Angiogenic Characteristics of Tumor-Associated Dendritic Cells in Ovarian and Breast Cancer Models

A Thesis Presented to
The College of Arts and Sciences
Ohio University

In Partial Fulfillment of the Requirements for Graduation with Honors in Biological Sciences

By
Deana Lewis
April 2016
Table of Contents

Abstract..................................................................................................................................................i

I. Introduction.......................................................................................................................................1
   a. General Characteristics of Dendritic Cells..........................................................2
   b. Use of Dendritic Cells as Vaccines for Tumor Therapies.........................5
   c. Ovarian and Breast Cancer..............................................................................9
   d. The 4T1 model of Breast Cancer and the ID8 model of Ovarian Cancer..................................................................................................................12
   e. Tumor Microenvironment of Ovarian and Breast Cancer..................13
   f. Leukocytes as Promoters of Angiogenesis in Cancer.............................15
   g. Characteristics of Tumor-Associated DCs..................................................16
   h. Dendritic Cells as a Source of Angiogenic Factors.................................17
   i. Hypothesis...........................................................................................................18

II. Materials and Methods...............................................................................................................19
   a. Reagents for molecular biology.............................................................19
   b. Polymerase Chain Reaction.................................................................19
   c. Gel Analysis of PCR fragments...............................................................21
   d. Reagents for flow cytometry analysis....................................................22
   e. Flow cytometry analysis procedure.......................................................22

III. Results.......................................................................................................................................25
   a. Expression of angiogenic molecules by ovarian cancer DCs at the level of RNA.................................................................................................................25
b. Analysis of chemokine molecules in DCs recovered from breast cancer tumors (solid tumor model) .............................................29

c. Flow Cytometry Analysis of Dendritic Cells in the Tumor Microenvironment ..........................................................32

IV. Discussion ..........................................................................................................................55

V. Acknowledgements .............................................................................................................61

VI. References ..........................................................................................................................62
Abstract

The tumor microenvironment (TME) contains different types of cells in addition to tumor cells. Among them, fibroblasts, endothelial cells and immune cells can be found in the TME of several types of cancers. In particular, regarding immune cells, infiltration of T cells (specific immune cells) and antigen presenting cells such as macrophages and dendritic cells (innate immune cells) has been observed. These immune cells, rather than fighting the tumor can contribute to tumor growth by suppressing the anti-tumor immune response or by promoting the generation of new vessels that can supply the growing tumor with nutrients. This process is named angiogenesis, and it has been reported that macrophages infiltrating tumor can produce angiogenic factors. Reports from our lab indicate that dendritic cells, another type of antigen presenting cell, can also contribute to tumor angiogenesis. The main function of dendritic cells in healthy individuals is to process antigen material (foreign material) and present it on the cell surface to the T-cells of the immune system. T cells are then activated and a specific immune response against this antigen is established. In this way, dendritic cells act as messengers between the innate and the adaptive immune systems. In this study, dendritic cells isolated from murine 4T1 breast and ovarian tumors were analyzed by molecular biology (polymerase chain reaction) and immunological techniques (flow cytometry) to identify the expression of angiogenic molecules and chemokine receptors. The main goal of the study was to highlight the potential of dendritic cells to migrate towards the TME and promote tumor growth by producing angiogenic factors.
Introduction

Dendritic cells (DCs) are a group of cells that provide critical information on the original antigen context to trigger a diverse spectrum of appropriate protective responses. These cells are specialized in antigen processing and can efficiently present endogenous and exogenous antigens in both major histocompatibility complex class (MHC)-I and MHC-II contexts respectively. DCs present antigens to T cells, the orchestrators of specific immune responses. The presentation happens through interaction of processed antigen (as peptides) associated to MHC molecules on the surface of the DCs and the receptor for antigen that T cells carry (TCR). In particular, MHC-I molecules present antigenic peptides that come from cytosolic antigen (for example due to a viral infection) to CD8 T cells; while MHC-II molecules present antigenic peptides that come from the outside of the cells and are captured by phagocytosis, to CD4 T cells. Other antigen presenting cells in our body are macrophages and B cells. Although all of our nucleated cells carry MHC-I molecules, only antigen presenting cells express MHC-II. In contrast to other antigen presenting cells such as macrophages, DCs can present exogenous antigen through MHC-I; and cytosolic antigen through MHC-II. The presentation of exogenous non-cytosolic antigens on MHC-I by dendritic cells relies on “cross presentation”, which is a phenomenon critical for immunity against viruses and intracellular bacteria. On the other hand, unconventional presentation of endogenous cytosolic antigens on MHC-II by dendritic cells relies in contrast on autophagy.
General Characteristics of Dendritic Cells

The main function of DCs is to present antigen to T cells, which leads to the activation of T cells that specifically recognize the antigen. These T cells then organize specific immune response against the pathogen (or infected cell, or tumor cell) that carries the same antigen. DCs also induce and maintain immune tolerance. This means that they educate the immune system to not respond to self-antigens. They appear to work to maintain immune memory in tandem with B-cells. Immunological memory is very important, because it allows our body to respond faster the second time a pathogen infects us. DCs are present in those tissues that are in contact with the external environment and the inner lining of certain parts of the body. Once activated, they migrate to lymph nodes where they interact with T-cells and B-cells to initiate and shape the adaptive immune response. Furthermore, DCs are responsible for initiating all antigen-specific immune responses. As such, they are master regulators of the immune response and serve this function by linking the microbial sensing features of the innate immune system to the exquisite specificity of the adaptive response. DCs help guide the immune system to respond to foreign antigens while avoiding generation of autoimmune responses to self. A diagram of some key agents and weapons of the immune system is set forth in Figure 1.

As mentioned above, DCs reside in peripheral tissues where they act as the sentinels of the immune system, continuously patrolling the environment in search of antigen. At this stage they possess an ‘immature’ phenotype, which is mainly characterized by a low surface expression of MHC class I and II molecules. In
addition, immature DCs express low levels of surface costimulatory molecules. The interaction of costimulatory molecules on DCs with costimulatory molecules carried by T cells is needed for full activation of T cells. This signal provided by DCs to T cells is named signal 2, in opposition to signal 1, which is the interaction of MHC molecules with TCRs. Immature DCs are specialized in the recognition and uptake of antigens. Exogenous antigens are internalized and processed by DCs, and the antigenic peptides are presented in the MHC class II complexes on the cell surface. Endogenous antigens, either self proteins or viral proteins, are cleaved into peptides by proteasomes and assembled into stable MHC class I–peptide complexes in the endoplasmic reticulum, which are subsequently transported to the cell surface. (Lesterhuis, 2004). Of importance for DC-based vaccines in cancer immunotherapy is the finding that internalized antigens from exogenous sources, such as apoptotic or necrotic tumor cells, may also be present in MHC class I. Thus, DC may present tumor antigens to both CD4 + and CD8 + T cells.

DCs prominently express both major histocompatibility complex class (MHC) I and II molecules (Nussenzweig et al., 1980, Steinman et al., 1979), which are potent stimulators of T cells in primary mixed leukocytes reactions (MLR) (Steinman and Witmer, 1978). In addition, all DCs share the capability to efficiently uptake and process antigens for presentation to naïve T-cells. The unconventional presentation of exogenous noncytosolic antigens on MHC-I by DCs relies on cross presentation (Bevan, 1976). Dendritic cells degrade their engulfed cargo slowly and can control lysosomal degradation potentially to preserve peptides for T-cell recognition (Savina
This activity is influenced by the maturation status of the DC, with lipopolysaccharide exposure enhancing lysosomal acidification and antigen proteolysis. The glycolytic rate of DCs is also affected by toll-like receptor stimulation triggering a circuit that ensures the de novo synthesis of fatty acids critical for proper DC activation (Everts et al., 2014). DCs are strategically positioned at body barriers and also organ entry ports, such as the splenic marginal zone. To ensure stimulation of naive T-cells, DCs require efficient directional migration toward T-cell zones either within their respective lymphoid organ of residence or toward remote tissue-draining lymph nodes (LNs).

Figure 1. Dendritic Cell Characteristics (Avigan, 2004).
Use of Dendritic Cells as Vaccines for Tumor Therapies

DCs are paradoxically important in cancer due to their capability to generate either immunity or tolerance. Given that their central role is controlling the immune response in patients with cancer, DCs are a critical cell type that must be considered in cancer immunobiology. DCs have become the natural agents for antigen delivery. Thus DCs are an essential target in efforts to generate therapeutic immunity against cancer. Vaccination strategies involving DCs have been developed owing to the special properties of these cells in coordinating innate and adaptive immune responses. The aim of DC vaccination is to induce tumor-specific effector T-cells that can reduce the tumor mass specifically and that can induce immunological memory to control tumor relapse (Paluka, 2012). In this process, the first step would be to provide the DCs with tumor-specific antigens which can be done by inducing DCs to take up the tumor-specific antigen in vivo. Tumor-specific antigens are molecules only expressed by tumor cells. Mouse models of cancer demonstrate that DCs can capture tumor antigens that are released from tumor cells and cross present these antigens to T-cells. This results in generation of tumor-specific cytotoxic T lymphocytes (CTLs) that contribute to tumor rejection.

The vaccines focusing on dendritic cells offer a broader potential immune response because dendritic cells are the master cells of the immune system. When the DC’s are activated against a particular pathogen the, in turn, mobilize all of the other agents (T-cells, B-cells) to attack that pathogen. Many of the therapeutic vaccines for
cancer have targeted existing dendritic cells *in situ* in a patient’s body, by administering various compounds or factors that are designed to attract dendritic cells to the tumor (Avigan 2004). The idea behind this “natural vaccination” is to put DCs in the presence of tumor antigens. Taking into account that the TME is very immunosuppressive, DC activating molecules are administered in this context to allow the DCs to migrate towards the lymph nodes once they capture tumor antigen (for example, from dead tumor cells). This need to activate DCs in the TME to make them act in a way that will activate T cells to attack the tumor is very relevant. Tumors attract immune cells and turn them into collaborators of tumor growth. In case of a DC, tumors can prevent their activation (maturation) but not their capability to engulf antigen. These immature DCs have low levels of costimulatory molecules as described above. If these DCs interact with T cells, they can induce tolerance. This means that the T cell will not attack the tumor cells.

With the emergence of the tumor microenvironment as an essential ingredient of cancer malignancy, therapies targeting the host compartment of tumors have begun to be designed and applied in the clinic. Neoplastic cells have been a focus of interest in cancer research for several decades. This approach led to the identification of key oncogenes or oncogenic signaling pathways targetable for therapeutic interventions (Sounni, 2012).

Therapies targeting cellular constituents of the tumor microenvironment, notably endothelial and inflammatory cells, have showed benefits but also have several limitations. When targeting angiogenesis, the anti-VEGF antibody increases
overall survival or progression-free survival of patients with metastatic colorectal cancer, non-small cell lung cancer and breast cancer when given in combination with conventional chemotherapeutic regimens (Ebos, 2011). Sunitinib, a multireceptor tyrosine kinase inhibitor, also offers a clinical benefit for patients with renal cell carcinoma and advanced gastrointestinal stromal tumors, a benefit that could be in part due to its c-KIT–inhibitory activity. Sorafenib, an antiangiogenic tyrosine kinase inhibitor that also targets Raf kinase activity, has been approved for the treatment of renal cell carcinoma and liver cancer. Overall, the survival benefits of antiangiogenic drugs have been rather modest thus far and most cancer patients stop responding or do not respond at all to the antiangiogenic therapy (Ebos, 2011). Antiangiogenic drugs used in the clinic are centered on the blockade of the VEGF-signaling pathway whereas VEGF-independent angiogenic factors such as fibroblast growth factor, angiopoietins, placental growth factor (PIGF), matrix metalloproteases (MMPs), and ECM molecules are worth considering (Barker, 2012 and Van de Veire, 2010).

There are therapies that target inflammation of the tumor microenvironment. Chronic inflammation contributes to cancer development and clinical and experimental data indicates that the presence and activation of chronic innate immune cell types like neutrophils, macrophages and mast cells (MCs) promote cancer development (Sounni, 2012). Advances in tumor immunology have highlighted a high diversity in tumor-infiltrating leukocyte subsets that can play antagonist functions. Depending on their polarization status, immune cells can exert either antitumor or protumor functions. Several tumor-promoting inflammation inhibitors are designed to:
inhibit signal transducers and transcription factors that mediate survival and growth, inhibit tumor-promoting chemokines and cytokines that promote tumor infiltration and deplete the tumor-promoting immune and inflammatory cells that promote development and progression (Sounni, 2012). Preclinical evidence supports the use of anti-inflammatory drugs in cancer prevention and therapy. Several anti-inflammatory drugs have been found to reduce tumor incidence when used as prophylactics, and to slow down tumor progression and reduce mortality when used as therapeutics, such as cyclooxygenase 2 inhibitors in colorectal cancer and in breast and colorectal cancer resistant to chemotherapy (Kang, 2011).

Carcinoma-associated fibroblasts (CAFs) are involved in cancer progression and metastasis through their ability to enhance tumorigenecity, angiogenesis and metastatic dissemination of cancer cells compared with normal fibroblasts. These CAFs contain a membrane-bound serine protease called fibroblast activation protein (FAP) that is not detected in normal fibroblasts. FAP expression has been associated with an overall poorer prognosis in several cancer types, including ovarian cancer but not in breast cancer (Brennen, 2012). Attempts to block enzymatic activity of FAP with small molecule inhibitors combined with docetaxel have resulted in lowered survival rates of lung cancer patients (Sounni, 2012). An alternative could be to use the enzymatic activity of FAP localized specifically in the tumor stroma to activate cytotoxic prodrugs. This strategy is expected to enhance drug efficacy delivered to the tumor microenvironment (Brennen, 2012).
Ovarian and Breast Cancer

Mouse models representing human spontaneous ovarian and breast cancer are needed to understand how epithelial ovarian and breast cancers differ from cell tumors. Ninety percent of ovarian cancer is thought to arise from the epithelium and its inclusion cysts due to multiple genetic changes (Fong, 2009). The BRCA1 gene increases risk of female breast and ovarian cancers. To address specific roles of BRCA1 in the normal development and cancer pathogenesis, mouse models were developed. It has been shown that mammary epithelium-specific activation of BRCA1 alone is insufficient for cancer induction. They can be induced by concurrent inactivation of BRCA1 together with p53 (Harlan, 2015). p53 is another tumor suppressor gene commonly inactivated in familial breast carcinomas. Mouse BRCA1/p53 deficient tumors are mainly basaloid, no expression of estrogen receptor or progesterone receptor (Liu et al., 2007). Pathogenic mutations in the BRCA1 and BRCA2 genes confer high risks of ovarian and breast cancer (Miki et al., 1994; Wooster et al., 1995). The Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA) has provided convincing evidence that variants identified through a genome-wide association study (GWAS) of breast cancer are also associated with the risk of developing breast cancer for BRAC1 and/or BRAC2 mutation carriers (Antoniou et al., 2008a).

Most ovarian tumors originate from the surface epithelial lining and can be categorized into four major types: serous, endometrioid, mucinous, or clear cell
tumors, (Auersperg, 2002), with the serous subtype being by far the most prevalent. Human ovarian surface epithelial cells are often found in the form of inclusion cysts and clefts, which may represent the earliest stage of neoplastic transformation leading to primary ovarian tumors (Auersperg, 2002). Dissemination of ovarian cancer cells within the peritoneal cavity is the result of the exfoliation of cells from the primary tumor, which subsequently disperse in the whole abdomen by the physiological flow of peritoneal fluid. The ability of ovarian tumor cells to metastasize requires reorganization of the actin cytoskeleton network, particularly at sites of focal adhesion complexes, and changes in cellular adhesion molecules, growth factor receptors and intracellular signaling kinases (Bast, 1993). In addition, among women, breast cancer is the most commonly diagnosed cancer after nonmelanoma skin cancer, and it is the second leading cause of cancer deaths after lung cancer. A possible genetic contribution to both breast and ovarian cancer risk is indicated by the increase incidence of these cancers among women with a family history and by the observation of some families in which multiple family members are affected with breast/ovarian cancer in a pattern compatible with an inheritance of autosomal dominant cancer susceptibility (American Cancer Society, 2016). Figure 2 and 3 depict some of the classical inheritance features of a deleterious BRCA1 and BRCA2 mutation, respectively.

Figure 2. Classic BRCA1 Pedigree

![Classic BRCA1 Pedigree](image-url)
This pedigree shows a family with a deleterious BRCA1 mutation across three generations, including affected family members with breast cancer or ovarian cancer and a young age onset. A deleterious BRCA1 mutation can be transmitted through maternal or paternal lineages.

Figure 3. Classic BRCA2 Pedigree (National Cancer Institute, 2016)
This pedigree shows a deleterious *BRCA2* mutation across three generations, including affected family members with breast (including male breast cancer), ovarian, pancreatic, or prostate cancers and a relatively young age at onset. A deleterious *BRCA2* mutation can be transmitted through maternal or paternal lineages.

**The 4T1 model of breast cancer and the ID8 model of ovarian cancer**

The 4T1 model of breast cancer and the ID8 model of ovarian cancer were used for the analyzing the phenotype of tumor-associated DCs. In the case of ovarian cancer, this model is based on a spontaneous *in vitro* transformation of mouse ovarian surface epithelium cells (MOSEC). The relevance of this model established by Roby and collaborators (*Roby et al*) is that the tumor develops in an immunocompetent mouse after injection of the tumor cells in the peritoneal cavity, which is the place where the tumor develops in humans. This model was established in the C57BL6 mouse strain.

The 4T1 mouse mammary tumor cell line is one of only a few breast cancer models with the capacity to metastasize efficiently to sites affected in human breast cancer (*BMC Cancer*, 2008). Once tumor cells are injected in the mammary fat pad of BALB/c mice, the tumor develops fast as a solid tumor but also can metastasize to lungs and bones at late stages, replicating the characteristics of human breast cancer.
Previous work from our lab shows that both mouse tumors are infiltrated by immune cells, among others DCs. In addition, both models can be used to test immunotherapeutic approaches.

**Tumor Microenvironment of Ovarian and Breast Cancer**

The tumor microenvironment of breast cancer has demonstrated the ability of stromal tissues to regulate the growth and differentiation state of breast cancer cells (DeCosse et al. 1973). Some recent studies like *in vivo* and *in vitro*, have demonstrated that the growth, differentiation, invasive behavior and polarity of normal mammary epithelial cells and breast cancer carcinomas are influenced by surrounding stromal cells including fibroblasts, myofibroblasts, leukocytes, and myoepithelial cells (Bissell and Radisky 2001, Radisky et al. 2001). In addition, certain histopathological features of breast tumors including lymphocytic infiltration, fibrosis, and lymphangiogenesis, have proven prognostic significance. In the past few years, the role of cellular microenvironment in tumorigenesis has become an intense area of research. Studies have demonstrated genetic abnormalities, such as heterozygosity (LOH), occur not only in cancer cells but in stromal cells as well (Kurose et al. 2001, Kurose et al. 2002, Lakhani *et al.* 1998, and Moinfar *et al.* 2002). To study optimal growth of tumor cells, many researchers have focused primarily on the tumor cells, however emerging evidence indicates that tumors are composed of tumor parenchyma and stroma two discrete but interactive parts that crosstalk to promote tumor growth. Cancer-associated fibroblasts (CAFs) are the most frequent component of tumor stroma, especially in breast and pancreatic cancer (Kalluri *et al.* 2006 and Ostman *et al.* 2009).
Increasing data indicates that the depletion of fibroblast activation protein (FAP)-expressing tumor stromal cells led to stunted tumor growth and improved response to tumor vaccination providing evidence that the tumor microenvironment is fertile ground for development of novel therapies with the potential of augmenting existing treatment and prevention options (Ostman et al. 2009 and Loeffler et al. 1955-1962).

The tumor microenvironment of ovarian cancer often presents as an advanced metastatic disease, however many patients are treated with a combination of major debulking surgeries and chemotherapy to achieve complete cytoreduction (no tumor residue). The heterogeneity of ovarian cancers among and within subtypes has been illustrated by genetic profiling (J. Farley, 2008). Recently, a broad study by The Cancer Genome Atlas (TCGA) has demonstrated among other findings that serous ovarian adenocarcinoma could be clustered in 4 different subtypes without being able to relay them to prognosis (D. Bell, 2011). The concept of cancer stem cells (CSCs) has emerged as an alternative to the clonal theory of tumor evolution. Among the heterogeneous populations constituting a tumor, a small proportion of cells (0.01% to 0.1%) have properties that mimic to certain extent normal stem cell biology: self-renewal with asymmetric and symmetric cell division; recapitulation of the tumor heterogeneity in immune-suppressed mice and the ability to undergo serial passages in vitro and in vivo due to unlimited division potential (M.R. Alison, 2012). In addition, ovarian cancers overexpress LL-37 (leucine, leucine 37) which is a member of the cathelicidin family of antimicrobial polypeptides. While LL-37 does not act directly
on ovarian cancer cells, it attracts mesenchymal stem cells (MSCs) into ovarian tumor xenografts (S.B. Coffelt, 2009).

**Leukocytes as Promoters of Angiogenesis in Cancer**

Although the normal role of immune cells is to fight tumors, it has been shown that tumor-associated leukocytes can promote angiogenesis. In this way, the TME changes the functions of immune cells to help with tumor growth. Infiltration of immune cells into a tissue can precede the development of a tumor and it has been proposed that chronic inflammation (mediated by leukocyte infiltration) is a risk factor for the development of cancer (Ruegg, 2006; Peek, 2006; Szabo, 2004). So, inflammatory conditions as those caused by some infections can be involved in the development of a tumor. For example, gastric carcinomas can arise in a *H. pylori* induced gastritis environment (Peek, 2006) or hepatitis B virus/hepatitis C virus can induce hepatic cancer (Szabo, 2004). In addition, chronic (but noninfectious) inflammatory conditions as in the case of smoking-related bronchial cancer can induce cancer (Williams, 2001). In the same way, chronic pancreatitis is considered a risk factor for pancreatic cancer, and many of the growth factors involved in tissue remodeling and regeneration in chronic pancreatitis are present in pancreatic cancer (Jura, 2005). In particular, infiltrating inflammatory cells secrete a diverse repertoire of growth factors and proteases that enhance tumor growth by stimulating angiogenesis (generation of vessels that will drive nutrient to tumor cells).
Characteristics of Tumor-Associated DCs

DCs can be found in the microenvironment of several types of cancer (Baleeiro, 2008; Shurin, 2006; Curiel, 2004; Whiteside, 2006; Mantovani, 2004). Factors produced by tumor cells or other cells in the TME such as vascular endothelial growth factor (VEGF), interleukin (IL)-10 and prostaglandin E-2 (PGE2) can affect the normal function of DCs (Liu, 2009). In this context, DCs attracted to the TME might become incapable of inducing specific T cell responses, or can deactivate specific T cell responses against the tumor. For example, DCs showing low levels of costimulatory molecules (which indicates that they can suppress T cell activation) have been found in tumors expressing high levels of VEGF, a molecule produced at high levels in breast and ovarian cancer (Gabrilovich, 1999).

Importantly, DCs might be able to promote angiogenic processes in the tumor microenvironment (Curiel, 2004; Mantovani, 2004; Markiewski, 2008; Fainaru, 2010). As mentioned above, tumors need blood supply to grow and some studies have proposed that antigen presenting cells incorporate into growing vessels in experimental models supporting their development (Rehman, 2003; Lewis, 2007; Kuwana, 2006). A study by Huarte et al. 2008 (Huart, 2008), demonstrated that DC can localize in a perivascular position in the ID8 model of ovarian cancer and help new vessels to develop in tumor. Then, DCs can contribute to angiogenesis by producing factors that promote growth of endothelial cells (Sozzani, 2007).
**Dendritic Cells as a Source of Angiogenic Factors**

Our lab has previously shown that DCs *in vitro* are able to produce angiogenic factors such as matrix metalloproteases, VEGF, angiogenin, heparanase, and basic fibroblast growth factors among others (Sprague, 2011). Our lab has also participated in a study showing that DC precursors contribute to tumor angiogenesis in the ID8 model of ovarian cancer (Conejo-Garcia, 2004). In addition, it has been shown that depletion of tumor–associated DCs *in vivo* reduces tumor growth and decreases angiogenesis in this mouse model of ovarian cancer (Bak, 2007; Huarte, 2008). In the same way, data from other labs (Fainaru, 2008) highlighted the contribution of DCs to angiogenesis in a murine model of endometriosis and in the peritoneal Lewis lung carcinoma tumor model.

In conclusion, considering all the information presented above it is clear that DCs are immune cells with a relevant in establishing specific immune response, among them anti-tumor immunity. On the other hand, tumors can modify this function of DCs and turn them in conspirators of tumor growth. Then, it becomes important to understand how the tumor microenvironment can attract these cells, and how they collaborate then with tumor growth. In this context this project investigates DCs cells isolated from the tumor microenvironment to determine the presence of receptors that can be targeted by the tumor for DC recruitment; the activation status of these cells; and the expression of factors by DCs that can promote angiogenesis.
Hypothesis

Taking into account previous studies from our lab and reports from the literature we hypothesize that DCs are attracted to the tumor microenvironment of ovarian and breast cancer and there they will show low capacity to induce immune responses (due to low expression of costimulatory molecules) and will also produce factors promoting angiogenesis.

Here we will investigate the expression of angiogenic molecules and chemokine receptors in these cells by a combination of immunological (flow cytometry) and molecular biology (polymerase chain reaction) techniques.
Materials and Methods

Reagents for Molecular Biology

The reagents used for molecular biology were:

1) Ethidium Bromide Solution (10 mg/ml; catalog # H5041, Promega, Madison, WI)

2) OmniPur Agarose (Catalog #, 9012-36-6 Millipore, Temecula, CA)

3) Sterile water, nuclease-free 1.25 ml (Catalog# AM9937; Thermo Fisher Scientific, Waltham, MA)

4) PCR-to-Gel TAQ Master Mix 2X (Catalog# 1B1409, Amresco LLC, Solon, OH)

5) Primers manufactured by Thermo Fisher Scientific

Polymerase Chain Reaction (PCR)

Tumor-associated DCs were analyzed at the level of RNA for expression of angiogenic molecules, growth factors, chemokine receptors and cytokine receptors by means of PCR. The angiogenic molecules analyzed were VEGF 164, TWEAK, Angiogenin, Heparanase, Fibroblast Growth Factor, Hepatocyte Growth Factor, Osteopontin, MMP2 and MMP9. Chemokine receptors analyzed were CCR1, CCR3, CCR5, CXCR3 and CD126, a cytokine receptor also involved in migration. Beta actin was used to determine cDNA load. The sequence of the primers is shown in Table 1:
Table 1: Primer Sequences

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Amplified product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenin</td>
<td>Forward 5'- CAG CAT GTG GAC CCT CAG GT -3’ Reverse 5’- TGA AGC GTT TGC ACT GGA CA -3’</td>
<td>96</td>
</tr>
<tr>
<td>CD126/IL6R</td>
<td>Forward 5’-CCG TCG GCT GCA CGC TGT TG -3’ Reverse 5’- GTG CCA TTT GCC ACC TCC AGC -3’</td>
<td>93</td>
</tr>
<tr>
<td>CCR1</td>
<td>Forward 5’- GGA CCT GCC CAT GCA GGT GA -3’ Reverse 5’-TCC AGA ACC GTT CAC CCA CAA AA-3’</td>
<td>91</td>
</tr>
<tr>
<td>CCR3</td>
<td>Forward 5’- CCA TGC TGT GTT TGC CCT TCG-3’ Reverse 5’- GCA ATG CTG CCA GTC CTG CAA-3’</td>
<td>91</td>
</tr>
<tr>
<td>CCR5</td>
<td>Forward 5’- GAA GAG GCA CAG GGC TGT GAG G -3’ Reverse 5’- TGG AAG GTG GTC AGG AGG AGG A -3’</td>
<td>98</td>
</tr>
<tr>
<td>CXCR3</td>
<td>Forward 5’- CGC CCT GCC CAC AGG ATT TC-3’ Reverse 5’- ACC GCC CCA TTG CCT AGC AG-3’</td>
<td>99</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Forward 5’-TGT GTG CCA ACC GGT ACC TT-3’ Reverse 5’-TTC CAG TCG TTC AAA GAA GAA ACA -3’</td>
<td>108</td>
</tr>
<tr>
<td>Heparanase</td>
<td>Forward 5’- GGG GCC GGA TGG ATT ACT TT-3’, Reverse 5’-CCA TGA AAA ACC CGT CTC CA-3’</td>
<td>103</td>
</tr>
<tr>
<td>HGF</td>
<td>Forward 5’-GGG ACG GTA TCC ATC ACT AAG A -3’ Reverse 5’-CTT TAC CGC GAT AGC TCG AA-3’</td>
<td>99</td>
</tr>
<tr>
<td>MMP2</td>
<td>Forward 5’-GCA TCG CTC AGA TCC GTG GT-3’ Reverse 5’-GAA TGT GGC CAC CAG CAA GG-3’</td>
<td>106</td>
</tr>
<tr>
<td>MMP9</td>
<td>Forward 5’-TAA AGG CCG CTC GGA TGG TT-3’ Reverse 5’-CCA ACT ACG GTC GCG TCC AC-3’</td>
<td>104</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Forward 5’- CCT CCT CCC TCC CGG TGA AA -3’ Reverse 5’- GGG TCA GGC ACC AGC CAT GT -3’</td>
<td>102</td>
</tr>
<tr>
<td>TWEAK</td>
<td>Forward 5’-CCG AGC TAT TGC AGC CCA TT-3’ Reverse 5’-GCC ACT CAC TGT CCC ATC CA-3’</td>
<td>84</td>
</tr>
</tbody>
</table>
The reagents used for the PCR were *Thermo Science* PCR water nuclease free, *Amresco* PCR-to-Gel TAQ Master Mix 2X, and a primer mix. The primer mix consisted of the *Thermo science* PCR water and forward and reverse primers.

The PCR amplification was performed in a thermal cycler (Multigene, Labnet International, Edison, NJ) under the following conditions:

1) One cycle at 94°C for 4 min, in order degrade all DNAses

2) 48 amplification cycles, each cycle consisting of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 15 seconds

3) One cycle at 72°C for 10 minutes in order to elongate any unfinished fragment

The products of amplification were separated using a 1.5% agarose gel electrophoresis and photographed under a UV light. The expected size of specific PCR amplification products was expected to be around a 100 bp for all primers.

**Gel Analysis of PCR Fragments**

PCR fragments were analyzed through gel electrophoresis. To prepare the gel, .75g of *OmniPur* agarose and *Quality Biological* 1X TAE buffer were used. A small amount of 5μl of Promega ethidium bromide was added. The addition of the ethidium bromide is crucial in that it glows under UV light, which inserts under base pairs and allows vision of the DNA because DNA is colorless. The mixture was then swirled and boiled for 1 minute until poured to solidify. Once solidified, the 1X TAE buffer
was poured to cover the gel completely and 20 μL from each PCR vial was extracted and added to the gel comb. The gel was run at 100 V for about 35 minutes.

**Reagents for Flow Cytometry Analysis**

The reagents used for flow cytometry analysis were:

1) 1X PBS (Gibco, Thermo Fisher Scientific)

2) FACS Blocking Buffer (0.05 Azide and 2% fetal bovine serum [Gibco] in PBS)

3) Flow Cytometry Fixation Buffer 1X (eBioscience Inc., San Diego, CA)

4) Antibody mixtures (1/100 dilution of each antibody in FACS buffer, all antibodies eBioscience)

**Flow Cytometry Analysis Procedure**

FACS mouse breast and ovarian tumor samples were used for flow cytometry analysis. For the breast tumor samples, 4T1 solid tumors were established in BALB/c mice, and then resected and mechanically disaggregated to obtain single cell suspensions (pools from 8 different tumors). Similarly, pool of spleens from non-tumor bearing mice were used as a source of normal DCs. These tissues were also disaggregated into single cell suspensions.

As for the ovarian tumor samples, the samples used were cell suspensions obtained from ascites induced by injection of ID8 tumor cells into the peritoneal cavity of C57BL/6 mice (pools from three different tumors in triplicate) and spleens (pool in duplicate). To stain our samples, these were divided into several fractions in order to perform several simultaneous staining. Our flow cytometry allows to analyze 10
parameters at the same time, 2 related to size and complexity of the cells, and 8 related to different fluorescent signals. In this way up to 8 different antibodies specific for molecules of interest and tagged with different fluorochromes could be used simultaneously. Samples were stained and analyzed in triplicate. When the fluorescent antibodies were added to the cell suspensions, the tubes were covered with aluminum foil to avoid any light from entering because the fluorochromes are photosensitive (they can break apart in the light). First, the samples to be stained were treated with Blocking Buffer (0.05% Azide, 2% FBS). The protein in the buffer provided by the FBS will help block all non-specific binding, assuring that the antibodies only interact with their specific receptors on the cell surface. Once the blocking buffer was added, the cell suspensions were kept on ice for 20 min to allow for the blocking to happen. Then, a mixture of specific antibodies as presented in Table 2 was added to the cell suspension which was then incubated on ice for 40 minutes. After the 40 minutes, the cells suspensions were washed with 2ml of FACS Buffer. To accomplish this, cell suspensions were centrifuged at 1200rpm for 5 minutes at 7°C. Then supernatants were eliminated and a second step of staining was used because one antibody in each mix was biotinylated, meaning it did not carry a fluorochrome, but is susceptible of interacting with a molecule such as streptavidin (SAV) that can carry it. This allows for amplification of the signal of that particular antibody. Thus, our samples were stained for 30 min on ice with SAV-Pe-Texas Red (a fluorochrome). After the 30 minutes, cell suspensions were washed again with FACS Buffer and then suspensions fixed with Flow Cytometry Fixation Buffer which
was added to each tube. Table 2 shows the individual stains used for this study. Each antibody is named after the molecule that it specifically recognizes. The cells were then analyzed using the flow cytometer, BD FACSARia (BD Biosciences, San Jose, CA). The flow cytometer allows single cell analysis of cellular suspensions and simultaneous acquisition of all signals derived from the antibodies bound to each cell.

Table 2. Antibody mixtures used for staining of tumor-associated DCs (ovarian and breast cancer model)

<table>
<thead>
<tr>
<th>Fluorochromes</th>
<th>FITC</th>
<th>PE</th>
<th>PE-Texas red</th>
<th>Percp</th>
<th>PeCy7</th>
<th>APC</th>
<th>APCCy7/AF700</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CD107b</td>
<td>PDL1</td>
<td>VEGFR2</td>
<td>CD8a</td>
<td>CD11c</td>
<td>SIRPα</td>
<td>CD45</td>
</tr>
<tr>
<td>B</td>
<td>CD86</td>
<td>CD107b</td>
<td>MHC-II</td>
<td>CD8a</td>
<td>CD11c</td>
<td>SIRPα</td>
<td>CD45</td>
</tr>
<tr>
<td>C</td>
<td>CD40</td>
<td>CD107b</td>
<td>OX40L</td>
<td>CD8a</td>
<td>CD11c</td>
<td>SIRPα</td>
<td>CD45</td>
</tr>
<tr>
<td>D</td>
<td>CD107b</td>
<td>VEGFR1</td>
<td>TIE2</td>
<td>CD8a</td>
<td>CD11c</td>
<td>SIRPα</td>
<td>CD45</td>
</tr>
<tr>
<td>E</td>
<td>CD107b</td>
<td>CD80</td>
<td>(CD144)VE-Cadherin</td>
<td>CD8a</td>
<td>CD11c</td>
<td>SIRPα</td>
<td>CD45</td>
</tr>
<tr>
<td>Control 1</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>CD45</td>
</tr>
<tr>
<td>Control 2</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
<td>Isotype</td>
</tr>
</tbody>
</table>
Results

Expression of Angiogenic Molecules by Ovarian Cancer DCs at the Level of RNA

In a first series of experiments we evaluated the expression of angiogenic factors in DCs that were purified from the tumor microenvironment of ovarian cancer. To this end, previous researchers in the lab induced the generation of ovarian cancer ascites in the mouse model. Once ascites was established in the peritoneal cavity, the
cell suspension was subjected to cell separation procedures to obtain pure DCs. To accomplish this, DCs in the suspensions were stained with specific antibodies, purified by fluorescence activated cell sorting and RNA extracted. Finally, RNA from these cells was reverse transcripted into complementary DNA (cDNA) which was the source for the current studies. Figure 4 shows the process for obtaining the cDNA samples used in our studies (both for ovarian cancer or breast cancer).

Here, we determined the expression of well-known angiogenic molecules by means of PCR technology. In particular, we evaluated the expression of Angiogenin, Osteopontin, Hepatocyte Growth Factor (HGF) (Kodelja, 1997), Heparanase (Gingis-Velitski, 2004), TWEAK (Donohue, 2003; Nakayama, 2003) and matrix metalloproteases (MMPs) 2 and 9 (Allavena, 2008). All of these molecules have been shown to collaborate with the angiogenic process in the context of cancer.

**Figure 5. Analysis of TWEAK expression in ovarian cancer DCs**
In Figure 5, the analysis of the expression of TWEAK in samples of DCs recovered from ovarian cancer ascites determines that all were positive. This is evidenced by the fluorescent band of approximately 100 bp in size observed in our gels. The primers were designed to amplify a product of such size if the gene was expressed in our samples. The lower, diffuse bands at the lower portion of the gel are caused by excess primer that did not react in our samples. The left lane shows a 100 bp DNA ladder that helps identify amplified fragments of the expected size (Molecular Marker, MM). To far the right, the negative sample, with an absence of a band at the 100 bp size is a negative control in which all reagents for the PCR reaction are included except the cDNA (non-template control, NTC).

**Figure 6. Analysis of Heparanase expression in ovarian cancer DCs**
Dendritic cells recovered from mouse ovarian cancer ascites. Here we observed that only two of our samples are positive for Heparanase. That indicates that the expression of this molecule on tumor infiltrating DCs is not an extended occurrence in the microenvironment of ovarian cancer. This also points out towards differences that can happen in the microenvironment of tumors, even in those generated in genetically homogenous mouse strains. These individual differences highlight the difficulty to generate a tumor therapy that efficiently affects all patients.

**Figure 7. Expression of Fibroblast Growth Factor in ovarian cancer DCs**

As observed above only few samples were positive for this angiogenic molecule among DCs recovered from ovarian cancer ascites. As mentioned above, this highlights the individual variations that can be observed in the microenvironment of ovarian cancer in different individuals.
On the other hand, we were not able to identify specific expression of angiogenin, HGF, MMP2 or MMP9 in our samples by means of PCR analysis.

**Analysis of chemokine molecules in DCs recovered from breast cancer tumors (solid tumor model).**

In the next series of experiments, we focused on the expression of chemokine receptors by tumor-associated DCs. In this case, we used a model of breast cancer. Why? Both our models are “orthotopic” models, meaning that the tumor is grown in the experimental animals in the same location as it regularly does in humans. The search for chemokine receptors in DCs can help determine how they migrate towards tumors. In the case of ovarian cancer, the tumor grows in the peritoneal cavity, which already harbors high levels of macrophages and dendritic cells, so an investigation on those receptors in that model is not that relevant. On the other hand, the orthotopic site of breast cancer in the mouse is the mammary fat pad, which is normally devoid from DCs. Thus, investigation of the chemokine receptors on DCs in this model will give a great insight on how these cells manage to migrate from other regions of the bodies to the growing tumor. This is very relevant for therapeutics, since as shown above, these cells in the context of a tumor can promote tumor angiogenesis. The chemokine and cytokine receptors investigated here were CCR1, CCR3, CCR5, CXCR3 and CD126. In this series of experiments, we analyzed samples from DCs recovered from 3 pools of tumors that represent (each pool) an independent experiment (breast cancer cells implanted in at least 4 different mice each time).
Figure 8. Analysis of CCR3 expression in breast cancer DC.

Here we investigated the expression of CCR3 on breast cancer-associated DCs. We observed a strong signal for CCR3 in all our samples. CCR3 is a receptor for several chemokines, among them RANTE, CCL6, CCL7, CCL8, CCL3, CCL15 and CCL24. Interestingly, complementary studies in our lab (not published) show that breast cancer cells can produce RANTES. So this could be a mechanism of attraction of these cells to the cancer microenvironment.
In this case we were also able to observe specific expression of the investigated gene in our samples. This receptor can interact with CCL9, CCL10 and CCL11, chemokines that our lab has also detected as products of breast cancer cells. On the other hand, we were not able to identify specific expression of CCR1, CCR5 and CD126 in our samples by means of PCR analysis.

Overall, the study on chemokine receptors on tumor-associated DCs revealed two molecules that are expressed by these cells, and can indicate a mechanism for attraction of these cells towards the tumor microenvironment.
Flow Cytometry Analysis of Dendritic Cells in the Tumor Microenvironment

In these series of experiments, we analyzed the expression of markers of angiogenic potential and costimulatory molecules in DCs present in the TME of mouse models of ovarian and breast cancer. DCs were identified due to the expression of surface markers (molecules expressed specifically by particular cells). Figure 10 shows the design of our flow cytometry experiments.

**Figure 10: FACS experimental design**
In these studies, we first define DCs as leukocytes (cells that carry the CD45 receptor) and within that population we investigated cells for expression of both CD11c (a typical mouse DCs marker) and CD8 (which helps define a population of DCs). In this way we determine the expression of angiogenic markers and costimulatory molecules in CD8 positive DCs and CD8 negative DCs. The CD8 positive DCs are considered to more efficiently activate the cytotoxic branch of specific immunity in healthy individuals, while the CD4 subpopulation helps better activate a B cell/antibody response. Controls using isotypes (antibodies that are non-specific but carry a fluorochrome) helped set the limits between positive and negative signals. Figure 11 shows the gating strategy used in our samples. A gate is a subpopulation of cells chosen due to the expression of particular markers and which is further studies for additional markers.

**Figure 11. Strategy to differentiate between positive and negative FASC samples for breast cancer.**
On the left of the Figure 11 we present a sample that has been stained 8 different isotypes (one for each fluorochrome) and a sample that has been stained with isotypes and a positive marker for leukocytes. By comparing both samples we can identify where the leukocyte population (CD45) lies. Within that population we plot different fluorochrome signals (all isotype signals). This helped us determine what would be a negative signal, and anything to the right of that in a 2D plot (dot plot) is considered positive. Since we plot the signal of two fluorochrome channels at the same time (right figures), we can define 4 populations: double negative for both colors, 2 single positive regions for each color and a double positive color region. Cells that fall in this last region will be expression of both markers analyzed.

**Figure 12. Gating strategy for flow cytometry analysis of breast cancer samples.**

**Breast Cancer FACS analysis: gating strategy**

In this figure, we show how we chose populations for analysis in our samples. As shown, we first gate on CD45 positive cells (leukocytes) and then we set up the limits for CD11c positive/CD8 negative and CD11c positive/CD8 positive cells. These
are the two DC populations we described above. Within those populations we analyzed the expression of costimulatory molecules and surface angiogenic markers.

Interestingly, as shown in Fig. 12, CD8 negative DCs are a more prevalent population of DCs in the breast cancer microenvironment than CD8 positive DCs, suggesting that the former population might contribute more to determining the characteristics of the tumor microenvironment.

**Figure 13. Expression of PDL1 and VEGFR2 in breast cancer DCs**

Here we analyzed the expression of pDL-1, a negative costimulatory molecule and VEGFR-2, a marker of pro-angiogenic leukocytes. This receptor is able to interact with VEGF, an angiogenic factor that also promotes an immunosuppressive state in DCs. As shown, these DCs recovered from the microenvironment of breast cancer show expression of both molecules. When analyzing the data, the numbers in each quadrant show the percentage of cells in each quadrant. In this case samples were
analyzed in triplicate, and represent a pool of 8 individual breast 4T1 tumors. As it can be observed in Fig. 13, CD8 negative DCs express a higher proportion of both markers. For example, we detected between 9 to 19% of double positive (DP) DCs for VEGFR-2 and PDL-1 among CD8 positive DCs, while for the same markers we observed that CD8 negative DCs had a percentage of DP cells ranging from 28 to 51%. This points out towards the complex nature of the DC population in the microenvironment of breast cancer, and the possibility of CD8 negative DCs to suppress immune responses and to be sensitive to the effect of VEGF.

**Figure 14. Expression of CD40 and OX40L in breast cancer DCs**

As above, we observed expression of both markers in these DCs. In this case, the values of DP cells were similar in both types of DCs, although CD8 negative DCs show a higher proportion of cells that are single positive for CD40. Both molecules are costimulatory molecules. It is important to comment that the stimulation of T cells by
DCs depend on the overall interaction of several costimulatory molecules between both types of cells.

**Figure 15. Expression of VEGFR-1 and TIE-2 in breast cancer DCs**

- **Breast Cancer**
- **CD8 positive DCs**
- **CD8 negative DCs**

Here we determine that DCs in the microenvironment of breast cancer express two angiogenic markers, VEGFR-1 (another receptor for VEGF) and TIE2, a molecule that has been associated with pro-angiogenic leukocytes.
Here we observed that both types of DCs express similar levels of CD80 and VE-Cadherin. CD80 is a costimulatory molecule while VE-Cadherin is another molecule associated with pro-angiogenic leukocytes.
These molecules are costimulatory. We observed that DCs (both CD8 positive and negative) show similar expression of MHC-2 (between 60 to 70% of all cells), this is restricted to a single positive type of expression because very low expression of CD86 was observed.

In order to better understand the levels of expression of the investigated markers in our cells we also stain DCs from spleens of healthy mice. DCs in healthy individual live in the tissues and the spleen is an organ that can be used to purify or analyze normal DCs.
As it can be seen, the populations of the spleen look very different from that of the tumor. In particular, the amount of CD45 cells (leukocytes) is much higher than that present in the tumor, and the limits between positive and negative signals are also different between both types of samples. The samples were run in duplicate and represent pools of spleen collected from healthy mice.
We observe expression of both markers in these cells, but interestingly, when focusing on the CD8 negative populations we can see that VEGFR-2 is expressed at lower levels than their tumor counterparts (Fig. 13). Although more experiments are needed to confirm this, this is in line to what is expected for tumor-associated DCs, and it points towards differences between DCs in healthy or malignant environments.
In this case, when we compare these cells with their tumor counterparts (Fig. 14), the CD8 DC spleen population express very high levels of CD40, a costimulatory molecule that through interaction with T cells, help stimulate DCs to be better at doing their immunological role. Again, these highlights the differences between DCs present in different environments.
Figure 21. Expression of VEGFR-1 and TIE-2 in BALB/c mice spleen DCs

BALB/c Spleen

CD8 positive DCs

CD8 negative DCs

Here again, our data show that most differences are observed when analyzing the CD8 negative populations. At the level of spleen, these cells express low levels of both angiogenic markers than their tumor counterparts (Fig. 15).
Figure 22. Expression of CD80 and VE-Cadherin in BALB/c mice spleen DCs

BALB/c Spleen

CD8 positive DCs

CD8 negative DCs

In this case, there were not evident differences between spleen and tumor DCs (Fig. 16) when we analyzed these markers.
Here we observed differences in the expression of both costimulatory molecules when comparing the CD8 DC populations. Spleen DCs express overall higher levels of MHC-II and CD86 than tumor CD8 DCs.

In all, tumor DCs in the breast cancer model expresses angiogenic markers, and in the case of CD8 DCs, they tend to express lower levels of costimulatory molecule than DCs present in the spleen of healthy mice.
As above, we gated on CD45 positive populations (CD45, defined by comparing with isotype controls) and we investigated DC populations that were CD8 positive and negative. The analysis proceeded in the same way as for breast cancer samples. Similar to what we saw in our breast cancer studies, CD8 negative DCs appear to be in higher numbers when compared to CD8 positive DCs.
As with breast cancer, we were able to detect expression of the negative costimulatory molecule PDL-1 and the angiogenic marker VEGFR-2 in DCs (both CD8 positive and negative) present in the microenvironment of ovarian cancer in the mouse model.
In this case we observed expression of the costimulatory molecule CD40 in both types of DCs, but low levels of OX40L in CD8 negative DCs.

**Figure 27. Expression of VEGFR-1 and TIE-2 in ovarian cancer DCs**

Here we observed that a great proportion of ovarian cancer DCs were double positive for both angiogenic markers investigated. In addition, almost all the cells were positive for VEGFR-1 (determined by adding the values of all cells positive for VEGFR1 [DP and SP]).
In this case we observed that great part of the DC populations were positive for the angiogenic marker VE-Cadherin and also express the costimulatory molecule CD80.
We also detected expression of MHC-2 by most of our cells and expression of the costimulatory molecule below the 50% in both type of DCs.

**Figure 30. Gating strategy for flow cytometry analysis of C57BL6 spleen DCs.**

**C57BL/6 spleen**

Similar to what we did in the breast cancer models, DCs from healthy mice (same strain used to develop the tumors) were analyzed in our studies. Here we show the gating strategy for this analysis. As described before, the spleen is characterized by having a lot of leukocytes which is reflected in high levels of CD45 in our staining.
We observed that spleen DCs in this mouse strain express the negative costimulatory molecule, PDL1, and the angiogenic marker VEGFR-2, at similar levels as those observed in tumor-associated DCs (Figure 25).
Here we observed that spleen DCs express both costimulatory molecule. In particular expression of OX40L was detected at lower levels in their tumor counterparts (Fig. 26).
Again, the phenotype of the splenic DCs resembled in average those of their tumor counterparts when compared to Fig. 27.
In this case, we observed that spleen DCs, in particular those that are CD8 negative, showed lower levels of the angiogenic marker VE-Cadherin than that observed in their tumor counterparts (Fig. 28).

**Figure 35. Expression of CD86 and MHC-2 in C57BL/6 mice spleen DCs**

Finally, an analysis of costimulatory molecules CD86 and MHC-2 in spleen DCs showed that these cells express higher levels of CD86 in both CD8 negative and positive subsets in comparison to their tumor counterparts (Fig. 29).

A brief conclusion regarding ovarian cancer DCs indicates that they resemble more their healthy counterparts than those analyzed in breast cancer. But nevertheless, some angiogenic markers are still expressed at higher levels than the spleen cells, and also some costimulatory molecules were expressed at lower levels.
**Discussion**

As described in the introduction, DCs are present in the microenvironment of different types of cancer (Baleeiro, 2008; Curiel, 2004; Mantovani, 2004; Shurin, 2006; Whiteside, 2006). Mouse DCs are divided into myeloid and plasmacytoid populations. The myeloid DCs, which are now named conventional DCs (cDCs), are further subdivided into subsets. CD11c is typically used as a marker of murine cDCs (Liu, 2010; Shortman, 2010). Two major DC subsets populations are distinguished by the molecules they express: CD11c\textsuperscript{hi}MHCII\textsuperscript{+}CD8\alpha\textsuperscript{+}CD205\textsuperscript{+}SIRP\alpha\textsuperscript{CD11b\textsuperscript{−}} and CD11c\textsuperscript{hi}MHCII\textsuperscript{+}CD8\alpha\textsuperscript{33D1\textsuperscript{+}}SIRP\alpha\textsuperscript{CD11b\textsuperscript{+}} (Liu, 2010; Shortman, 2010). Not all those markers are used at the same time; in our case as in other studies we used CD11c and CD8 to define the two populations. Although we stained for SIRP\alpha in our studies, we have a non-specific signal from that antibody so we decided not to include the marker for analysis. That did not modify our data since in several studies it focuses on CD8 positive and negative DCs. For example, studies performed by Dudziak et al. 2007, (Dudziak, 2007), show that the CD8\alpha\textsuperscript{+} DC subpopulation is specialized in primarily presenting peptides associated to MHC-I antigens, while the CD8\alpha\textsuperscript{−} subpopulation is involved in presenting MHC-II associated peptides.

Within the tumor microenvironment, cytokines such as VEGF, IL-10 and PGE-2 can profoundly affect the nature of DCs. In particular, DCs showing low levels of costimulatory molecules have been detected in tumors expressing high levels of VEGF (Gabrilovich, 1996; Gabrilovich, 1999) an angiogenic molecule which has been proposed as a target for antitumor therapies (Ferrara, 2004; Ferrara, 2005; Kenny,
VEGF is a soluble molecule that can interact with DCs through receptors on their surface (VEGR-1 and 2). As we have shown in our data, DCs both in the tumor microenvironment and in healthy tissues express these molecules, but only in the tumor environment these cells might find high and consistent levels of VEGF that can affect their function. VEGF can induce an immunosuppressive profile in DCs. Meaning, these cells might not be able to activate, and rather suppress T cells that can fight the tumor. As we mention in the introduction, tumors can express specific molecules that can be a target of T cells. For example, it has been shown that cancer patients treated with anti-VEGF antibody showed a decrease in the levels of immunosuppressive circulating DCs (Osada, 2008). Moreover, a therapeutic agent endothelial cell-produced antiangiogenic cytokine vascular endothelial growth inhibitor which blocks the effects of VEGF can improve DC function in vivo (Tian, 2007). The literature indicates that VEGF blocks maturation of DCs.

Immature DCs can detect foreign (microbial infection) or tumor antigen, take it up by phagocytosis and process them into peptide fragments (Banchereau, 2000). Peptides can also be produced intracellularly due to virus infections. Antigenic peptide fragments are presented on the DC surface by MHC molecules. These MHC molecules can present the antigenic peptides to T cell receptors in order to activate T cells (Banchereau, 2000). An immature DC which has processed a pathogen will undergo maturation and migrate to lymphoid regions where it can present the antigen peptide to naïve T lymphocytes (Banchereau, 2000; Timmerman, 1999). The maturation process involves upregulation of MHC class II molecules, costimulatory
molecules such as CD40, CD80, CD86; and OX40L. Upon maturation, DCs increase their efficacy to present processed antigens in the context of MHC molecules, and consequently an improved capability to activate T cells. As we show in our results section, we investigated all of these molecules in our cells. We observe that in general tumor-associated DCs overall express lower levels of costimulatory molecules when compared with healthy tissues counterparts. It is important to note that those healthy DCs were not activated by pathogens; in that case those molecules got to very high levels (higher than the ones we observed here). That means also that we do not detect any immune activation on DCs present in the tumor site. So, if they encounter T cells they in turn will not be activated. So our studies are in line with literature indicating that in the context of the tumor microenvironment DCs are not efficient in activating T cells, or can induce suppression of their activity. If DCs present tumor antigen that they acquire through phagocytosis to T cells without upregulating costimulatory molecules, this can lead to the deactivation of specific T cells, a process named anergy. That could happen in our models, but to know that future studies need to focus on purifying the tumor DCs and culturing them together with T cells to see the effects on these last cells.

But DCs can help tumor growth in another fashion, for example promoting angiogenesis. Tumors require a blood supply to expand, so they promote the induction of new vessels, a process that is named angiogenesis (Patan, 2000). These vessels are different from vessels of normal tissue regarding molecular structure (Djonov, 2003; Papetti, 2002). Some studies suggest that recruitment of endothelial progenitors that
differentiate into endothelial cells, might contribute to the formation of tumor vessels
(Bailey, 2003). For example, monocytes (a precursor of macrophages) can act as
endothelial cell progenitors and incorporate into growing vessels (Rehman, 2003;
Lewis, 2007; Kuwana, 2006). In addition, DCs cultured in the presence of tumor
factors, can undergo an endothelization process characterized by the display of
endothelial markers such as VEGFR-2 and VE-Cadherin (Fernandez Pujol, 2001;
Gottfried, 2007; Conejo-Garcia, 2004; Lu, 2011; Lu, 2010). In our studies we observe
that both tumor-associated DCs and healthy DCs express angiogenic markers such as
VEGFR-1 and -2, VE-Cadherin or TIE-2. And we found that in general, some of these
markers were more highly expressed in tumor-associated DCs. As described above,
DCs are able to express endothelial markers but it is a matter of debate whether
leukocytes such as DCs or monocytes can directly incorporate to blood vessels, these
cells can promote angiogenesis by producing angiogenic factors. Here, focusing in the
ovarian cancer model we were able to detect by PCR analysis that DCs purified from
the ovarian cancer microenvironment express some angiogenic molecules, particularly
TWEAK. This molecule has multiple functions, and among them is the stimulation
and growth of endothelial cells. So, DCs producing TWEAK in the tumor
microenvironment might help promote tumor growth. In this way, the present study is
a starting point to study the role of TWEAK produced by tumor-associated DCs on
ovarian cancer grows. Highlighting the relevance of DCs in tumor angiogenesis, our
lab and others have reported that DCs or macrophages (another antigen presenting
cell), can collaborate with angiogenesis in ovarian and other human cancers and in
mouse tumor models (Fainaru, 2008; Coukos, 2005; Curiel, 2004; Mantovani, 2004). For example, eliminating DCs from the tumor microenvironment reduced tumor growth in a mouse model of ovarian carcinoma (Huart, 2008).

Finally, we also investigated the potential of DCs to migrate towards tumors or remain capture in their midst. For that purpose, we used a breast cancer model, a solid tumor model that will induce migration of infiltrating DCs. As shown in our studies’ DCs in the tumor microenvironment express CCR3 and CXCR3 (receptors for chemokines that can be produced by the tumor). These receptors could direct these cells towards the tumor where they can be subjected to the influence of VEGF for example. Future studies from our lab will investigate the effect of blocking these receptors on DCs on the overall growth of a tumor. These receptors can be blocked using specific antibodies.

For future experiments the angiogenic molecules that were detected by PCR need to also be investigated at the level of protein. This will allow determining if the molecules that we observed expressed at the level of the RNA are also present as proteins, which are the ones that will carry the angiogenic properties in the tumor. These proteins can be investigated by means of western blot analysis. To do this, DCs need to be isolated from tumors and proteins extracted from those cells. Similarly, the chemokine receptors detected in our cells at the level of RNA, flow cytometry should be used to analyze the presence of those molecules as proteins on the surface of the cells. In addition, we can investigate how the purified cells migrate towards their
specific ligands (chemokines) by using an immunological assay named chemotaxis assay.

In conclusion, our work suggests that tumor-associated DCs can be attracted to the tumor microenvironment through CCR3 and CXCR3; and that there they can be subjected to the influence of VEGF due to the expression of VEGFR which can account for the low levels of costimulatory molecules that we observed in these cells. In addition, we demonstrated that tumor-associated DCs in a model of ovarian cancer express TWEAK, an angiogenic molecule which can be considered as a target for future therapies.
Acknowledgements

I would like to sincerely thank Dr. Fabian Benencia and Michelle Pate for teaching me many of the techniques needed to complete my thesis project. I also wish to thank Dr. Christine Griffin for her suggestions and guidance while putting together my paper and associated presentation. I am so grateful to Ohio University’s Department of Biological Sciences at the College of Arts and Sciences for providing me with the opportunity to complete an honors thesis.
References


Benencia, F. 2016. Personal communication


D. Radisky, C. Hagios, M.J. Bissell Tumors are unique organs defined by abnormal signaling and context Semin. Cancer Biol., 11 (2001), pp. 87–95


Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas Kurose K., Gilley K., Matsumoto S., Watson P.H., Zhou


Harlan, Blaine, and Alexander Nikitin. "A Quest for Better Mouse Models of Breast and Ovarian Cancers."


Lihong, Mo. "Syngeneic Murine Ovarian Cancer Model Reveals That Ascites Enriches for Ovarian Cancer Stem-Like Cells Expressing Membrane GRP78."


Rehman, J., et al., Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation,


