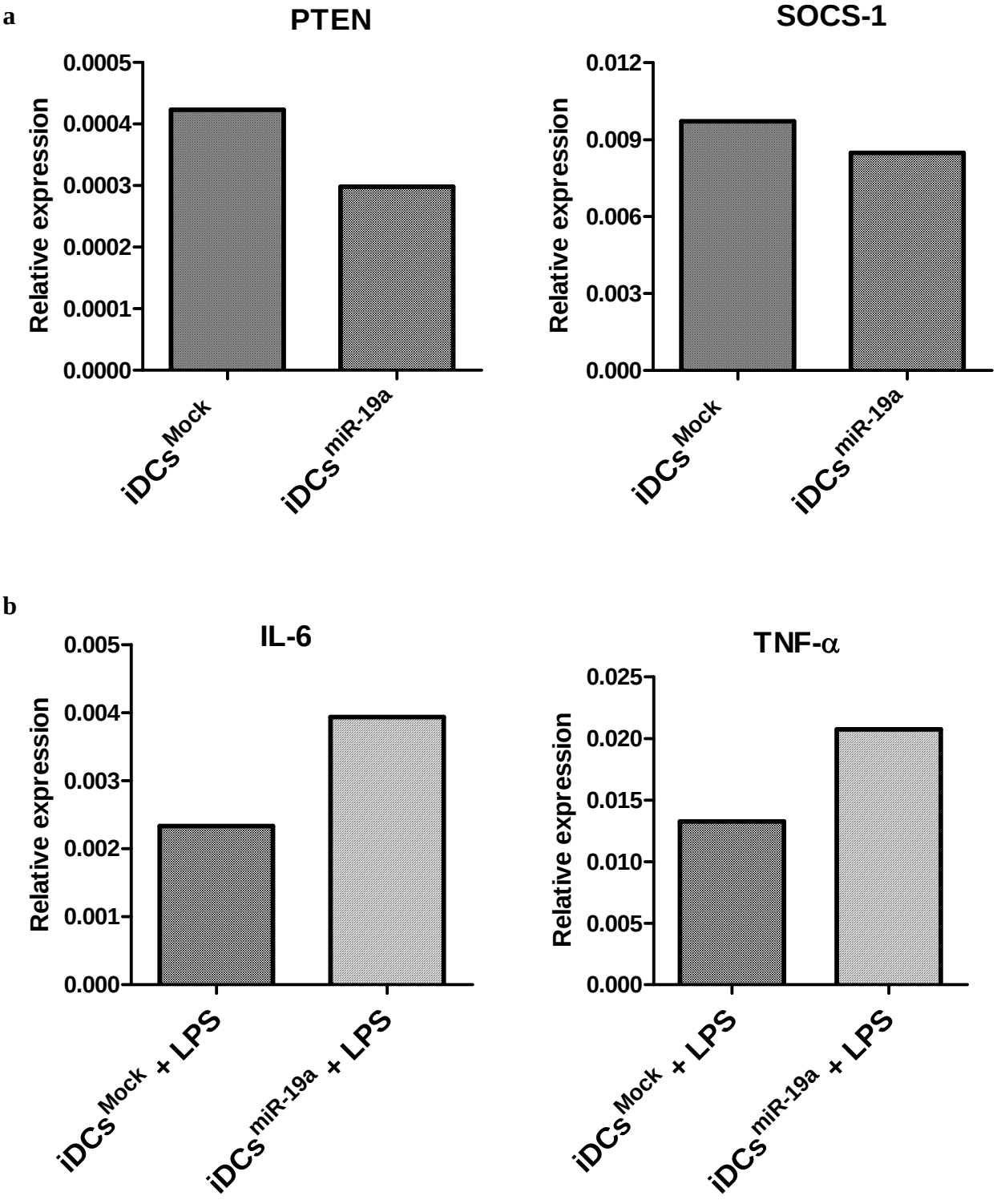
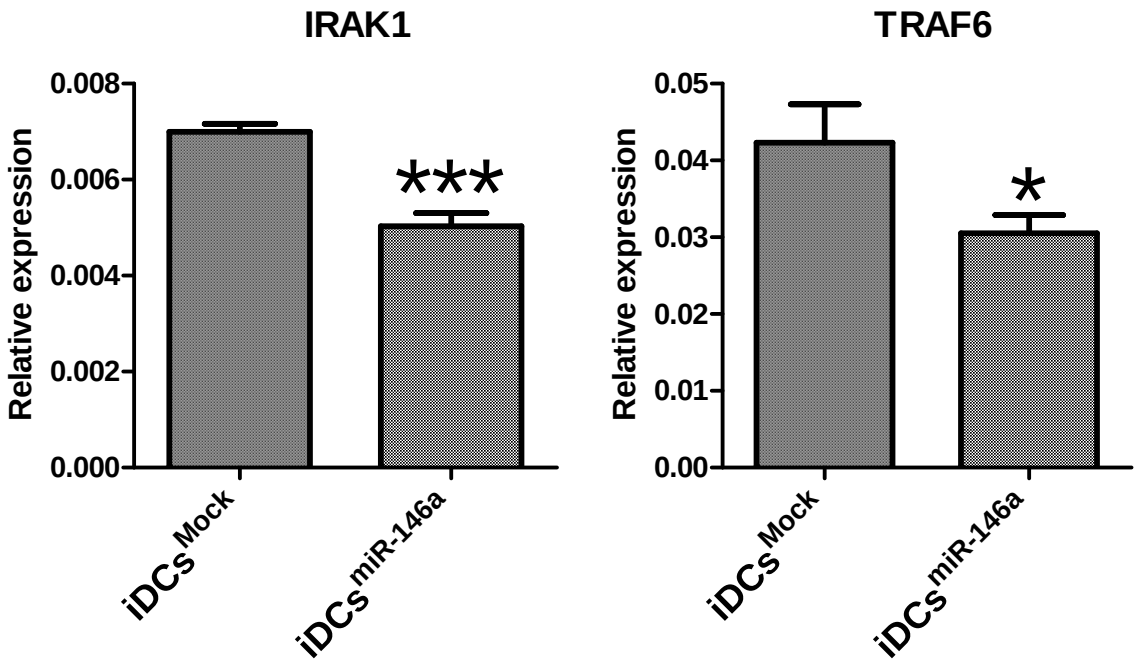

a
PTEN
Relative expression
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
iDCs Mock
iDCs miR-19a
SOCS-1
Relative expression
0.012
0.009
0.006
0.003
0.000
iDCs Mock
iDCs miR-19a
b
IL-6
Relative expression
0.005
0.004
0.003
0.002
0.001
0.000
iDCs Mock + LPS
iDCs miR-19a + LPS
TNF-α
Relative expression
0.025
0.020
0.015
0.010
0.005
0.000
iDCs Mock + LPS
iDCs miR-19a + LPS

Fig. 16. Effect of transfection with miR-19a mimic on PTEN and SOCS-1 mRNA levels and on IL-6 and TNF-a mRNA levels in DCs

Expression levels of PTEN and SOCS-1 mRNA in iDCs transfected with miR-19a in (a) and the levels of IL-6 and TNF-α mRNA after LPS stimulation in (b). The results are from the most representative experiment.

a


IRAK1
Relative expression
0.008
0.006
0.004
0.002
0.000
***
iDCs Mock
iDCs miR-146a
TRAF6
Relative expression
0.05
0.04
0.03
0.02
0.01
0.00
*
iDCs Mock
iDCs miR-146a

b

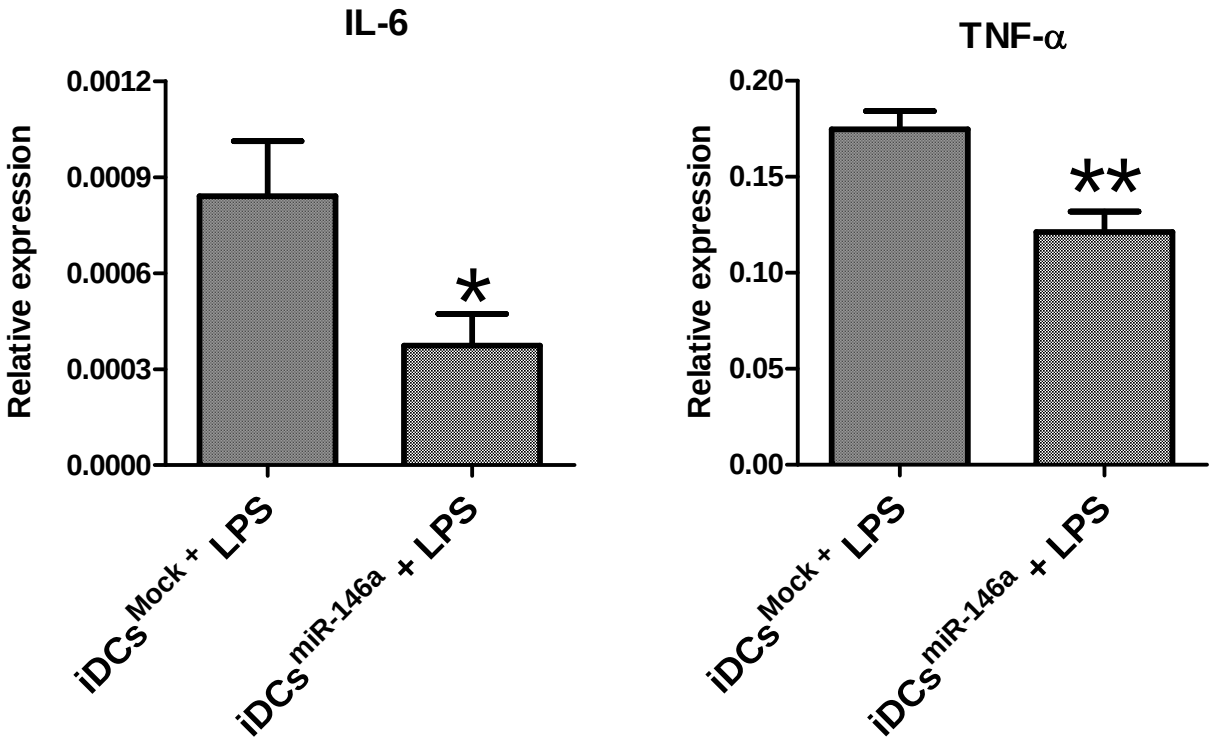

IL-6
Relative expression
0.0012
0.0009
0.0006
0.0003
0.0000
*
iDCs Mock + LPS
iDCs miR-146a + LPS
TNF-α
Relative expression
0.20
0.15
0.10
0.05
0.00
**
iDCs Mock + LPS
iDCs miR-146a + LPS

Fig. 17. Effect of transfection with miR-146a mimic on IRAK1 and TRAF6 mRNA levels and on IL-6 and TNF-a mRNA levels in DCs

Expression levels of IRAK1 and TRAF6 mRNA in iDCs transfected with miR-146a in (a) and the levels of IL-6 and TNF-α mRNA after LPS stimulation in (b). The results represent the mean ± standard deviation of one experiment performed in triplicate (*p<0.05; **p<0.01; ***p<0.001).

Chapter 9:

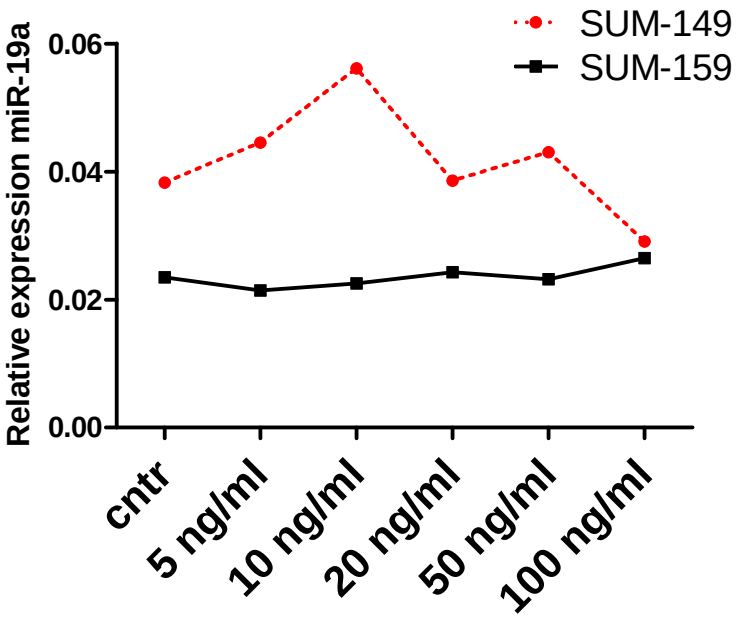
Effect of IL-6 on miR-19a and miR-146a levels in SUN-149 and SUM-159 cells

Effect of IL-6 on miR-19a and miR-146a expression levels in SUM-149 and SUM-159

As SUM-149 and SUM-159 cells could influence the synthesis of pro-inflammatory cytokines in $mDC^{SUM-149}$ and $mDC^{SUM-159}$, we wanted to asses if those cytokines could affect in turn the levels of miR-19a and miR-146a in SUM-149 and SUM-159 cells. IL-6 could increase the levels of miR-19a in SUM-149 cells, with the strongest induction at 10 ng/ml, but it had no effect in SUM-159 cells (Fig. 18a). On the other hand, IL-6 induced a modest increase of miR-146a levels in SUM-149 cells, that remained ~37-fold lower than those in SUM-159, while in SUM-159 IL-6 (5 ng/ml) could upregulate the expression levels of miR-146a to a values ~208-fold higher than those in SUM-149 and this effect decreased with the increase of IL-6 concentration (Fig. 18b).

We can conclude that IL-6 had different effect on the expression of miR-19a and miR-146a in SUM-149 and SUM-159. It can be hypothesized that in the SUM-149 model of IBC tumor microenvironment, the IL-6/miR-19a axis represents a self-sustaining loop able to maintain a pro-inflammatory status of DCs. While in the SUM-159 model of non-IBC tumor microenvironment, IL-6/miR-146a may represent a self-limiting loop that maintains an immune suppressed status of DCs.

a


SUM-149
SUM-159
Relative expression miR-19a
0.06
0.04
0.02
0.00
cntr
5 ng/ml
10 ng/ml
20 ng/ml
50 ng/ml
100 ng/ml

b

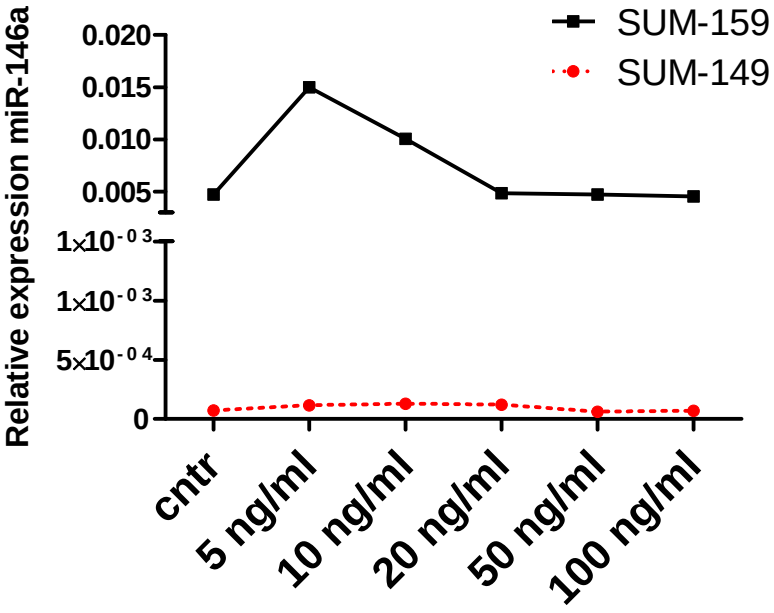

SUM-159
SUM-149
Relative expression miR-146a
0.020
0.015
0.010
0.005
1x10-03
1x10-03
5x10-04
0
cntr
5 ng/ml
10 ng/ml
20 ng/ml
50 ng/ml
100 ng/ml

Fig. 18. Effect of IL-6 on miR-19a and miR-146a expression levels in SUM-149 and SUM-159 cells

Dose-dependent effect on the expression levels of miR-19a (a) and miR-146a (b) in SUM-149 and SUM-159 after 24 h of IL-6 stimulation. The results are from the most representative experiment.

Chapter 10:

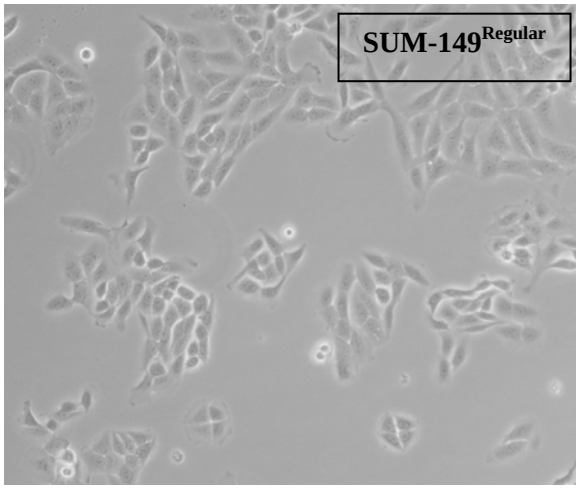
miR-19a levels in SUM-149 with EMT and CSC-like phenotype

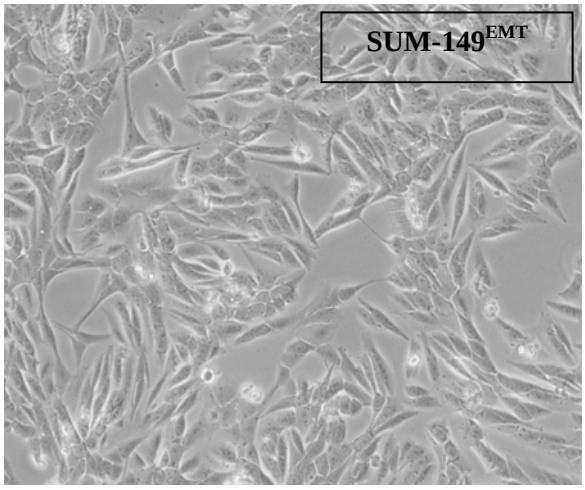
Increased levels of miR-19a in SUM-149 cells are associated with EMT and CSC-like phenotype

SUM149 is a mixed cell line exhibiting two distinct CD44/CD24 populations, $CD44^{+}CD24^{-}$ and $CD44^{+}CD24^{+}$, that are characterized by basal-like (spindle-like appearance) or luminal-like (epithelial-like appearance) features respectively [79]. As IL-6 is produced by SUM-149 (99), induced EMT in breast cancer (53, 54), regulated the survival and growth of breast cancer CSCs (100), and induced miR-19a synthesis in SUM-149, we assessed if increased levels of miR-19a can be associated with an enrichment of CSCs ($CD44^{+}CD24^{-}$ $ALDH^{+}$) with EMT characteristics (expression of EMT-regulating gene: Twist1, Snail1, Snail2, TG2, FOXC2, ZEB1),.

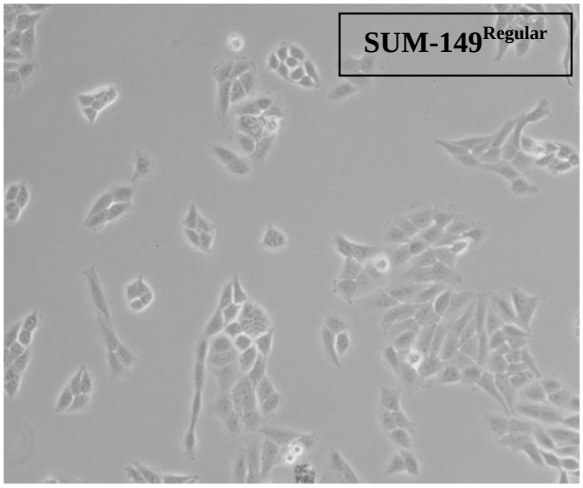
When they were allowed to grow to 80-90% of confluence in a long-term cell culture (5-7 days) without changing the culture medium, SUM-149 cells acquired spindle-like appearance ($SUM\text{-}149^{EMT}$ cells) resembling cancer cells with EMT phenotype, compared with SUM-149 ($SUM\text{-}149^{Regular}$ cells) that are grown in canonical conditions (40-50% confluence for 3 days)(Fig. 19). Furthermore, $SUM\text{-}149^{EMT}$ cell culture was enriched in the $CD44^{+}$ population (Fig. 20a) and in cells with increased ALDH activity ($ALDH1^{+}$)(Fig. 20b). As IL-6 regulates the growth and survival of $CD44^{+}CD24^{-}$ breast cancer stem cells and IL-6 is produced by SUM-149, we assumed that the long-term cell culture could have increased levels of IL-6. Thus, we measured IL-6 levels in the $SUM\text{-}149^{EMT}$ and $SUM\text{-}149^{Regular}$ cell culture supernatants. We found that $SUM\text{-}149^{EMT}$ cell culture had higher levels of IL-6 compared with those of $SUM\text{-}149^{Regular}$ and HMLE used as control (Fig. 21).

Canonical **Long-term**





Canonical **Long-term**



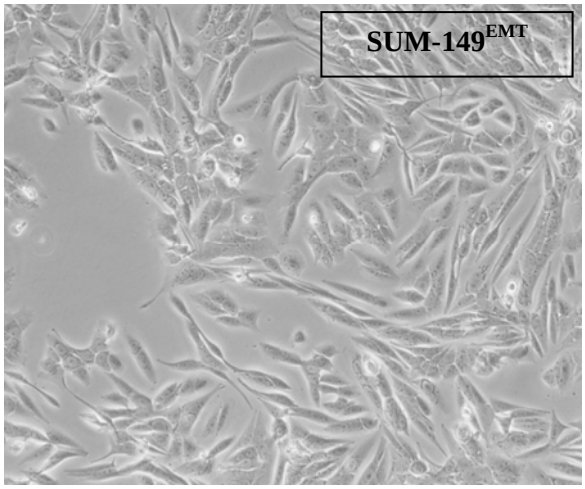
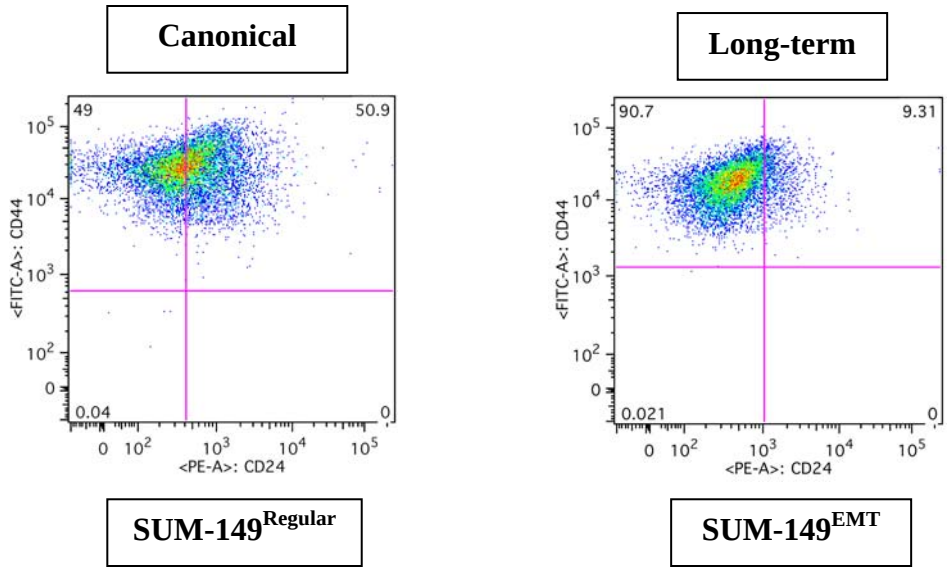


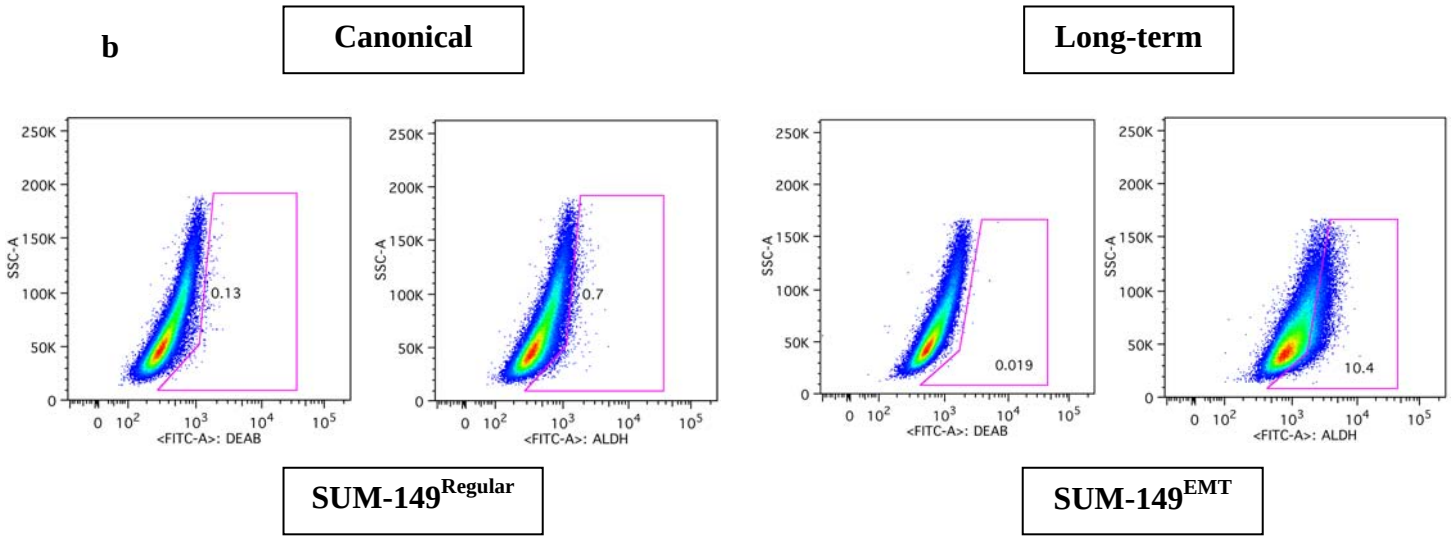
Fig. 19. Induction of EMT phenotype in SUM-149 cells by long-term cell culture

SUM-149 cells cultured under different cell culture conditions: canonical (3 days and 40-50% confluence) and long-term culture without changing culture medium (5-7 days and 80-90% confluence). Four representative pictures are shown.

a


Canonical
Long-term
49
50.9
0.04
0
90.7
9.31
0.021
0
<FITC-A>: CD44
<PE-A>: CD24
SUM-149Regular
SUM-149EMT

b


Canonical
Long-term
0.13
0.7
0.019
10.4
SSC-A
<FITC-A>: DEAB
<FITC-A>: ALDH
SUM-149Regular
SUM-149EMT

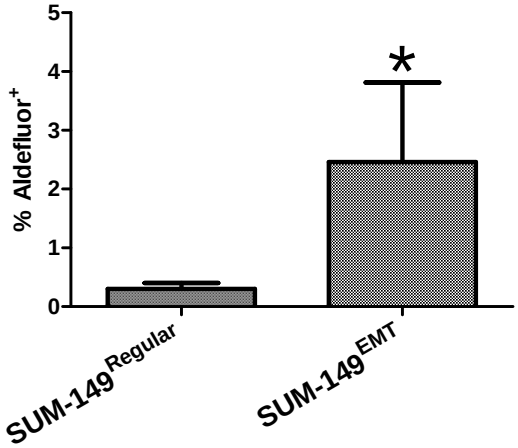

% Aldefluor+
*
SUM-149Regular
SUM-149EMT

Fig. 20. Enrichment of SUM-149 cell subset with CSC-like phenotype by long-term cell culture

Enrichment of the population with cancer stem cell (CSC)-like phenotype ($CD44^+CD24^-$ and $ALDH1^+$) in SUM-149 cell culture under different conditions: canonical (3 days) and long-term (6-7 days, without changing of culture medium). In (a) it is shown that the long-term cell culture condition induced an enrichment of the $CD44^+CD24^-$ population and in (b) it is shown that the long-term cell culture induce the enrichment of tumor cells with increased ALDH activity. The doplot in (a) and (b) are from the most representative experiment. The Aldefluor chart results represent the mean ± standard deviation of 3 independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

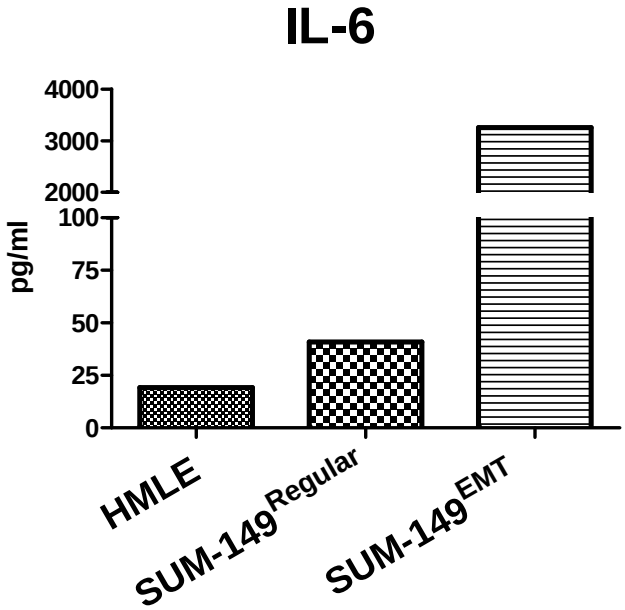
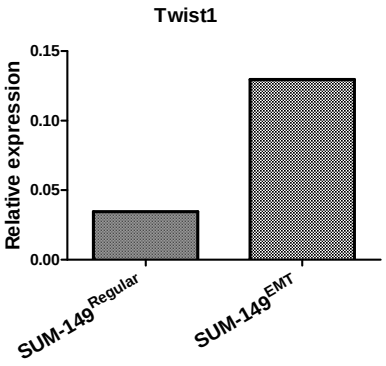

IL-6
4000
3000
2000
100
75
50
25
0
pg/ml
HMLE
SUM-149Regular
SUM-149EMT

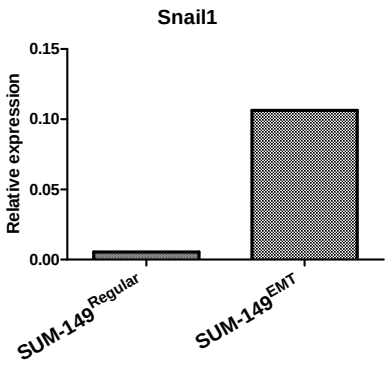
Fig. 21. Increased levels of IL-6 in the supernatant of SUM-149^{EMT} cells from long-term cell culture

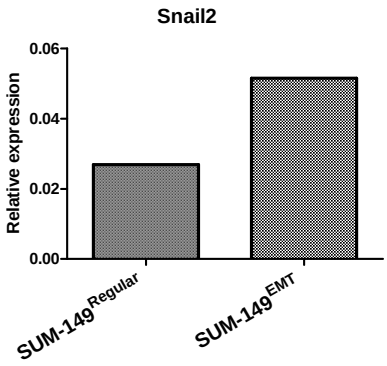
The supernatant of SUM-149^{EMT} cells from ling-term cell culture Levels had increased levels of IL-6 compared with the supernatant of HMLE (human mammary epithelial cells) and SUM-149^{Regular} cells.

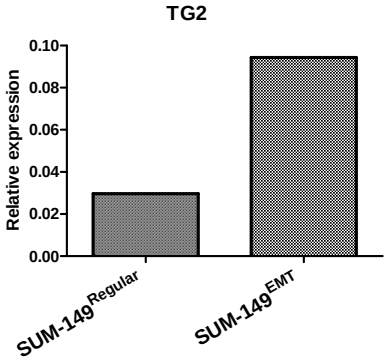
It was previously reported that IL-6 can induce EMT in breast cancer cells (53, 54) and that EMT breast cancer cells have CSCs phenotype (101). As in our study, we showed that IL-6 induced miR-19a synthesis, we evaluated if SUM-149^{EMT} cells, beside an enrichment of tumor cells with CSC phenotype, had also EMT characteristics. We found that SUM-149^{EMT} cells expressed higher levels of the master regulator of EMT (Twist1, Snail1, Snail2, TG2, FOXC2), the EMT markers (N-cadherin, vimentin), EGFR, E-cadherin and most importantly higher levels of miR-19a compared with SUM-149^{Regular} cells (Fig. 22). It is noteworthy that EGFR-mediated signaling was shown to be involved in regulating EMT in IBC (102) and E-cadherin plays important role in tumor emboli formation (103) and is associated with poor outcome in IBC (103, 104). Finally, IL-6 could also increase the expression levels of miR-155 in SUM-149 (Fig. 23), and miR-155 was reported to regulates TGF-β-induced EMT (105) and induce chemoresistance in breast cancer (106).

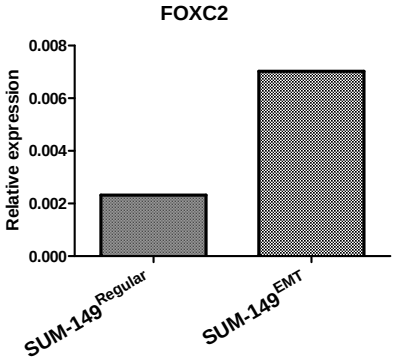
We can conclude that increased levels of miR-19a might be associated with the enrichment in the population of SUM-149 cells with EMT and CSC phenotype and that IL-6 might play an important role in regulating this phenomenon. Therefore, in the tumor microenvironment the increased levels of IL-6 might drive the development of tumor cells responsible of the more aggressive behavior of IBC. Further studies are required to assess the role of IL-6 in the induction of EMT and CSC phenotype in SUM-149 cells.

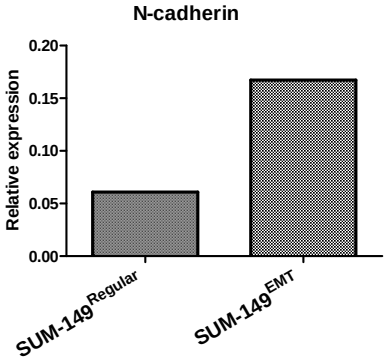

Twist1
Relative expression
0.15
0.10
0.05
0.00
SUM-149Regular
SUM-149EMT

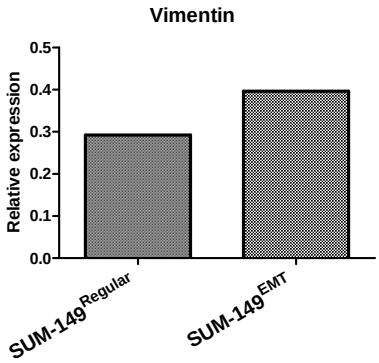

Snail1
Relative expression
0.15
0.10
0.05
0.00
SUM-149Regular
SUM-149EMT

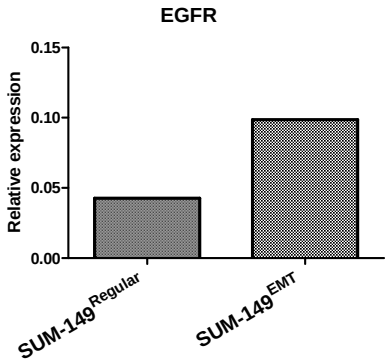

Snail2
Relative expression
0.06
0.04
0.02
0.00
SUM-149Regular
SUM-149EMT

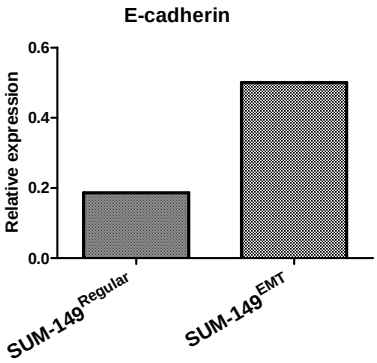

TG2
Relative expression
0.10
0.08
0.06
0.04
0.02
0.00
SUM-149Regular
SUM-149EMT


FOXC2
Relative expression
0.008
0.006
0.004
0.002
0.000
SUM-149Regular
SUM-149EMT


N-cadherin
Relative expression
0.20
0.15
0.10
0.05
0.00
SUM-149Regular
SUM-149EMT


Vimentin
Relative expression
0.5
0.4
0.3
0.2
0.1
0.0
SUM-149Regular
SUM-149EMT


EGFR
Relative expression
0.15
0.10
0.05
0.00
SUM-149Regular
SUM-149EMT


E-cadherin
Relative expression
0.6
0.4
0.2
0.0
SUM-149Regular
SUM-149EMT

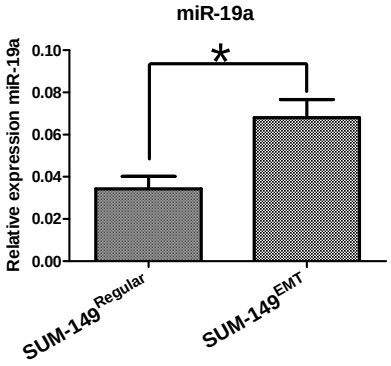

miR-19a
Relative expression miR-19a
*
0.10
0.08
0.06
0.04
0.02
0.00
SUM-149Regular
SUM-149EMT

Fig. 22. Increased expression of EMT-regulating genes and miR-19a in SUM-149 cells from long-term cell culture

SUM-149^{EMT} cells from ling-term cell culture expressed higher levels of the EMT-regulating genes (Twist1, Snail1, Snail2, TG2, FOXC2), the EMT markers (N-cadherin, vimentin), EGFR and E-cadherin in SUM-149^{EMT} compared with SUM-149^{Regular} cells. Results of gene expression levels are from the most representative experiment. The expression levels of miR-19a in SUM-149^{EMT} and SUM-149^{Regular} cells represent the mean ± standard deviation of 3 independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

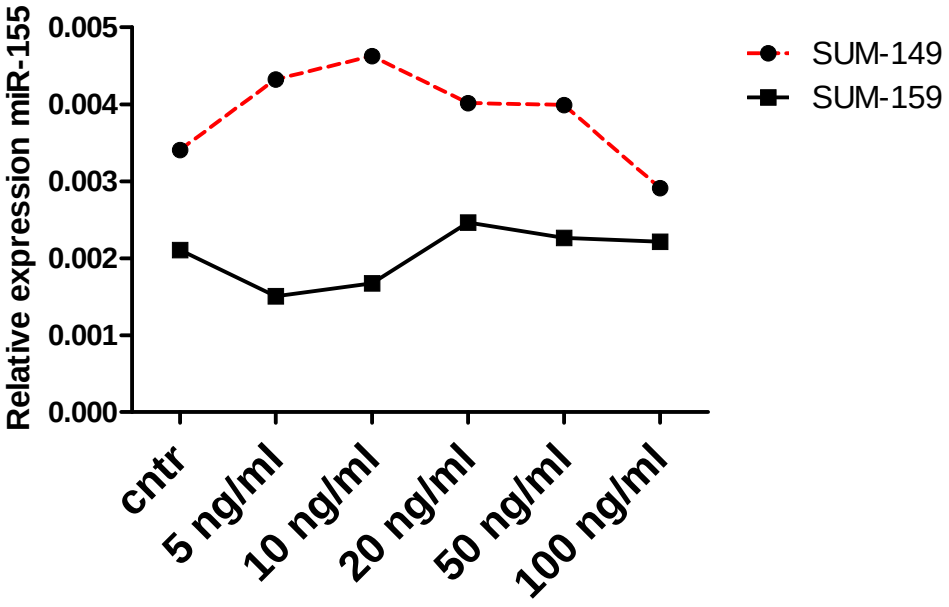

Relative expression miR-155
0.005
0.004
0.003
0.002
0.001
0.000
cntr
5 ng/ml
10 ng/ml
20 ng/ml
50 ng/ml
100 ng/ml
SUM-149
SUM-159

Fig. 23. Effect of IL-6 on miR-155 expression levels in SUM-149 and SUM-159 cells

Dose-dependent effect on the expression levels of miR-155 in SUM-149 and SUM-159 after 24 h of IL-6 stimulation. The results are from the most representative experiment.

Chapter 11:

Serum levels of miR-19a and clinical outcome in breast cancer patients

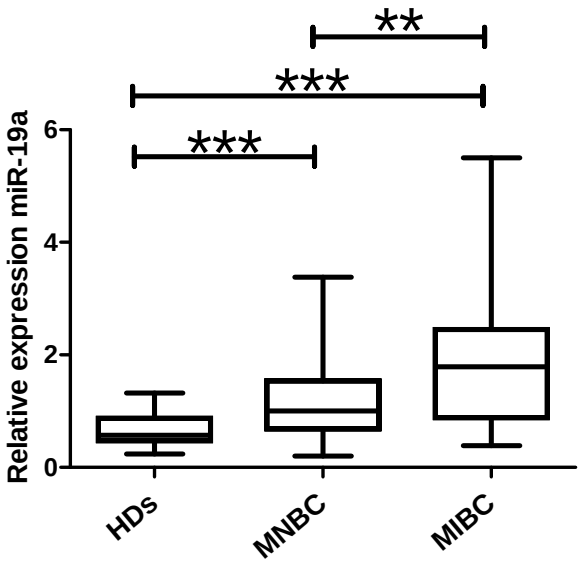
MiR-19a serum levels are predictive of favorable clinical outcome of metastatic IBC patients

MiRNAs released by tumor cells in the tumor microenvironment can gain access to the bloodstream through the newly formed blood vessels around the tumor. Consequently, tumor cell-derived miRNAs can be detected in the serum as circulating miRNAs and may reflect the dysregulated pattern of the parental tumor. Therefore, circulating miRNAs can be used as serum biomarkers for IBC.

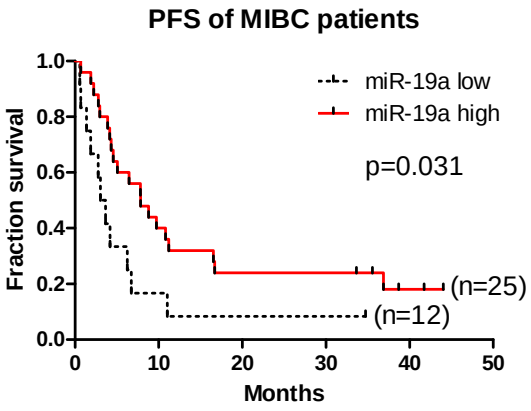
As miR-19a is differentially expressed and secreted by SUM-149 and SUM-159 and had a significant different impact on DC functions, we wanted to assess if differences in levels of miR-19a were also present between the serum of IBC and non-IBC patients and if they correlated with clinical outcome. We evaluated the serum levels of miR-19a in 35 non-metastatic (16 locally advanced breast cancer [LABC], and 16 IBC) and 64 metastatic (27 metastatic non-IBC [MNIBC], and 37 metastatic IBC, [MIBC]) breast cancer patients. While in M0 cohort we did not find significant differences between IBC and LABC patients, in M1 cohort MIBC had higher median levels of miR-19a than MNIBC ($p=0.010$) (Fig. 24a). Then, we evaluated if there was a correlation between the serum levels of miR-19a and the clinical outcome. In M0 cohort there was no correlation; however, in the M1 cohort MIBC patients with high serum levels of miR-19a had longer progression-free survival (PFS: 7.9 vs. 3.0 months; $p=0.031$) and overall survival (OS: 24.6 vs. 11.7; $p=0.018$) than patients with low levels of miR-19a (Fig. 24b). Similar pattern of survival was found also in MNIBC but the differences in the survival curve were not statistically significant. These results apparently seem to

contradict our *in vitro* model, in which SUM-149 secreted higher level of miR-19a and induced higher levels of pro-inflammatory cytokines responsible of the development of tumor cells with aggressive features. However, increased miR-19-mediated activation of DCs leads to increased levels of costimulatory molecules that are necessary for T cell activation. Consequently, a more effective T cell mediated immune response can be achieved. Depending on which T cell mediated immune response is elicited (Th1, Th2, Th-17), different results can occur with different effects on clinical outcome: 1) Th1 lymphocytes can induce an anti-tumor responses mediated by $CD8^+$ CTL and NK cells; 2) Th2 lymphocytes inhibit the anti-tumor responses induced by Th1 lymphocytes; and 3) Th-17 lymphocytes mediate a tumor promoting pro-inflammatory responses. Several factors can affect miR-19a serum levels in different ways and they will be examined in the discussion.

a


**
***
***
Relative expression miR-19a
6
4
2
0
HDs
MNBC
MIBC

b


PFS of MIBC patients
Fraction survival
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Months
miR-19a low
miR-19a high
p=0.031
(n=25)
(n=12)

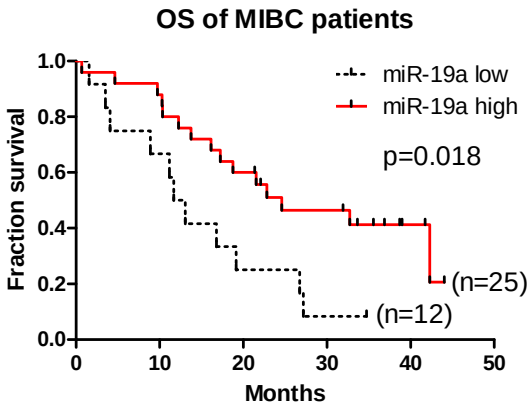

OS of MIBC patients
Fraction survival
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Months
miR-19a low
miR-19a high
p=0.018
(n=25)
(n=12)

Fig.24. Levels of miR-19a in the serum of breast cancer patients and clinical outcome

In a) levels of miR-19a in the serum of MNIBC and MIBC compare with HDs; b) Kaplan-Meier plots of MIBC patients according to the serum levels of miR-19a. Patients with high levels of serum miR-19a had longer PFS (7.9 vs. 3.0 months; p=0.031); and OS (24.6 vs. 11.7; p=0.018).

Table 1. Clinical characteristics of breast cancer patients at the beginning of the study and their association with serum miR-19a levels.

		miR-19a, n (%)		
Characteristic	**n**	**Low**	**High**	***P***
Age at sample collection (years)	54			
< 45	21	7 (15)	14 (26)	.221
≥ 45	78	39 (84)	39 (74)	
Race				
Asian	5	4 (9)	1 (2)	.348
African-American	5	3 (7)	2 (4)	
Hispanic	7	4 (9)	3 (6)	
Non-Hispanic white	82	35 (76)	47 (89)	
IBC				
Yes	56	21 (46)	35 (66)	**.045**
No	43	25 (54)	18 (34)	
Stage				
M0	35	17 (37)	18 (34)	.834
M1	64	29 (63)	35 (66)	
Grade				
1	3	2 (5)	1 (2)	.292
2	26	15 (34)	11 (21)	
3	67	27 (61)	40 (77)	
Hormone receptor				
Positive	63	34 (74)	29 (55)	.060
Negative	36	12 (26)	24 (45)	
HER2				
Positive	51	24 (52)	27 (51)	1.000
Negative	48	22 (48)	26 (49)	
Triple-negative status				
Yes	18	4 (9)	14 (26)	**.035**
No	81	42 (91)	39 (74)	

DISCUSSION

IBC is a very aggressive form of locally advanced breast cancer (LABC) characterized by high ability to invade, grow and spread in the dermal lymphatics of the skin of the breast. Its higher aggressiveness is responsible of worse prognosis compared to non-IBC. So far, little is known about the molecular mechanism that can explain such differences in clinical outcome. Indeed, recent studies performing gene expression profiling by the World IBC Consortium could not identify a definitive molecular signature able to explain the difference in the behavior of tumor cells between IBC and non-IBC. Moreover, the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) reported that contrary to the decreasing trend of non-IBC, the incidence of IBC is continuously increasing (19). Therefore, the discovery of new biomarkers and molecular targets for IBC are needed.

In the tumor microenvironment, the complex network of interactions between cancer cells and tumor associated cells play a central role in driving tumor development and progression. Particularly, paracrine crosstalk between cancer and bystander cells in the tumor microenvironment could regulate tumor dissemination at early stage, even before the primary tumor is clinically detectable. Indeed, tumor associated immune cells can regulate the dissemination of tumor cells in two opposite ways. They can limit the spread of tumor cells by activating an anti-tumor immune response (43, 44), or they can promote tumor progression by their chronic activation and secretion of pro-inflammatory cytokines (IL-6, IL-8, TNF-α) responsible of the development and expansion of tumor cells with CSC-like and EMT characteristics.

As DCs play a central role in the regulation of the immune response, we hypothesized that the cell-free miRNAs released from IBC cells can be taken up and affect the functions of TADC in term of increased migratory ability toward IBC cells and production of pro-inflammatory cytokine IL-6, IL-8, TNF-α.

In an *in vitro* model of tumor microenvironment, we found that the TN IBC cells SUM-149 could induce an increased migration, activation and production of IL-6, IL-8, and TNF-α compared with the TN non-IBC SUM-159 cells. This was mediated by the differential expression and release into the culture medium of miR-19a and miR-146a by SUM-149 and SUM-159 respectively. The uptake of miR-19a had a pro-inflammatory effect, while that of miR-146a had an anti-inflammatory effect. MiR-19a can target PTEN in B cells and induce its downregulation PTEN (85). It was shown that in DCs the downregulation of PTEN by siRNA regulated the PI3K/Akt-mediated signaling and promoted maturation, activation, migration and survival of DCs (95, 96). Furthermore, PI3K/Akt signaling regulated the generation and differentiation of immature monocytes-derived dendritic cells (MoDCs) by GM-CSF and IL-4 signaling (107) and the increased survival by the anti-apoptotic signal of adipocyte-derived hormone leptin (108). This may be relevant in IBC, as obesity was found to be associated with an increased risk of IBC patients (109, 110) and breast cancer patients with high body mass index had high levels of leptin (110).

In our study, we showed that miR-19a could reduce the expression of PTEN and increase the activation of DC in term of pro-inflammatory cytokine production. Furthermore, PI3K signaling can mediate a negative feedback regulation of production

of IL-12 by DC (111). IL-12 plays a central role in the induction of an anti-tumor immune response (Th1-mediated immune response). Therefore, in the IBC tumor microenvironment, the uptake of miR-19a released from tumor cells may have a dual effect on tumor-associated DCs. Indeed, the downregulation of PTEN in tumor-associated DCs can induce an increased activation and production of pro-inflammatory cytokine, but on the other hand it can limit an effective anti-tumor response by reducing the levels of IL-12.

MiR-146a can target the two adapter molecules IRAK1 and TRAF6 that regulate IL-1, TLR and CD40 signaling (91, 112). IL-1-, TLR- and CD40-mediated signaling pathways are important in the process of maturation and activation of DCs. Therefore, the downregulation of IRAK1 and TRAF6 hamper the full activation of DC and accordingly the pro-inflammatory responses (113-115). Therefore, in the non-IBC tumor microenvironment, the uptake of miR-146a released from tumor cells may induce a downregulation of DC functions resulting in reduced ability to produce pro-inflammatory cytokines and stimulate an effective anti-tumor response.

Which role do the pro-inflammatory cytokines (IL-6, IL-8, TNF-α) have in the tumor microenvironment? In the IL-6 signaling cascade, the binding of IL-6 to its receptor IL-6R activates STAT3 (116) and STAT3 is a transcription factor that regulate miR-19a synthesis (117). We showed that IL-6 stimulation was able to induce an upregulation of miR-19a synthesis in SUM-149. Therefore, in the IBC tumor microenvironment, the increased activation of DC by the uptake of miR-19a can induce an upregulation of IL-6 production by DCs that in turn can further stimulate SUM-149 to

synthesize IL-6 in a self-sustaining loop. Moreover, miR-19a can induce downregulation of SOCS-1 (98), a negative regulator of IL-6R, determining and increase in IL-6R-mediated signaling in cancer cells. Recently, it was reported that IL-6 is able to induce EMT in breast cancer (53, 54) cells and sustain growth and proliferation of $CD44^+CD24^+$ stem cell-like breast cancer cells (100). We showed that IL-6 could also increase the synthesis of miR-155 in SUM-149. It was reported that the pro-inflammatory cytokines IFN-γ and IL-6 induced upregulation of miR-155 expression (118) and miR-155 regulates TGF-β-induced EMT (105) and induces chemoresistance (106). Moreover, miR-155 overexpression promotes breast cancer cell proliferation, migration, invasion both *in vitro* and *in vivo* and tumor development in nude mice (61). As we showed that increased levels of miR-19a were associated to the expansion of $CD44^+CD24^+$ population with EMT-like characteristics both miR19a and miR-155 might represent a marker for EMT in this population of cells. Indeed, we showed that transcription factors regulating EMT were upregulated in $CD44^+CD24^+$ cells of SUM-149 and also E-cadherin, an important adhesion molecule responsible of the tumor emboli formation (103). Moreover, IL-6 can induce the expression of the chemokine receptor CXCR4 in breast cancer and the CXCR4/CXCL12 axis may be involved in driving the metastatic process to the bone (119-121). It was reported that, as breast cancer start to disseminate to the bone there is an increase in DC population within the bone that induce tumor progression and bone loss (122). So, DCs might have a role in inside the bone that sustains the survival and proliferation of TN IBC cells with CSC/EMT-like characteristics.

Therefore, the crasstalk between IBC cells and DCs mediated by IL-6 and miR-19a in the TN IBC tumor microenvironment might be responsible of the induction of a population of cells with CSC/EMT-like characteristics that can disseminate even at early stage of tumor development and be responsible of the poor prognosis.

Also IL-8 is involved in the regulation of CSCs. Indeed, it can regulate the self-renewal of breast CSCS and PTEN is negative regulator of IL-8R signaling (51). Therefore, the IL-6-mediated increase of the levels of miR-19a in IBC cells can sustain the proliferation and survival of CSCs by downregulating PTEN and increasing IL-8 signaling. It was reported that IL-8 is also involved in regulation of angiogenesis, invasion in breast cancer (123) and in the retention of DCs at tumor site, that prevents the migration to lymph nodes and the induction of an anti-tumor immune response (124). We showed that SUM-149 and SUM-159 can produce IL-8 and that the culture medium of SUM-149 could induce a higher degree of migration in iDCs. It can be hypothesized that IBC cells can attract iDCs present in tissue surrounding the tumor more efficiently and enhance their activation and functions through the release of miR-19a inside the exosomes. It can also be hypothesized the exosomes may have an effect also at level of lymph nodes. Indeed, exosomes can flow inside the lymphatic vessels and reach either locoregional or distant lymph nodes, where they can induce activation of dendritic cells. Particularly, we showed that $mDC^{SUM-149}$ could produce higher levels of IL-8, compared $mDC^{SUM-159}$. It was reported that SUM-149 cells express the receptors for IL-8 (CXCR1 and CXCR2) and that IL-8 signaling may function as chemotatic factor, induce fibronectin expression, induce morphology changes towards a migratory spindle-like

phenotype through PI3K/Akt signaling pathway (125). Therefore, $mDC^{SUM-149}$ present in the lymph nodes might play a role in the dermal lymphatic invasion.

Besides regulating IL-8 signaling, increased levels of miR-19a might also play a direct role in the regulation of neoangiogenesis in IBC. Indeed, it was reported that overexpression of miR-19a can induce higher levels of neovascularization in Kras-transformed mouse colonocytes, by targeting the anti-angiogenic regulator thrombospondin-1 (Tsp1) (84). Therefore, increased levels of miR-19a in IBC cells might have a role in regulating neoangiogenesis in the TN IBC tumor microenvironment by targeting Tsp1 and inducing higher level of IL-8 in TADC.

Also TNF-α was reported to induce the EMT and enhance invasiveness and migratory properties of breast cancer cells (126-130). Indeed, chronic stimulation with TNF-α can induce EMT and CSC properties in normal breast epithelial and cancer cells by the upregulation of Twist1 and Snail through NF-kB activation (130, 131). Moreover, TNF-α can increase the levels of ceramide in MCF-7 (132, 133) and ceramide was found to trigger the secretion of exosomes (64). Therefore, the increased levels of TNF-α in the TN IBC microenvironment might contribute to the development of breast cancer cells with CSC/EMT-like characteristics and increased the secretion of exosome from IBC cells.

MiR-19a released in the tumor microenvironment may reach the peripheral blood circulation through the newly formed blood vessels around the tumor. Therefore, high levels of miR-19a can potentially be measured in the serum of IBC patients with tumor expressing high levels of miR-19a and correlate with poor outcome. However, besides

tumor cells, other cell type can contribute to the levels of miRNAs in the serum. Indeed, it should be observed that most of the circulating miRNAs originate from blood cells, particularly immune cells (77), and may be expressed also in tumor. Thus, it is not easy to distinguish the individual contribution to the serum levels of miRNAs. It can be assumed that the levels of circulating miRNA may represent the general clinicopathological condition of the cancer patients and originate from both blood and tumor cells.

Several factors may affect serum levels of miR-19a. We showed that miR-19a released from SUM-149 cells can induce an increased activation and maturation of DCs and to secrete higher levels of pro-inflammatory cytokines that may be responsible of poor IBC outcome. On the other hand increased activation and maturation of DCs is accompanied by upregulation of costimulatory molecules that consequently leads to an increased ability to activate different subset of T lymphocytes: Th1, Th2, and Th17. In turn, T lymphocytes can migrate to tumor site where they carry out their effector functions as tumor-associated (TAL) or tumor-infiltrating lymphocytes (TIL). Th1 lymphocytes can induce an anti-tumor responses mediated by $CD8^+$ CTL and NK cells and the presence of tumor-associated $CD8^+$ T cells is a favorable prognostic factor in breast cancer (134, 135). Recently, it was reported that antigen-specific effector $CD8^+$ CTL and Th1 lymphocytes upregulate miR-19a upon activation (136, 137) and lymphocytes can release miRNAs through exosome secretion (73). Therefore, increased serum levels of miR-19a can result from an effective Th1-induced anti-tumor immune response that may be responsible for the good prognosis. Moreover, an effective anti-

tumor response mediated by $CD8^+$ CTL and NK cells can determine an extensive IBC tumor cell death, causing an increased release of cellular miRNA and therefore contributing to high levels of miR-19a in patients with good prognosis. Indeed, we showed that high levels of miR-19a in the serum of MIBC patients correlated with a better clinical outcome. It can be hypothesized that IBC patients with poor outcome are unable to develop an effective anti-tumor immune response, evidenced by low levels of miR-19a in the serum. Moreover, the miR-19a detected in the serum might originate in part by IBC cells with aggressive features, such CSC/EMT-like phenotype.

CONCLUSION

We concluded that the TN IBC cell line SUM-149 can represent a valuable model to study the effect of tumor microenvironment crosstalk between IBC and tumor associated DCs. In particular, SUM-149 can affect the function of DCs through the release of miR-19a that subsequently can be taken up by DCs, leading to a higher activation status and responsiveness to stimulatory signals by downregulating PTEN and SOCS-1 (PI3K/Akt, CD40, IL-6, IL-8 and TNF-α signaling). In turn, the increased activation of DCs determined a higher secretion of pro-inflammatory cytokines (IL-6 and TNF-α) that induced the development of tumor cells with aggressive characteristics (EMT/CSC-like phenotype) responsible of tumor progression and poor prognosis in IBC patients. On the other hand, serum miR-19a may represent a valuable prognostic serum biomarker for MIBC patient with good clinical outcome.

CAVEAT AND WEAKNESS

Transfer of miRNAs from breast cancer cells to iDCs

We hypothesized that miRNAs are released by breast cancer cells and are taken up by iDCs. Several studies showed that miRNAs can be physiologically released by normal and tumor cells through three mechanisms such apoptosis, necrosis, and active secretion (68, 69, 133) either encapsulated inside exosomes or in a complex with Ago2. Our *in vitro* model is a controlled system in which the viability of breast cancer cell lines was always > 90% across different experiments. The caveat is that we can exclude that the mechanism of miRNA release in our model is necrosis or apoptosis. This can represent a weakness, as our *in vitro* model may represent only partially the overall mechanism of miRNA transfer in the tumor microenvironment. Indeed, apoptosis and necrosis can have a relevant impact on extracellular miRNA levels *in vivo*, as multiple factor can influence the tumor cell viability (hypoxia, nutrient availability, chemo- and radiotherapy, cellular and soluble apoptotic signals delivered by immune cells).

To further prove our hypothesis of miRNA transfer, several approaches may be used. Particularly, miRNA transfer can be inhibited by targeting either the secretion or the uptake of miRNAs. The process of secretion through exocytosis can be inhibited by culturing breast cancer cells at $4^{o}C$, or using secretion inhibitor chemicals, like brefeldin A (BFA) or cytochalasin. Furthermore, recently it was

reported that miRNA can be secreted through ceramide-dependent pathway (64), thus this strategy to inhibit the secretion of miRNA can be pursued. On the other hand, the uptake of miRNAs can be targeted by EDTA (reduction of free $Ca2^{+}$), fixation with paraformaldehyde.

Effect of miRNA uptake on DC functions

The second point of our hypothesis was that the transfer of miRNAs can affect DC function (activation, maturation, migration and cytokine synthesis). The weakness of our experimental design is that we did not completely prove that miR-19a and miR-146a, released from SUM-149 and SUM-159 respectively, were the major player in regulating DC functions. Indeed, we only used mimic miR-19a and miR-146a to increase their levels on DCs, but other miRNAs may be involved. Furthermore, in addition to miRNAs, exosomes contain mRNA, DNA and proteins with regulatory functions (70, 72, 138). Therefore, it is necessary to assess if the knocking down miR-19a and miR-146a can inhibit respectively the pro- and anti-inflammatory functions of DCs.

Another weakness is that we evaluated the effect of the miR-19a and miR-146a overexpression only on cytokine synthesis. To fully demonstrate the effect of breast cancer miRNA transfer on DC functions, it is necessary to assess the effect of both mimic and inhibitor miR-19a and miR-146a on the expression of costimulatory/activation, maturation surface markers and migration of DCs.

Finally, additional breast cancer cell lines could be used to prove the role of miR-19a and miR-146a in the regulation DC functions. As MCF-7 and MDA-231did not

affect DC functions when co-cultured in transwell and expressed low levels of miR-19a and miR-146a compared with SUM-149 and SUM-159, they can be transfected with miR-19a and miR-146a mimic and co-cultured with iDCs.

SUM-149 and SUM-159 are not representative of all IBC and non-IBC

In our study, we proposed SUM-149 as a model for IBC. It should be pointed out that SUM-149 is not representative of all IBC, as this is TN IBC. Thus, SUM-149 may be a valuable model for patients with TN IBC with subtype. Indeed, when we used the $HER2^{+}$ IBC cell lines KPL-4, SUM-190, and IBC-3, we did not observe significant changes in DC functions (costimulatory/activation and maturation surface markers). Furthermore, due to tumor heterogeneity, this model could be valid only for a subset of patients within the same subtype. In support of this hypothesis, the two non-IBC TN breast cancer cell lines SUM-159 and MDA-231 had significant different effects on DC functions. Further studies are required to establish the molecular signature of SUM-149 and SUM-159 that are responsible of the different effects on DC functions. This would help the identification of subsets of patients within the TN subtype (IBC and non-IBC) with considerably different clinical outcome.

IL-6-mediated induction of EMT

We showed that long-term cell culture induced the enrichment of SUM-149 cells with EMT and CSC phenotype. This process was associated to an increase in the levels of IL-6 in the cell culture supernatant. A caveat is that we did not demonstrate the direct

effect of IL-6 in the induction of EMT and CSC expansion. Indeed, it may be an effect rather than the cause. Therefore, it is required to prove the direct effect of IL-6 in this setting, by the use of either recombinant IL-6 and anti-IL-6 blocking antibody in the SUM-149 cell culture.

MATERIAL AND METHODS

Material and Methods

Cell lines

The human breast cancer cell lines MCF-7 (ER/PR$^+$), MDA-231 (ER/PR$^-$, HER2$^-$ triple negative: TN), KPL-4 (HER2$^+$, inflammatory breast cancer) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture with DMEM/F-12 medium supplemented with 10% FBS (Tissue Culture Biologicals, Seal Beach, CA) and 1% of Antibiotic-Antimycotic 100X (Gibco, Carlsbad, CA). The two TN cell lines SUM149 (IBC) and SUM-159 (non-IBC), SUM-190 (HER2$^+$, inflammatory breast cancer), kindly provided by Dr. N. Ueno (The Morgan Welch Inflammatory Breast Cancer Research Program and Clinic, The University of Texas MD Anderson Cancer Center, TX) and IBC-3 (HER2$^+$, inflammatory breast cancer), kindly provided by Dr. W. Woodward (Radiation Oncology, The University of Texas MD Anderson Cancer Center) were maintained in culture with Ham's/F-12 medium supplemented with % FBS (Tissue Culture Biologicals, Seal Beach, CA), 5 μg/mL insulin, 1 μg/mL hydrocortisone and 1% of Antibiotic-Antimycotic 100X (Gibco, Carlsbad, CA). The immortalized human mammary epithelial cells (HMLE) were kindly provided by Dr. S. Mani (Molecular Pathology, The University of Texas MD Anderson Cancer Center) and were maintained in DME: F12 media (1:1) supplemented with insulin, EGF, hydrocortisone and 5% calf serum.

Generation of DCs from monocytes

Immature dendritic cells (iDCs) were generated from monocytes isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors. Particularly, PBMCs isolated from total blood by Ficoll-Hypaque density centrifugation were plated in T-75 flasks (Costar Corning, Corning, NY) at a density of 4×10^6 cells/mL of RMPI 10% FBS and allowed monocytes to adhere to the plastic of the flasks. After ~4 h at 37°C, non-adherent cells were removed with two washes and monocytes were cultured 5 days in RMPI 10% FBS supplemented with rhIL-4 1000 U/ml (R&D Systems, Minneapolis, MN) and GM-CSF 1000 U/ml (Leukine, Berlex, Richmond, CA). At day 2, 2 mL of spent culture medium were replenished with 2 mL of fresh RMPI 10% FBS with rhIL-4 1000 U/ml and GM-CSF 1000 U/ml. At day 5 iDCs were harvested and used for co-culture experiments. To induce maturation, iDCs were stimulated with LPS 100 ng/ml (Lipopolysaccharide from E. coli J5, Sigma, St. Louis, CA).

FACS analysis

The differentiation and the maturation of iDCs were evaluated assessing the expression of CD80, CD86, CD83, CD40, HLA-DR and CD11c (BD Biosciences, San Jose, CA) using phycoerythrin-coupled (PE) antibodies anti-CD80, CD86, CD83, CD40, HLA-DR and allophycocyanin-coupled (APC) antibody anti-CD11c. SUM-149 cells were evaluated for the presence of breast cancer stem cells (BCSCs) by expression of the CD44 and CD24 surface markers and the ALDH enzymatic activity using fluorescein isothiocyanate-coupled (FITC) antibody anti-CD44, PE antibody anti-CD24 (BD

Biosciences) and ALDEFLUOR™ Kit (Stem Cell Technologies). FACS analysis was carried out using BD™ LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software Version 8.8.6 (TreeStar).

Transwell co-culture

To prevent cell-to-cell contact and evaluate the effect of soluble factors released by breast cancer cells on DC functions, cultures in transwell were performed. Transwell with 1.0 μm pore size membrane (Millipore, Temecula, CA) were used and 0.3 X 10^6 breast cancer cells/well were plated in the upper chamber and 0.1 x 10^6 iDCs/well were plated in the lower chamber of 24-well plate (Corning Costar, NY). RPMI 10% FBS was used as culture medium in all co-culture experiments. The cells were cultured at 37^{o}C for different time (24 or 48 h) according to the experimental setting.

Exosome and miRNA transfer

To assess the transfer of miRNAs from breast cancer cells to iDCs, SUM-149 and SUM-159 cells were labeled with PKH67 green fluorescent membrane linker-dye (Sigma) following the manufacturer's instructions and transfected with Dy547-labeled meridian mimic negative control (Dharmacon, Lafayette, CO). After extensive washing steps, PKH67-labeled SUM-149 and SUM-159 cells (0.3 X 10^6 cells/well) were plated in the upper chamber of Transwell (1.0 μm pore size membrane) and 0.1 x 10^6 iDCs/well were plated in the lower chamber of 24-well plate. After 24 h of culture, iDCs were harvested and analyzed for fluorescent exosome uptake. Briefly, iDCs cells were fixed

with 1 mL of PBS 4% paraformaldehyde for 1 h at 4^{o}C, then the cell suspension was washed in PBS and centrifuged onto microscope glass slides using cytospin centrifuge (Cytopro, Wescor Inc., Logan, UT) at 400 rpm for 5 minutes. Finally, SlowFade Gold Antifade reagent with DAPI (Invitrogen) was added to the slides to stain nuclei and protect fluorescent dye from photo-bleaching. Pictures were captured by either Nikon Eclipse 80i fluorescence microscopy or Olympus FV1000 laser-scanning confocal microscopy and the images were analyzed with NIS-Elemnts BR 3.1 and Olympus Fluoview (Ver.3.0 Viewer) respectively. The transfer of exosomes to iDCs was also evaluated by FACS analysis. To further confirm the transfer from breast cancer cells to iDCs, exosomes were isolated from the supernatants of PKH67-labeled SUM-149 and SUM-159 cells using exosome precipitation solution (Exo-Quick, System Biosciences) according to the manufacturer's instructions, added to iDCs and cultured for 24 h. Then, iDCs were harvest an analyzed for fluorescent exosome uptake with laser-scanning confocal microscopy (Olympus FV1000).

RNA extraction and qRT-PCR

Total RNA was isolated from DCs, breast cancer cell lines, exosomes, cell culture supernatant, and breast cancer patient serum using the Total RNA Purification Kit (Norgen Biotek Corporation, Thorold, ON, Canada) following the manufacturer's instructions. For the miRNAs extracted from supernatants, it was used the culture medium of cell line with 70-80% confluence and viability >90%. Supernatants were centrifuged at 3000 rpm for 15 minutes and than filtered through 0.2 mm filter to remove

cellular debris. The concentration of total RNA was measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and then RNA was immediately stored at -80^{o}C. Total RNA isolated was reversed transcribed to cDNA using either TaqMan MicroRNA Reverse Transcription Kit or High Capacity cDNA Reverse Transcription Kit for miRNA and gene expression analysis respectively (Applied Biosystems, Foster City, CA). Briefly, for miRNA expression analysis 10 ng of total RNA were reverse transcribed in a total volume reaction of 15 μL, following the manufacturer's instructions. The reaction was performed using the Veriti Thermal Cycler (Applied Biosystems) at 16 ^{o}C for 30 minutes, 42^{o}C for 30 minutes and 85^{o}C for 5 minutes. For gene expression analysis, 300 ng of total RNA were reverse transcribed in a total volume reaction of 50 μL and the reaction was performed at 25^{o}C for 10 minutes, 37^{o}C for 120 minutes and 85^{o}C for 5 minutes. The expression levels of miRNAs and genes were measured by qRT-PCR using the TaqMan MicroRNA or Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The reaction was performed using the 7900HT Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA) at 95^{o}C for 10 minutes and 40 cycles at 95^{o}C for 15 seconds and 60^{o}C for 60 seconds. The relative expression of miRNAs and genes was calculated using the equation $2^{-\Delta Ct}$, where ΔCt = mean $Ct_{miRNA/gene}$ – mean $Ct_{U6\ snRNA/GAPDH}$, Ct = threshold cycle.

Breast cancer cell line and iDC transfection

To confirm the transfer of miRNAs from breast cancer cell lines to iDC, SUM-149 and SUM-159 cells were transfect with 100 nM miRIDIAN mimics has-miR-19a and hsa-miR-146a respectively (Dharmacon) at the concentration of 0.1 X 10^6 cells/well in 24-well plats using DharmaFECT transfection reagent (Dharmacon). After 24 h at 37^{o}C transfected cells were extensively washed and maintained in culture for additional 24h in their culture medium. Then, 0.1 X 10^6 cells/well of transfected SUM-149 and SUM-159 cells were plated in the upper chamber of Transwell (1.0 μm pore size membrane) and 0.1 X 10^6 iDCs/well were plated in the lower chamber of 24-well plate and cultured for 24 h at 37^{o}C. The levels of miR-19a and miR-146a were evaluated in iDCs by qRT-PCR.

To evaluate the effect of miR-19a and miR-146a on the expression levels of their target genes and on cytokines production, iDCs were transfected with 50 nM miRIDIAN mimics has-miR-19a and 100 nM miRIDIAN mimics hsa-miR-146a for 24-48 h and then PTEN, SOCS-1, IRAK1 and TRAF6 mRNA levels were evaluated by qRT-PCR. IL-6 and TNF-α mRNA levels were measured after 3 h stimulation with LPS 100ng/ml.

Luminex Bead Array Assay for cytokine detection

To evaluate the effect on the levels of secreted cytokines, iDCs were co-cultured with SUM-149 and SUM-159 cells in Transwell (1.0 μm pore size membrane). Briefly, 0.3 X 10^6 cells/well of either SUM-149 or SUM-159 cells were plated in the upper chamber and 0.1 X 10^6 iDCs/well were plated in the lower chamber of 24-well plate for

24 h. To induce maturation and synthesis of cytokines, iDCs were stimulated with LPS 100 ng/ml for 18 h. The supernatants were collected, centrifuged and cytokine levels were evaluated using Milliplex MAP Human Cytokine/Chemokine Panel following the manufacturer's instructions (EMD Millipore, Billerica, MA) and analyzed by a Luminex 100 Analyzer running BioPlex 4.2 Software (Bio-Rad).

Cell migration assay

The migratory ability of DCs was evaluated in Transwell Chamber with 8.0 μm pore size membrane. Briefly, 5 X 10^5 cells/well were plated in the upper chamber in RPMI serum-free and culture for 16 h. The conditioned media from MCF-7, SUM-149 and SUM-159 cells were used as migration stimuli. Conditioned media were prepared by culturing MCF-7, SUM-149 and SUM-159 cells in RMPI 10 FBS for 24-48 h until 70-80% cell confluence. Then the supernatants were centrifuged 2000 g for 10 minutes and filtered to remove cellular debris. The iDCs that migrated into the lower chamber were counted under microscopy using Neubauer chambers and the chemotactic index was calculated as number of iDCs that migrated to MCF-7, SUM-149 and SUM-159 conditioned media divided by number of iDCs that migrated to RPMI 10%. We also evaluated the migratory ability of iDCs that were previously co-culture with MCF-7, SUM-149 and SUM-159 cells in Transwell (1.0 μm pore size membrane) using the same experimental setting used for iDCs.

Detection of miR-19a in the serum of breast cancer patients

Ninety nine breast cancer patients were enrolled in 2 laboratory-based protocols approved by the institutional review board at The University of Texas MD Anderson Cancer Center (Houston, Texas) consisting in: 35 non-metastatic (16 locally advanced breast cancer [LABC], and 16 IBC) and 64 metastatic (27 metastatic non-IBC [MNIBC], and 37 metastatic IBC, [MIBC]) breast cancer patients. Serum was collected, after written informed consent was obtained, from all patients at the beginning of the study (before a new line of therapy for patients with M1 disease and before first-line therapy for patients with M0 disease). Serum were also collected from age-matched healthy donors (HDs) and used as a control. Patients' clinical and histopathological information is summarized in Table 1.

The serum was isolated from 10 mL of peripheral blood of breast cancer patients and HDs in BD Vacutainer serum tubes (Becton Dickinson Vacutainer, Franklin Lakes, NJ). Thirty minutes at ambient temperature were used to allow the blood to clot, and then the serum was separated by centrifugation within 3-4 h after phlebotomy and stored at -80°C in 1 mL aliquots. Total RNA was isolated using the Total RNA Purification Kit (Norgen Biotek Corporation, Thorold, ON, Canada) following the manufacturer's instructions, starting with 100 μL of serum.

Statistical analysis

The differences in the expression levels of CD80, CD86, CD40, CD80, HLA-DR, ALDH1, miRNAs, and genes were evaluated using GraphPad Prism 5.04 software

(GraphPad Software Inc., San Diego, CA). The two-tailed Student's paired and unpaired t test was used for the statistical analysis. The non-parametric 2-tailed Mann Whitney-U test was used to compare serum levels of miR-19a in the different groups of patients. The Kaplan-Meier method was used to evaluate the differences of progression-free survival (PFS) and overall survival (OS) durations of breast cancer patients that were divided in two groups according to levels of miR-19a: patients with high and low levels of miR-19a. To define high and low levels of miR-19a, we used a cut-off that corresponded to the mean values of each miRNA in the serum of HDs plus 2 standard deviations. The survival duration was calculated from the date of sample collection at the beginning of the study. A log-rank test was used to analyze the differences between groups and analyzed using GraphPad Prism 5.04 software. The p-values <0.05 were considered statistically significant ($p<0.05$ is indicated with: *; $p<0.01$ is indicated with: **; $p<0.001$ is indicated with: ***).

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VITA

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