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Regulation of inflammatory factors by double-stranded RNA receptors in breast cancer cells

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ABSTRACT

Malignant cells are not the only components of a tumor mass since other cells (e.g., fibroblasts, infiltrating leukocytes and endothelial cells) are also part of it. In combination with the extracellular matrix, all these cells constitute the tumor microenvironment. In the last decade the role of the tumor microenvironment in cancer progression has gained increased attention and prompted efforts directed to abrogate its deleterious effects on anti-cancer therapies. The immune system can detect and attack tumor cells, and tumor-infiltrating lymphocytes (particularly CD8 T cells) have been associated with improved survival or better response to therapies in colorectal, melanoma, breast, prostate and ovarian cancer patients among others. Contrariwise, tumor-associated myeloid cells (myeloid-derived suppressor cells [MDSCs], dendritic cells [DCs], macrophages) or lymphoid cells such as regulatory T cells can stimulate tumor growth via inhibition of immune responses against the tumor or by participating in tumor neoangiogenesis.

Herewith we analyzed the chemokine profile of mouse breast tumors regarding their capacity to generate factors capable of attracting and sequestering DCs to their midst. Chemoattractants from tumors were investigated by molecular biology and immunological techniques and tumor infiltrating DCs were investigated for matched chemokine receptors. In addition, we investigated the inflammatory response of breast cancer cells, a major component of the tumor microenvironment, to double-stranded RNA stimulation. By using molecular biology techniques such as qualitative and quantitative PCR, PCR arrays, and immunological techniques (ELISA, cytokine immunoarrays) we examined the effects of dsRNA treatment on the cytokine secretion profiles of mouse and human breast cancer cells and non-transformed cells.

We were able to determine that tumors generate chemokines that are able to interact with receptors present on the surface of tumor infiltrating DCs. We observed that PRR signaling is able to modify the production of chemokines by breast tumor cells and normal breast cells, thereby constituting a possible player in shaping the profile of the leukocyte population in the TME.

1. Introduction

Breast cancer is the second leading cause of cancer mortality in women and the American Cancer Society predicts that nearly 200,000 new cases of invasive breast cancer will be diagnosed in women with around 40,000 deaths each year despite advances in early detection, and treatments involving surgical procedures followed by administration of one or more chemotherapeutic agents. In view of this situation it becomes necessary to generate innovative approaches for the treatment of this disease. To this end, we consider it necessary to investigate in depth the biology of these tumors, and particularly the role of the microenvironment in sustaining tumor growth and impairing anticancer therapies. Tumors are composed by cancer cells and other cellular types such as fibroblasts, endothelial cells and infiltrating

Abbreviations: APCs, antigen presenting cells; BM-DCs, bone marrow-derived DCs; DCs, dendritic cells; DAMPs, danger associated molecular patterns; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDA-5, melanoma differentiation antigen 5; MDSCs, myeloid-derived suppressor cells; MHC, Major histocompatibility complex; PAMPS, Pathogen associated molecular patterns; PCR, polymerase chain reaction; PKR, protein kinase R; PRR, pathogen recognition receptors; RIG-I, petinoic acid-inducible gene I; TA, pumor-associated; TCM, pumor-conditioned medium; TLR, poll-like receptor; TME, Tumor microenvironment

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immune cells that together with extracellular matrix components constitute the tumor microenvironment (TME). The relevance of the TME as a driving force in tumor development and resistance to therapy has gained increasing recognition in the last years as we and others have reviewed (Benencia et al., 2012; Block et al., 2015; Pitt et al., 2016).

Many studies highlight the protective role of the immune system against tumors. For example, tumor-infiltrating lymphocytes (TILs) have been associated with improved survival of patients with melanoma, non-small cell lung, breast, colorectal, esophageal, and ovarian cancers among others (Zhang et al., 2003; Wang et al., 2016; Kilvaer et al., 2016; Noble et al., 2016; Bosmuller et al., 2016; Fortes et al., 2015; Maker et al., 2015). In particular for breast cancer, TILs count has been associated with increased survival in patients with triple-negative breast cancer (Garcia-Teijido et al., 2016; Denkert et al., 2010; Matsumoto et al., 2016). Indeed, the existence of anti-tumor immune responses is the basis for cancer immunotherapies, such as adoptive T cell therapies, engineering of chimeric antigen receptor T cells; dendritic cell (DC) vaccination, or blocking immune checkpoint molecules. Not surprisingly, a focus on TME is postulated in order to improve the efficacy of some of these therapies (Beavis et al., 2016; Munn and Bronte, 2016).

On the other hand, tumor-associated leukocytes such as regulatory T cells (Treg) or myeloid-derived suppressor cells can promote tumor growth by impairing antitumor immune responses (Munn and Bronte, 2016; Condamine and Gabrilovich, 2011; Kumar et al., 2016). Indeed, we have previously demonstrated the relevance of the TME in attracting MDSCs by a complement-mediated process (Markiewski et al., 2008). It has been shown that complement also participates in the development of the metastatic niche in breast cancer, by promoting MDSC-mediated induction of Tregs (Vadrevu et al., 2014). DCs with the capability to suppress T cells responses via indoleamine 2,3-dioxigenase (IDO) expression have been described in tumor-draining lymph nodes (Munn and Bronte, 2016; Baban et al., 2005). Importantly, tumor-associated macrophages (TAM) have also been shown to impair the efficacy of antitumor therapies in some settings (Belgiovine et al., 2016).

However, the mechanisms by which inflammatory cells can be attracted to and shaped by the TME are not fully understood. As described above, inflammation may be a pre-existent condition for some malignancies, but also tumor cells can produce cytokines and chemotactic factors thereby attracting inflammatory cells, which in turn can synthesize additional cytokines, growth factors, and proangiogenic factors that contribute to cancer progression. In this context, the expression and activation of pathogen recognition receptors (PRRs) on tumor cells might trigger the production of chemotactic molecules and growth factors. Among other inflammation-related molecules, toll-like receptors (TLRs) have emerged as important molecules related to inflammatory process. TLRs are a family of cell surface receptors that recognize microbial (exogenous) or cellular (endogenous) products and initiate innate immune responses (Underhill, 2003; Sloane et al., 2010). In particular, TLR3 recognizes double-stranded (ds)RNA, *i.e.* from virus-damaged cells, and activates genes important for immune cell interactions and antiviral responses (Underhill, 2003). Interestingly, the expression of TLR3 has been described in thyroid, pancreatic, melanoma, ovarian and breast cancer cells (Schwartz et al., 2009; McCall et al., 2007; Conforti et al., 2010; Friboulet et al., 2010). Activation of TLRs by endogenous ligands is suggested to be responsible for the chronic inflammation associated with the growth of certain cancers (Tsan, 2006; Wang et al., 2014). In addition, other PRRs that are activated by dsRNA are cytoplasmic molecules such as melanoma differentiation antigen (MDA)-5, retinoic acid-inducible gene (RIG)-I and protein kinase R (PKR) (Kalali et al., 2008).

Herewith we determined the potential of murine and human breast cancer cells to attract immune cells upon dsRNA PRR stimulation.

2. Materials and methods

2.1. Mice

Female BALB/c (H-2Kd) and C57BL/6 mice (H-2Hb) (6-8 week old) purchased from Charles River Laboratories (Wilmington, MA), were used for the experiments described here. Protocols for animal use in these experiments were sanctioned by the Ohio University Institutional Animal Care and Use Committee. C57BL/6 mice were used as a source of bone marrow cells for DC preparation and splenocytes.

2.2. Primary cultures of DCs

Bone marrow precursors recovered from femurs and tibiae of 4–8 week old female C57BL/6 or BALB/c mice were differentiated into DCs by culturing them with granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described (Lutz et al., 1999; Lutz and Schuler, 2002). To this end, cell suspensions isolated from bone marrow were cultured in RPMI-1640 supplemented with antibiotics, 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), and murine recombinant GM-CSF (20 ng/ml; Peprotech Inc., Rocky Hill, NJ). The cultures were maintained for 8 days, and GM-CSF replenished on days 3 and 6 of culture.

2.3. Tumor cell lines and tumor implantation

For mouse experiments we used the 4T1 cell line of breast cancer. These cells were maintained in RPMI plus L-glutamine (2 mM), penicillin (100 μg/ml), streptomycin (100 U/ml) and FBS at 10% (all Invitrogen). Solid breast tumors were implanted in BALB/c mice by injection of 4×10^5 tumor cells in the mammary fat pad. Tumors were measured by an external caliper and had a diameter ranging from 0.5 to 0.9 cm at the time of excision.

BT20, MDA-MB231, MDA-MB468, ZR75-1, HTB133, and MCF-7 human breast cancer cells (ATTC, Manassas, VA) were cultured in high glucose DMEM plus antibiotics, glutamine and FBS as above. For some studies we used primary human mammary epithelial cells (HMEPC, Lonza) as a control. Cells were cultured following the manufacturer's instructions.

2.4. Isolation of dendritic cells by magnetic activated cell sorting (MACS)

In some experiments DCs were recovered from tumors or spleens by magnetic sorting. To accomplish this, single cell suspensions of these tissues prepared as we previously described (Conejo-Garcia et al., 2005). Briefly, upon excision in aseptic condition, tumor were minced with scissors in PBS and the resulting suspension then filtered through 70 μM cell strainers (BD Biosciences, San Jose, CA) to eliminate debris. Red blood cells were then lysed by hypotonic shock and the resulting cells were collected and thoroughly washed in PBS in preparation for further studies. DCs were isolated from single cell suspensions using a CD11c cell isolation kit together with MS columns and an octoMACS magnet (all Miltenyi Biotec, Auburn, CA).

2.5. Treatment of cancer cells

Poly(I:C) (Invivogen, San Diego, CA), a synthetic compound that is structurally analogous to double-stranded RNA, was used to treat mouse and human breast cancer cells and human breast epithelial cells. Cells were exposed to different concentrations of dsRNA (0–10 μg/ml) admixed with Lipofectamine 2000 transfecting reagent (Invitrogen). Supernatants were collected and tested for the presence of several chemokines by ELISA. Treated cells were also harvested for RNA extraction, followed by PCR analysis.