Impact Of Oxybenzone On Innate Immune Signaling

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Impact of Oxybenzone on Innate Immune Signaling

A thesis presented

by

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Public Health
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EDCs are commonly thought to bind or interfere with estrogen, androgen, progesterone, thyroid, and retinoid receptors. Oxybenzone is considered to be an endocrine disrupting chemicals and approximately 97% of people in the United States were found to have BP3 in their urine. This thesis will address how BP3 effects the innate immune system, in particular myeloid cells. My Master’s thesis aims to address two main overarching questions. Does BP3 alter macrophage polarization, cytokine/chemokine secretion, viability in vitro? Does exposure to BP3 in vivo during pregnancy/lactation affect the RNA expression of cytokines and immunosuppressant factors associated with myeloid population? It is unknown how BP3 impacts immune subpopulations in a neoplastic setting. Additionally, it is important to consider how these effects may contribute to malignant behaviors. My thesis evaluates the effects of BP3 on the Raw 264.7 cell lines as well as tumor tissues from mice exposed to BP3 during pregnancy and lactation. We hypothesized that BP3 exposures induce changes in myeloid cell interactions in the immune system through ER mediated mechanisms. We anticipated that BP3 would increase growth and migration of 4T1 cells through indirect signals imparted by myeloid cell populations. We also hypothesized that there will be a decrease in T cell proliferation
following BP3 exposure and an alteration in gene expression consistent with a shift from Th1 to Th2. Finally, we expected that BP3 exposure would increase the number of myeloid cells in mouse tumors. Our research shows that oxybenzone appears to enhance the pro-inflammatory state of RAW264.7 cells and may result in release of unidentified factors that can impact 4T1 cell anchorage independent cell growth in these pro-inflammatory conditions. BP3 may also impact the metabolic activity of recovering RAW264.7 cells following LPS induced activity. Additionally, BP3 may impact the release of factors from macrophages that control T cell activation induced proliferation. By using the p53/- mouse tumors we found that exposure to 3mg/kg/day BP3 during pregnancy and lactation did alter IDO1 RNA expression but this was not associated with markers of immunosuppressive cell types.
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CHAPTER 1

ENDOCRINE DISRUPTING CHEMICALS AND THE IMMUNE SYSTEM

Endocrine Disrupting Chemicals

Endocrine disrupting chemicals (EDCs) are substances that interfere with hormone biosynthesis, metabolism, or action, leading to a disruption in homeostasis or reproduction. They are thought of as any natural or synthetic compound that interferes with the hormonal or homeostatic system’s ability to communicate with its environment (Diamanti-Kandarakis et al. 2009). Endocrine disruptive chemicals spread through food consumption, water, air, contaminated soil, and the use of consumer products. These chemicals are found in pesticides, fungicides, industrial chemicals, plasticizers, nonylphenols, metals, pharmaceutical agents and phytoestrogens (Yilmaz et al. 2020). The U.S. Environmental Protection Agency (EPA) defines EDCs as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process.” By interacting with hormone receptors they can alter hormone synthesis and even the transport of metabolic processes that are essential during development (Arya et al. 2020).

EDCs are commonly thought to bind or interfere with estrogen, androgen, progesterone, thyroid, and retinoid receptors. EDCs can be categorized as androgenic and antiandrogenic as well as a thyroid hormone receptor agonist or antagonist. They have been shown to affect both the female and male reproductive system in animal models (Diamanti-Kandarakis et al. 2009). However, they can act not only through nuclear hormone receptors, but also nonnuclear steroid and nonsteroidal hormone receptors.
including, membrane ERs, neurotransmitter receptors, and even the aryl hydrocarbon receptor (AhR) (Diamanti-Kandarakis et al. 2009). Perhaps of greater concern is the observation that some EDCs can modify DNA methylation or histone acetylation resulting in transgenerational affects (Anway & Skinner, 2006). A cross-sectional study examined the connection of individual and combined EDCs with infertility. They found that women have 5-10 times higher EDC levels than men (Calafat et al. 2008).

The endocrine system is highly conserved among mammals allowing for translatable research done in animal models, however there has also been increasing numbers of epidemiological studies that aim to discover the EDC burden of disease in humans (Gore, 2015). Specific effects to the female reproductive system include impairing processes in ovarian development, disrupting steroid hormone levels, reduced fertility, and even abnormal puberty. In relation to the male reproductive system, studies have shown that hormone disruption can influence semen qualities, lead to testicular abnormalities, and is associated with human testicular cancer (Gore, 2015).

**Oxybenzone (BP3)**

The first sunscreen was developed in the 1930s to prevent UV-B and in the 1970s sunscreen further developed to protect against UV-A (Rebut, 1990). In the 1980s oxybenzone (Benzophenone-3/BP3) was approved by the FDA to be used in sunscreens. BP3 is used as a short wave UVB and UVA light absorber at concentrations up to 6% in sunscreens.

In terms of broad-spectrum performance (UVA) BP3 just meets the FDA cutoff with a critical wavelength absorbance of 370 nm. This suggests that BP3 may not be the most effective chemical for protection against UVA (Dinardo 2018). It is also used in plastics.
as a UVB absorber and stabilizer (Dinardo 2018) and is approved by the FDA as an indirect food additive (Calafat et al. 2008) to prevent polymer or food photodegradation (Calafat et al. 2008). Plastic products that can contain oxybenzone include, food packaging, medical devices, vinyl floors, cosmetics, and even cleaning supplies (Arya et al. 2020). The prevalence of sources and pollution have resulted in detectable levels of BP3 in surface water, drinking water, and wastewater (Calafat et al. 2008). Today BP3 is an emerging human and environmental contaminant (Dinardo, 2018).

EDCs can have longer or shorter half-lives depending on the compound and the model system. The half-life of BP3 is 15-18 hours in rats (Arya et al. 2020). BP3 is conjugated with glucuronic acid in the liver, which makes it water-soluble and able for excretion through urine (Gonzalez et al. 2006). In the body, BP3 undergoes 2 phases of biotransformation. In rats, after oral and dermal administrations of 100 mg BP-3/kg body weight (Kadry et al. 1995; Okereke et al. 1993, 1994, 1995), the parent compound and three oxidative metabolites (2,4-dihydroxylbenzophenone, 2,2´-dihydroxy-4-methoxybenzophenone, and 2,3,4-trihydroxybenzophenone) were detected in plasma, tissues, and urine. Urine was the major route of excretion; BP-3 and its metabolites were excreted mainly as glucuronide conjugates (Kadry et al. 1995; Okereke et al. 1993).

In humans, the rate of systematic absorption of BP3 has been estimated as 1-2% via topical application (Hayden, 1997). Repeated application of sunscreens with BP3 increases the concentration of BP3 in urine and shows greater systemic absorption (Gonzalez et al. 2006). In some cases, as much as 10% of the applied dose can be absorbed systemically. (Jiang,1999).
Approximately 97% of people in the United States were found to have BP3 in their urine based on a study that examined 2,517 urine samples from the United States general population (older than 6 years) in 2003-2004 (Calafat 2008). Additionally, BP3 can react with chlorine and produce hazardous by-products (Dinardo, 2018). These oxidative metabolites identified in the rat study were also found in human urine. (Felix, 1998).

A study of urine BP3 in the NHANES population found the limit of detection (LOD), calculated as 3S0, where S0 is the standard deviation as the concentration approaches zero (Taylor 1987), was 0.34 µg/L. We detected BP-3 in 96.8% of the 2,517 samples at concentrations ranging from 0.4 to 21,700 µg/L. In particular, females were 3.5 times more likely than males, and non-Hispanic whites were 6.8 times more likely than non-Hispanic blacks to have BP-3 concentrations above the 95th percentile (Calafat et al. 2008). In a cohort of 304 women undergoing infertility treatment, BP3 exposure was positively associated with women who did moderate/heavy outdoor work which confirms sunscreen as a source for BP3 exposure (Minguez-Alarcon et al. 2019).

Research has raised concern of whether BP3 should continue to be used in personal care products although many unknowns remain to be explored to understand its effects in the human body. In particular, a recent study assessing the systemic absorption of 6 sunscreen active ingredients, including BP3, found that these chemicals surpassed the FDA threshold plasma concentration (0.5ng/mL) after systemic absorption and remained in the plasma for at least 3 days after last use (Matta 2020). These results suggest that exposures that occur after dermal exposures are longer lasting than previously estimated, and that BP3 metabolism is less efficient, allowing it to remain in
circulation for days or even weeks in some individuals. Furthermore, among 48 participants, 14 also reported rashes after application. The effects of BP3 once it is systemically absorbed are not fully understood, therefore there remains a need for research to determine its effects and the mechanisms involved in any hazardous outcomes that are observed.

**Adverse effects associated with BP3**

Research has shown that BP3 may be acting as a xenoestrogen; this means that it can bind to the estrogen receptor (ER) and mimic estrogen to produce harmful effects. BP3 was found to skew phenotypic sex of 12 day old zebrafish, from less male to more female. In addition to this BP3 also interfered with the maturation stages of zebrafish gonads. Although concentrations of BP3 used were above those found in the aquatic environment, this reveals an estrogenic or antiandrogenic effect of BP3 on zebrafish (Kinnberg et al. 2015).

A study conducted with MCF-7 breast cells found that BP3 is a bioactive chemical that is weakly estrogenic and can also exhibit anti-estrogenic and anti-androgenic effects in vitro (Schlumpf, 2001; Schreurs, 2004). Of particular interest is that BP3 has been demonstrated to induce DNA damage in several cell types including breast epithelial cells (Majhi, 2020 Kim, 2018). This effect was dependent on the ER, implicating the receptor in its mechanism of action, but did not depend on strong transcriptional activity of the receptor. Additionally, BP3 has been negatively associated with morphological changes in the mammary gland in numerous studies using rodents. In the first study, exposure to BP3 during pregnancy and lactation was found to alter mammary gland morphology and function in mice. Exposure to 3000ug BP3/kg/day
during this critical period produced morphological changes that were observed long after exposures ended, suggesting these effects were permanent. This study suggests that exposures to xenoestrogens during pregnancy and lactation can have long-lasting effects on morphology of the mammary gland, consistent with other recent studies that have found that this period of life is vulnerable to environmental agents (LaPlante, 2018).

Additional studies have revealed that the offspring from BP3-exposed dams exhibited similar results. In females exposed to low doses during perinatal development, BP3 reduced mammary cell proliferation and decreased the number of cells expressing ERα (Matouskova, 2020). Combined, these studies suggest that BP3 may have adverse effects on ductal development during critical windows of susceptibility and may also produce permanent effects on sons and daughters exposed during perinatal development (Matouskova, 2020).

Epidemiological studies have also revealed associations between BP3 exposures and harmful health outcomes in human populations. A case-control study was conducted using a Nanjing Medical University Birth Cohort (China) with 101 patients with Hirschspung’s disease - a neonatal intestinal abnormality derived from the failure of enteric neural crest cells migration to hindgut during embryogenesis - and 322 controls. Women submitted questionnaires and spot urine samples that were used to quantify BP3 exposures. The urine samples taken after pregnancy were assumed to represent consistent levels of BP3 in that individual that would be similar to their BP3 exposures during weeks 5–12 of pregnancy (the time period where intestinal development occurs), although this was not actually demonstrated to be true. Additionally, 101 patients and 103 controls, who were chosen from university affiliated hospitals and had received surgical
and non-surgical treatments, provided colon tissue samples for analysis. BP3 exposure was found to be associated with Hirschsprung’s disease (Huo 2016).

Another study examined urinary BP3 metabolites from 404 multiethnic women in NYC who participated in the Children’s Environmental Cohort study from Mount Sinai hospital. Samples were taken in their third trimester, along with infant size at birth. Statistical analyses revealed that high concentrations of BP3 were associated with a decrease in birth weight among girls (Wolff, 2008). This supports the notion that chemicals with endocrine disrupting effects can alter the prenatal development of progeny (Ngalame et al., 2013; Veiga-Lopez et al., 2013). Collectively, these studies support the characterization of BP3 as a xenoestrogen, as well as an endocrine disruptor (La Merrill, 2020) with activities of concern that warrant further study.

**The Immune System**

The function of the immune system is to prevent or limit infection. It can detect and differentiate between self and foreign, as well as healthy and unhealthy cells. Each cell type in the immune system has a different function to produce the correct immune response (NIH, 2021). The immune system can be divided into two categories, innate and adaptive immunity. Innate immunity occurs immediately as genetically encoded receptors detect pathogen-associated patterns. Specific cell types that belong to the innate category include neutrophils, eosinophils, basophils, mast cells, monocytes, dendritic cells, and macrophages. Innate cells can initiate adaptive immune responses, which include activation of B and T cells. These cells recognize specific antigens to eliminate foreign or unhealthy tissues. B cells make antibodies and T cells have several functions including killing infected cells and initiating additional immune responses.
This thesis project will focus on the innate cell types, in particular myeloid cells. I have chosen to focus on these cell populations because their activity can be controlled by estrogen; they are also responsible for orchestrating the trafficking of other cell populations, as well as providing signals to control the type of activity and they have been demonstrated to interact with tumor cells to affect survival migration and cancer stem cell activity. Specific cell types of interest to this thesis project are monocytes, macrophages and dendritic cells.

Monocytes are immature cells that can differentiate into macrophages or dendritic cells depending on the cytokines present. Both cell types are important for antigen presentation to the adaptive immune cells, however the dendritic cell is the most potent initiator and modulator of the immune response (Banchereau, 1998). Dendritic cells sample large areas of the tissue through their extensive dendritic network to capture and process antigens and display large amounts of major histocompatibility-peptide complexes (MHC) at their surface. They also upregulate stimulatory molecules and migrate to lymphoid organs, the spleen, and lymph nodes where they interact with antigen-specific T cells (Banchereau, 1998). An increased presence of these immune cells indicate stimulation of the immune system. On the other hand, dendritic cells can also be tolerizing, which is important to control inflammation, but may help tumors evade the immune system.

Macrophages have a strong phagocytic activity necessary to remove dead and dying cells as well as detect bacteria. They are important for releasing signals involved in wound healing and can also polarize towards a tolerizing or immune suppressive phenotype. Since these cells are derived from the same precursor cell they often express
similar proteins, but particular membrane molecules such as F4/80 and differences in the levels of other proteins can distinguish them.

Once T cells are activated by dendritic cells, they can interact with other cells including B cells to initiate antibody formation, macrophages which enable cytokine release, and other targets specific for lysis (Banchereau, 1998). Dendritic cells process molecules into antigens that can be recognized by B cells. B cells are the precursors of antibody-secreting cells and directly recognize native antigens through B-cell receptors (Banchereau, 1998). Additionally, they can activate T cells by using a specific MHC. T lymphocytes require the antigen to be processed by an antigen presenting cell (APC) (Banchereau, 1998). T-cell antigen receptors recognize fragments of antigens bound to molecules of the MHC on the surface of APCs (Banchereau, 1998).

T cells can be divided into two categories, CD8+ T cells and CD4+ T cells. The major subsets of interest of CD4+ T cells are T helper cells, TH1 and TH2 cells (NIH, 2021). Extracellular antigens that have entered the endocytic pathway of the APC are processed and presented to T-helper cells by the MHC class II molecules. T-helper cells have the potential to have a profound immune-regulatory effect (Banchereau, 1998). Specifically, TH1 cells coordinate immune responses against intracellular microbes and produce molecules to activate additional immune responses such as macrophages. TH2 cells coordinate immune responses to combat extracellular pathogens. Regulatory T cells, also known as Tregs, monitor and inhibit the activity of other T cells (NIH, 2021).

**Immune Responses to BP3**

Many cell types within immune populations express ERs and this may explain how exposure to BP3 could alter immune activity. *In vivo* and *in vitro* mouse model
experiments showed that pregnancy levels of estrogen typically induce FoxP3+ T regulatory cells in splenocytes as well as ovarian tissue (Tai, 2008). Increases in estrogen are also associated with changes in T cell polarity. Th2 cytokines (TGFβ and IL4) are upregulated whereas Th1 cytokines (IFNγ and IL2) are decreased by estrogen (Salem, 2004). Finally, estrogen is associated with changes in chemokine related gene expression. For example, 17β-estradiol has been demonstrated to increase chemokine receptors (CCR1-5 in T lymphocytes) and CXCL12 and CXCR4 in breast cancer cell lines (Mo, 2005). If BP3 is acting as an estrogen receptor agonist (or antagonist), we may expect to see alterations in these pathways.

BP3 has the potential to cause harm to the immune system as an allergen, but the mechanism of action for the effects of BP3 on cells in the immune system is not fully understood. In a study assessing allergens due to sunscreen application, it was found that among 23,908 patients, 70% observed an inflammatory effect when they were administered a topical cream with a 10% concentration of BPS. The inflammatory response remained relatively high (64% of patients) after application of a 3% concentration of BP3 (Warshaw, 2013). Another study explored the inflammatory effect of four UV filters, including BP3, and observed a 1.1-1.5-fold increase in various inflammatory cytokines associated with macrophages such as TNFα and IL6. In an in vitro study using THP-1 cells that had been differentiated into macrophage-like cells, BP3 influenced cytokine gene expression. This study further supports research showing the allergy risk of BP3 by suggesting that the release of inflammatory cytokines may aggravate allergic or asthmatic symptoms (Ao, 2018).
BP3 increases the expression of genes associated with M2 macrophage polarization in a subset of patient-derived breast tissue explants (Gregory, 2020). BP3 has the potential to stimulate cytokines and chemokines associated with myeloid populations that are polarized towards M2. M2 macrophages are known to secrete proteins important for wound healing and these proteins can promote the migration of tumor cells. BP3’s increasing effect on M2 polarization suggests that this endocrine disrupting chemical may cause a pro-metastatic signal (Gregory, 2020).

Finally, there is also evidence that BP3 can adversely affect other tissues and organs of the body, including tissues that are strongly impacted by immune cell function. For example, the adverse effects of BP3 on the gut were shown in a study using *Carassius auratus*, a species of goldfish. The fish were exposed to varying concentrations of BP3, and it was observed that BP3 accumulation in the liver and intestine is time and dose dependent (Zhang, 2020). BP3 can induce effects in the immune, endocrine, and metabolic systems and increase the risk of fish being infected by pathogenic microorganisms. This study also showed that short term BP3 exposure causes a significant increase in biomarkers specific to endocrine disrupting compounds (Zhang, 2020).

My Master’s thesis aims to address two main overarching questions. Does BP3 alter macrophage polarization, cytokine/chemokine secretion, viability in vitro? Does exposure to BP3 in vivo during pregnancy/lactation affect the RNA expression of cytokines and immunosuppressant factors associated with myeloid population? It is unknown how BP3 impacts immune subpopulations in a neoplastic setting. Additionally, it is important to consider how these effects may contribute to malignant behaviors.
**Hypothesis statement**

We hypothesized that BP3 exposures induce changes in myeloid cell interactions in the immune system through ER mediated mechanisms. We anticipated that BP3 would increase growth and migration of 4T1 cells through indirect signals imparted by myeloid cell populations. We also hypothesized that there will be a decrease in T cell proliferation following BP3 exposure and an alteration in gene expression consistent with a shift from Th1 to Th2. Finally, we expected that BP3 exposure would increase the number of myeloid cells in mouse tumors. My thesis evaluates the effects of BP3 on the Raw 264.7 cell lines as well as tumor tissues from mice exposed to BP3 during pregnancy and lactation.

**Chapter Study Objectives**

Chapter 2: AIM 1; In this chapter I will discuss the BP3 effect on a triple negative breast cancer cell line (4T1) and a monocyte cell line (RAW 264.7). The following questions will be addressed:

2.1 Question: Does BP3 affect the growth, migration, or gene expression in a model of triple negative breast cancer (ERalpha negative)?

2.2 Question: Does a direct exposure to BP3 alter anchorage independent growth or stem like gene expression in 4T1 cells? Does BP3 enhance the secretion of factors from macrophages that facilitate anchorage independent growth of 4T1 cells?

2.3 Question: Does BP3 alter the cytokine or chemokine expression of the RAW 264.7 macrophage cell line? Does BP3 affect polarization in the absence of other signals? Does it alter LPS induced pro-inflammatory activity? Does BP3 affect tumor associated gene expression in RAW264.7 cells?
2.4 Question: Does exposure to BP3 affect the recovery of RAW264.7 macrophages following a pro-inflammatory signal?

2.5 Question: Does indirect BP3 exposure with secretion factors from 4T1s affect cytokine gene expression?

2.6 Question: Does BP3 affect the release of proteins from RAW264.7 which can impact CD4+ T cell activation?

Chapter 3: AIM 2; In this chapter I will discuss the impact of BP3 exposure during pregnancy and lactation in a p53/-/- tumor model. The following questions will be addressed:

3.1 Question: Does exposure to BP3 during pregnancy and lactation impact the gene expression related to immune endpoints in a p53/-/- tumor model.

3.2 Question: Does exposure to BP3 during pregnancy and lactation impact the population of GR-1 expressing cells (neutrophils or myeloid derived suppressor cells) in a p53/-/- tumor model?

3.3 Does exposure to BP3 during pregnancy and lactation impact the population of CD31 expressing cells (labeling vascular cells) in a p53/-/- tumor model? We anticipated this to be upregulated because large vessels were increased and IDO1 is associated with increased angiogenesis

3.4 Does exposure to BP3 during pregnancy and lactation impact the population of CD25 expressing cells (T cells and myeloid tolerogenic cells) in a p53/-/- tumor model?

3.5 Does exposure to BP3 during pregnancy and lactation impact the population of ARG1 expressing cells (M2 polarized macrophages) in a p53/-/- tumor model?
3.6 Does exposure to BP3 during pregnancy alter the number of FoxP3 expressing cells in tumors of mice implanted with P53 ko epithelium?

Chapter 4: Discussion and Conclusion; In this final chapter I will provide a brief discussion of the results and provide a conclusion that includes future direction.
CHAPTER 2

BP3 EFFECT ON A TUMOR CELL LINE AND A MONOCYTE CELL LINE

**Macrophages**

Macrophages are central regulators of organ development, disease progression, and tissue growth after an injury occurs (Rehman et al. 2021). There are two origins of macrophages, tissue-resident macrophages which develop during embryogenesis or monocyte derived macrophages which originate from bone marrow derived hematopoietic stem cells (Rehman et al. 2021).

Macrophages are one of the first cells to activate in response to inflammation. The monocytes and macrophages respond to chemokines through specific receptors on their membranes. The chemokines help them traffic to areas of concern. They function as an alert system during a microbial invasion and are essential in adaptive immune responses (Shi & Pamer, 2011). The structure of a macrophage resembles an amoeba so they are readily able to move and phagocytose dying cells and foreign bodies (Rehman et al. 2021). The digested proteins are then displayed on the cell surface in the context of the major histocompatibility complex. This antigen presentation allows for determination by adaptive immune cells as to whether an attack should be mounted or the proteins are “self” antigens and should be ignored (Jakubzick et al. 2017).

In addition to presenting an antigen, macrophages also secrete cytokines (i.e. TNFα, IL6 and IL-1b) and other chemokines (i.e. CCL2, CCL3, CXCL12) in this initial inflammatory response. Once the T helper cells are called to the area they are able to activate cytotoxic T cells and others in order to fight against the infection (Jakubzick et
al. 2017). From this point the T cell can also call in B cells to secrete antibodies which can target cells presenting the antigen in the future (Shi & Pamer, 2011).

**Plasticity of macrophages**

Macrophages exhibit plasticity and can be polarized towards 3 overarching phenotypic and behavior categories, M0, M1, and M2. These categories are different endpoints on a spectrum. However, it is important to note that it is a spectrum and the macrophage can also present a hybrid phenotype (Rehman et al. 2021). M1 macrophages are categorized as proinflammatory. These macrophages have enhanced antigen presenting capabilities, produce an abundance of reactive oxygen intermediates, and rely on anaerobic glycolysis for energy metabolism (Rehman et al. 2021). Interferon-gamma, a Th1 cytokine, lipoproteins, and intracellular pathogens all promote M1 differentiation (Roszer, 2015). Once polarized towards this phenotype they are able to generate Nitric Oxide from L-arginine via iNOS activity (Rehman et al. 2021).

They also secrete important proinflammatory cytokines including IL-1beta, TNFα, IL-12, IL-18, and IL-23 to direct the activity of other immune cell types towards the best function to clear the problem. M2 macrophages can be divided into subcategories of M2a, M2b, and M2c, and M2d each exhibiting their own activation markers. Polarization towards the classic M2a phenotype is achieved through exposure to Th2 cell cytokines IL-4, and IL-13. M2b polarization is achieved through exposure to LPS together with these cytokines. M2c polarization results from exposure to IL4 and IL13 together with glucocorticoids and TGF-beta. Finally, macrophages are polarized towards the M2d phenotype when adenosines and IL6 are also present.
M2a macrophages are important for wound healing and tissue repair via initiation of collagen synthesis and cell movement (Rehman et al. 2021). M2c macrophages are important for resolution of inflammation. They produce IL-10 and TGF-beta which are able to further increase macrophage differentiation towards the M2 phenotype and suppress T cell activity.

**Macrophages and tumors**

Although M2 can be beneficial for pathogen defense, mitigating inflammatory responses, and promoting wound healing, but they are also known to be involved in more nefarious health/disease presentation such as aiding in allergic inflammation, tumor growth, and play a role in endocrine signaling (Roszer, 2015). Tumor and stromal derived factors from the tumor microenvironment (TME) divert macrophages towards yet another phenotype which is similar, but distinct from M2 macrophages (Williams et al. 2016). The tumor associated macrophage (TAM) behaviors are manipulated to support tumor growth and metastasis (Williams et al. 2016). They promote cancer cell intravasation and together with fibroblasts promote cleaving of the extracellular matrix which is essential for cancer cells to have a path to promote tumor growth and the development of new blood vessels, known as angiogenesis, which is a prerequisite for metastatic disease.

Macrophages function to promote tight intercellular junctions with endothelial cells, and also provide survival cues to these endothelial cells. TAMs facilitate metastasis by reorganizing the extracellular matrix and degrading the basement membrane. This allows for tumor cell extravasation. During metastasis TAMS are observed to directly interact with tumor cells (Majidpoor & Mortezaee, 2021), an association that is encouraged through production of chemo-attractants such as CSF1, CCL2, CCL3,
CXCL12, VEGF, and SEMA3a. An upregulation of CCL2 is correlated with tumor vascularization, when blood vessels invade tumors.

**Chemokines**

Signals released from monocytes, macrophages and dendritic cells are responsible for trafficking and the activity of other immune cells. Immune cells communicate via cell-to-cell contact or secreted signaling molecules. Cytokines are proteins important for immune cell activation and function. Chemokines are ligands for CC motif- or beta chemokine receptors. A CC chemokine has two cysteines (amino acids) adjacent to the amino terminus and are crucial for the rapid response and specific chemotaxis of the cells. Chemokines can show both anti-cancer and pro-cancer properties (NIH, 2021).

Specific chemokines of interest to this thesis are CCL2, CCL3, and CCL4.

CCL2 is one of the key chemokines regulating migration and infiltration of macrophages. CCL2 is produced by many cell types (Fujimura 2015), such as endothelial, fibroblasts, epithelial, smooth muscle, mesangial, astrocytic, monocytic, and microglial cells. The main source of CCL2 however is macrophages (Fujimura 2015). The receptor for CCL2, CCR2, is mainly expressed in hemopoietic cells, monocytes, and a small population of T cells. In humans, CCR2 is highly expressed in chronic inflammatory diseases, such as arthritis or kidney disease. This is similar in mice, where CCR2-positive T cells accumulate after an inflammatory stimulus (Mack 2001).

CCL2 recruits and activates pro-tumor macrophages although its effect is mediated by neutrophils and macrophages (Lavender, 2017). The actions of CCL2 can vary depending on the presence of TGF-B signaling (Fridlender, 2009). It is also triggered by exposure to IL-4 and IFNγ (Gschwandtner, 2019). The CCL2 crosstalk
between macrophages and breast cancer cells is also believed to increase the breast cancer stem like activity. (Zhang 2021). An *in vivo* mechanistic study in mice, found that a CCL2 triggered chemokine cascade promoted the metastatic seeding of breast cancer cells (Kitamura, 2015). The CCL2 amplified and activated inflammatory macrophages to produce more CCL3, which facilitated the macrophage interaction with the breast cancer cells.

CCL3 has the ability to activate both innate and adaptive immune responses. CCL3 binds to CCR1, CCR3, and CCR5 which are expressed in immature myeloid cells, neutrophils, eosinophils, T cells, and monocytes among other cell types. *In vitro* studies have also revealed that CCL3 secretion induces chemotaxis of CCR1 and CCR5 expressing cells (Rabin 2003). Interestingly CCL3 plays a large role in the mobilization of myeloid precursor cells and is able to stimulate mature myeloid precursor cells as well. CCL3 works to sustain the recruitment of cells during an inflammatory response (Schaller, 2018). There is conflicting data on how CCL3 affects tumors. In several studies, CCL3 was found to reduce tumorigenicity and extend survival (Schaller, 2018), however, in other mouse studies CCL3 has contributed to cancer growth (Kitamura, 2015).

CCL3 is produced in humans and mice by various cell types, including Tregs, T cells, macrophages and some cancer cell types (Wang, 2013). Increased CCL3 expression was found to be higher in basal-like invasive human breast carcinoma associated with poor prognosis (Kitamura, 2015).

CCL4 is a macrophage inflammatory protein that induces the recruitment of dendritic cells, neutrophils, monocytes, macrophages, and T cells, including T regulatory
cells to inflammatory sites. It also acts on CD4+ T cells. The primary receptor for CCL4 is CCR5 and serves as a chemoattractant for CCR5 expressing cells. The expression of CCR5 and CCR1 on T cells suggest that CCL3 and CCL4 may play a role in cell-mediated immunity (Rabin 2003).

In addition, tissue environment plays a critical role in activating macrophages towards M2. Conditioned media as well as cell to cell interactions can determine the level of M2 markers and there are also tissue-derived endocrine signals that have the potential to promote M2 activation regardless of the IL-4/STAT6 receptor pathway.

**Macrophages and breast development**

Macrophages serve various functions in different endocrine organs of the body. Macrophage-endocrine cell interactions determine the function of endocrine tissues through the hypothalamus-pituitary-thyroid axis (Rehman et al. 2021). The breast is a central location where hormones can impact macrophage function. In mammary development, macrophages are recruited during ductal structure growth in the mammary glands. They play an important role in tissue patterning, branching morphogenesis, and regulate vascular growth (Lin et al., 2002). They also help guide the fusion of endothelial cells which is necessary for vascular sprouting (Qui et al. 2018). By producing matrix metalloproteinases (MMP), macrophages assist in the remodeling of the extracellular matrix during the outgrowth of ductal structures (Qui et al. 2018). Macrophages that reside in the tissue of the mammary gland maintain the viability and function of mammary stem cells (Gyorki et al. 2009: Williams et al. 2016). These tissue-resident macrophages are maintained and proliferate due to a colony stimulating factor (CSF1) produced in the local tissue stroma, which is a key growth factor that dictates
macrophages proliferation and survival (Williams et al. 2016). These macrophages are indefinitely replenished by the continued recruitment of circulating monocytes.

A surplus of macrophages in breast cancer is generally associated with decreased overall survival and is an independent prognostic indicator of breast cancer (Majidpoor & Mortezaee, 2021). Additionally, chronic inflammation develops when the body is unable to regulate immunostimulatory signals and they are left activated for a prolonged period of time. Low levels of reactive oxygen species, which aren’t enough to cause death may lead to increased mutagenesis if DNA repair pathways are compromised. During these periods of high inflammation macrophages can undergo apoptosis in order to suppress persistent immunity and facilitate wound healing. However, this can also in turn support tumor growth by facilitating angiogenesis and showing trophic macrophage characteristics. When macrophages are exposed to poorly vascularized tumor regions, HIF1a and HIF2a are upregulated to provide metabolic adaptation in a poor oxygen environment and further an immuno-suppressive environment. The production of IDO1 by macrophages represents an inflammatory induced response which in turn suppresses T cell activity.

The body requires different immune responses in order to adapt to its exposures to disease (Olsen, 1996). Inflammatory responses could benefit during an infection but if prolonged could increase likelihood of autoimmune diseases (Olsen, 1996). In women this is more common than in men (Olsen, 1996). This suggests that changes to female sex hormones play a role in chronic inflammatory diseases and periods of vulnerability such as menopause, when estrogen levels lower, wearing off its anti-inflammatory effect
which in turn can leave a woman more susceptible to chronic inflammation (Olsen, 1996).

Specifically in breast cancer, elevated body mass index (BMI) during menopause increases the risk of developing hormone receptor-positive breast cancer (Qiu et al. 2018). These overweight conditions contribute to chronic inflammation in the mammary tissue even if there is no cancer present. When examining these fatty tissues they have been found to contain macrophage-associated inflammatory pathways, including IL6, IL-8, CCR5, and PPARα (Qiu et al. 2018). In these dysfunctional adipose tissues known as, adipocyte hypertrophy, macrophages are recruited due to an abundance of apoptotic-induced cell death and establish inflammatory foci called, crown-like structures (CLS). This results in the release of free fatty acids that stimulate macrophages and lead to an upregulation of TNFα, IL1β, IL6, COX2, and PGE2 (Qiu et al. 2018). This can lead to an upregulation of CYP19, which is a gene that can induce estrogen production (Qiu et al. 2018) creating a positive feedback loop where chronic inflammation is sustained.

**Endocrine Disrupting Chemicals (EDCs) and macrophage interactions**

Environmental estrogens are ubiquitous worldwide including the arctic leading to unavoidable exposure (Chighizola, 2012). Environmental estrogens can be found in plants, fungi, meat and dairy products, and synthetic chemicals (Chighizola, 2012). Synthetic chemicals with estrogenic properties are known as xenoestrogens and are found in plastics, detergents, pesticides, and industrial chemicals (Chighizola, 2012).

Xenoestrogens are synthetic chemicals that have a diverse group of hydrocarbons and exhibit estrogen like activity (Chighizola, 2012). Xenoestrogens can vary by having different numbers of aromatic rings and some can be negatively charged if they are
chlorinated (Chighizola, 2012). Once xenoestrogens are bound to estrogen receptors, they are able to translocate the receptor-ligand complex from the cytoplasm to the nucleus, stimulating the synthesis of estrogen-regulated proteins (Chighizola, 2012). They are also able to antagonize endogenous hormones and alter the pattern of estrogen synthesis and metabolism (Chighizola, 2012). It has been proposed that environmental estrogens may mediate immunologic changes directly by binding to immune cells or indirectly by acting on several other tissues, modulating cytokine production (Chighizola, 2012).

Many studies examining hormone or xenoestrogens utilize a mouse macrophage cell line called RAW264.7. In RAW264.7 E2 treatment suppressed the ability of LPS to cause pro-inflammatory M1 polarization, inhibiting nitrite production and TNFα secretion (Kim, 2003; Tomaszewska, 2003). Pregnancy levels of E2 inhibit proinflammatory pathways such as, TNFα, IL-1b, and IL6 in M0 and dendritic cells while stimulating cytokines including IL-10 in M0 and dendritic cells (Straub, 2007).

Using RAW 264.7 cells stimulated with LPS, a select group of 21 endocrine disrupting chemicals were analyzed to determine their immunologic and cytotoxic effects (Kim, 2015). Some key endocrine disrupting chemicals were fenitrothion (FTH), pthalates, estriol, estradiol, and phenols (Kim, 2015). Fenitrothion is a common insecticide that inhibited mRNA expression of iNOS which reduced NO production. Additionally, pthalates and estriol also reduced NO production but this was not dependent on iNOS expression (Kim, 2015). In contrast, benzo(a)pyrene, a hydrocarbon produced during combustion, increased NO in RAW264.7 cells (Kim, 2015). The chemicals studied altered macrophage function in a dose dependent manner (Kim, 2015).
Immune cells undergo death through apoptosis or necrosis. Necrosis can induce inflammation while apoptosis can induce a tolerogenic response (Kim, 2015). When RAW264.7 cells were treated with estradiol or phenol, there was an increase in apoptosis (Kim, 2015). Benzo(a)pyrene caused an increase in cell death that was independent of apoptosis (Kim, 2015). These results exemplify that effects of estrogenic EDCs on macrophages may be greatly diverse depending on the nature of the chemicals (Kim, 2015).

**BPA and macrophages**

Although BP3 is not as extensively studied as BPA, studies regarding this endocrine disrupting chemical emphasizes the need to evaluate potential adverse immune effects that could be associated with BP3. Here I discuss adverse effects of BPA relating specifically to macrophages which further prompts the need to expand on BP3 studies to provide a comprehensive assessment of its effects on the innate immune system.

As a common chemical used in plastics, BPA has been extensively studied and shown to cause adverse immune effects, particularly involving macrophages. BPA has been shown to aggravate atherosclerosis, a cardiovascular disease (Sui et al. 2018). Macrophages are known to play a critical role in the development of atherosclerosis. Mice that were chronically exposed to BPA through drinking water led to enhanced production of inflammatory cytokines and macrophage infiltration. The macrophages isolated from the BPA exposed mice demonstrated a pro-inflammatory phenotype and impaired DNA-repair capacity. The DNA repair play critical for BPA enhancement of macrophage inflammation and atherosclerosis involved sirtuin I (Yang et al. 2021).
In addition to DNA repair mechanisms that impact BPA’s inflammatory effect, dysregulation of non-coding RNAs (LncRNAs) can change gene expression and influence the cross-talk between signaling pathways through network gene interactions which leads to adverse macrophage effects. The study analyzed red common carp, an aquatic species, HK macrophages and found the pro-inflammatory cytokine, IL1β to be significantly upregulated. Analysis of pathways suggested that BPA used NF-kB, TLR, HIPPO, and MAPK signaling pathways to cause the dysregulation and adverse immune responses in the primary HK macrophages (Liu et al. 2020).

BPA can induce the production of cytokines by instigating an immune response. Using mouse spleen and thymus cells in vitro, BPA, stimulated macrophages to secrete various cytokines including, IL-1, IL6, IL-12, TNFα, and MCP-1 (Yamashita, 2005). This was also seen using THP-1s, a human macrophage cell line. BPA increased the expression of pro-inflammatory cytokines, TNFα and IL6 while decreasing the expression of anti-inflammatory cytokines, including TGF-b and IL-10 (Liu, 2014). It was found that this occurs through an estrogen-receptor (alpha and beta) dependent mechanism. This evidence suggesting that BPA stimulates a pro-inflammatory response prompts the need to investigate whether BP3, an endocrine disrupting chemical that is also capable of mimicking estrogen, has adverse effects on macrophages as well.

**Results**

2.1 Question: Does a direct exposure to BP3 alter anchorage independent growth or stem like gene expression in 4T1 cells? Does BP3 enhance the secretion of factors from macrophages that facilitate anchorage independent growth of 4T1 cells?

**Triple Negative Model**
4T1 murine mammary carcinoma cells are highly tumorigenic, with low immunogenicity and characteristics that resemble stage IV human mammary gland tumors (Garcia et al. 2014). When injected into mice, malignant epithelial growth presents in a solid arrangement and it is known to spontaneously metastasize to other organs (Garcia et al. 2014). The 4T1 line does not have an estrogen, progesterone, or epidermal growth factor receptor 2 (ErbB2) protein receptor (Schrors et al. 2020) making them a model system for mimicking humans diagnosed with a triple negative breast cancer (Schrors et al. 2020). It has been established that the 4T1 mammary carcinoma is an adequate model of human TNBC in relation to tumor behavior and potential drug targeting therapies (Garcia et al. 2014).

Numerous studies have suggested that estrogenic EDCs can drive the proliferation of ERα positive breast cancer cell lines (Shanle & Wu, 2011). The Jerry lab has demonstrated that BP3 in particular has a weak estrogenic effect on T47D cells. Triple negative breast cancer is particularly difficult to treat because it does not exhibit expression of any of the standard receptors which can be targeted (ER, PR, Her-2). We are interested in whether BP3 could impart pro-tumorigenic activity on triple negative breast cancer lines through its interaction with stromal cells and crosstalk with the breast cancer cell line.

To accomplish the overall goal I first needed to establish whether BP3 could affect growth or migration through an ER independent direct effect on the cells. 4T1 cells, were treated with increasing doses of BP3 or the vehicle DMSO and the number of viable cells were assessed using an MTS assay.
We found that there is no change in migration when 4T1s are treated with BP3 at 10 or 30uM for 48 hours. There was also no change in proliferation after 48 hours when 4T1s were treated at varying doses, 0.3,1,3,10,30uM (Figure 1f).

To determine whether BP3 could impact the migration of cells, I utilized scratch wound assays. Cells were plated and allowed to grow to confluence. A pipet tip was used to scratch a void through the cells. The ability of cells to migrate back across the void was monitored via microscopy over time on the 8th and 24th hour of cell growth. The width of the scratch was measured using image J and graphed. We found that the scratch closed at rates similar to the DMSO control treated cells suggesting that BP3 did not affect migration (Figure 1g).

To determine whether BP3 exposure could alter gene expression. 4T1 cells were treated with 30uM BP3 or DMSO (vehicle) for 48 hrs. The cells were lysed in Trizol and the RNA purified. PCR was performed using Agilent’s Brilliant III ultrafast QRTPCR kit to examine the impact of BP3 exposure using primers specific to chemokines, chemokine receptors, growth factors, cytokines as well as genes associated with stemness or EMT.

We found that among chemokines Ccl2, Ccl3, Ccl4, Ccl5, and CCL22, CCL2 expression is significantly induced 4T1s treated with BP3 (Figure 1a). We also followed this up by examining the expression of their corresponding chemokine receptors, including, Ccr1, Ccr3, Ccr4, and Ccr5 (Figure 1b). We found that there is no change in expression between the control and exposed group (Figure 1). IL1β is a key cytokine in the immune environment. It can be expressed by tumor cells as well as macrophages and typically facilitates tumor growth. When analyzing its expression we found that BP3 decreases IL1β expression (Figure 1d).
We also examined expression of a several genes encoding secreted protein important for regulating immune function $TGF\beta 1$, an immune suppressive growth factor important in development, $Cox2$, an inflammatory related gene and $IL6$, a cytokine released from tumor cells and macrophages. $TGF\beta$ and $Cox-2$ were not significantly affected following BP3 exposure whereas $Il6$ demonstrated inconsistent results (Figure 1d).

To determine if BP3 exposure altered the expression of genes involved in cancer stemness, qRT-PCR was performed to analyze Sox4 and Prom1. Sox4 is a transcription factor that regulates survival, differentiation and epithelial to mesenchymal transition. Prom1 is a transmembrane glycoprotein that inhibits differentiation of stem cells (Figure 1e). CDH1, an adhesion related protein. It is downregulated when epithelial cells transition into more migratory cells (Figure 1e). We did not observe a significant change in mRNA expression of any of these genes following exposure to BP3, however slight trends up in Prom1 and Sox4 were of interest (Figure 1e).

Based on these interesting trends in cytokine RNA expression, we collected conditioned media off of 4T1 cells treated with BP3 and examined the level of secreted proteins using a Mesoscale Discovery pro-inflammatory protein analysis panel (MSD). We found that protein expression of KC/GRO, a neutrophil chemoattractant, is significantly decreased in the BP3 exposed 4T1 cells, however there is no change in IL6 protein expression, which agrees with the gene expression analysis (Figure 1i). These results suggest that the behavior (proliferation and migration) of the ER negative 4T1 tumor cells are largely unaffected by direct exposure to 30uM BP3. However, CCL2
RNA appears to be upregulated and KC/GRO is decreased following BP3 exposure in an
ER independent manner.

2.2 Question: Does a direct exposure to BP3 alter anchorage independent growth or
stem-like gene expression in 4T1 cells? Does BP3 enhance the secretion of factors
from macrophages that facilitate anchorage independent growth of 4T1 cells?

The ability of normal epithelial or tumor cells to survive and grow anchorage
independently is a marker of stem-like behavior. One can enrich populations of stem-like
or cancer stem-like cells by growing them in conditions where they can’t form good
interactions with the plastic tissue culture dish (anchorage independent growth). In
tumors, this behavior is a marker for an aggressive cell death resistant characteristic.
Since there was a trend towards elevated Sox4 and Prom1 mRNA expression following
4T1 exposure to BP3, we investigated whether BP3 affects 4T1 anchorage independent
growth.

We plated 4T1 cells at a low density on low attachment cell culture plates and
looked for growth of primary and secondary colonies. 4T1s were plated as single cells
and cultured for 1 week. After 1 week the cells, now in colonies, were resuspended and
re-plated in the same treatment groups for another week. After week 2 colonies were
measured and any colony above 40um was quantified in each treatment group.

We found that both 4T1s treated with BP3 through direct treatment and 4T1
conditioned media showed a decreasing trend in secondary colony growth (Figure 1c).
This was not what we expected given the upwards trend of Prom1 and Sox4, but it was
still interesting.
Since macrophages can secrete factors that can facilitate the cancer stem-like activities in breast cancer we also asked whether exposure of the RAW264.7 macrophage cell line to BP3 could result in the secretion of factors that would in turn enhance anchorage independent growth of tumor cells. We set up an anchorage independent assay where 4T1s were treated with varying conditioned medias containing macrophage secreted proteins. The 4T1s treated with M0 conditioned media demonstrated a significant increase the ability to grow anchorage independently and then form secondary colonies (Figure 2g). This suggests that BP3 causes macrophages to secrete factors that can in turn alter 4T1 behavior towards more stem-like growth/survival abilities.

2.3 Question: Does BP3 alter the cytokine or chemokine expression of the RAW 264.7 macrophage cell line? Does BP3 affect polarization in the absence of other signals? Does it alter LPS induced pro-inflammatory activity? Does BP3 affect tumor associated gene expression in RAW264.7 cells?

Monocyte Model Raw 264.7

The RAW264.7 cell line is an immortalized monocyte/macrophage-like that originates from an Abelson leukemia virus (MuLV) transformed cell line from male Balb/C mice (Taciak et al. 2018). They are a traditional model when studying macrophages and are able to be manipulated to express a more M1 phenotype (Taciak et al. 2018). Upon LPS stimulation, RAW 264.7 cells display an M1 phenotype and when co-cultured with 4T1 cells they exhibit the phenotype of tumor associated macrophages (TAMs) (Kong et al. 2019). The RAW264.7 cell line is unique because its phenotype can change depending on its passage or micro-environment (Kong et al. 2019).
To examine gene expression of the RAW264.7 cells the M0 cells were treated with DMSO (control) or 30uM BP3 for 48 hours. Once the cells were lysed in Trizol and the RNA purified, PCR was performed using Agilent’s Brilliant III ultrafast QRTPCR kit. We then analyzed gene expression and determined statistical significance using Graphpad Prism.

Among the chemokines examined (Ccl2, Ccl3, Ccl4, Ccl5, and Ccl22) Ccl2 has an increasing trend in gene expression (Figure 2a). Interestingly there is contradicting results in CCL4 expression. This is consistent with a decrease in Ccl4 noted in lungs of animals exposed to high levels of BP3. However, the results were not always consistent from one experiment to another. Among one set of samples, Ccl4 appears to have a slight increasing trend, and in another set of samples there was a significant decrease in Ccl4 gene expression. Among their corresponding receptors, Ccr1, Ccr2, Ccr3, Ccr4, and Ccr5, Ccr1 shows an increasing trend and CCR2 shows a decreasing trend, but there was no change in expression amongst Ccr3, Ccr4, or Ccr5 (Figure 2b).

ARG1 expression was examined because it has been shown to function as a tumor suppressor in breast cancer and we wanted to determine if BP3 altered its expression in immortal monocyte cells (Figure 2c). BP3 appeared to decrease expression compared to the vehicle group. Additionally to determine whether BP3 altered macrophage function, we examined Mrcl expression (Figure 2c). This gene codes for a protein that mediates the endocytosis of glycoproteins by macrophages. We found that BP3 increases Mrcl expression in RAW264.7 cells.

IL10 is an anti-inflammatory cytokine and our results show a minimal decrease in Il10 expression in the BP3 exposed cells (Figure 2c). We found no change in IL6
expression and a non-significant increase in expression in Mrc1. To further determine if 
BP3 impacts the gene expression of immune related cytokines we examined IL1β and 
TNFα expression and found no change (Figure 2d).

Based on these interesting trends in cytokine RNA expression, we collected 
conditioned media off of RAW 264.7 cells exposed to BP3 and examined the level of 
secreted proteins using a Mesoscale Discovery pro-inflammatory protein analysis panel 
(MSD).

At the protein level we also found no change in IL1β, IL6, IL-10, or TNFα 
expression (Figure 2e and 2f). The MSD showed high expression levels of IL6, IL-10, 
and TNFα in both treatment groups. Additionally the pro-inflammatory cytokine IL12p70 
showed no change in expression (Figure 2e). These results are interesting because BP3 
may be enhancing the immune response of RAW264.7 cells. This lead us to wonder how 
BP3 may effect RAW 264.7 cells stimulated with LPS.

**M1**

By treating RAW264.7 cells with LPS, macrophages were polarized towards the 
M1 phenotype. First we wanted to determine whether BP3 affected M1 proliferation. 
Using an MTS assay we determined that there is no change in proliferation of M1 
macrophages when treated with varying doses of BP3, 0.3uM, 3uM, and 30uM, for 48 
hours.

Following this, to examine gene expression of the RAW 264.7 cells stimulated 
with LPS, cells were exposed to DMSO (control) or 30uM BP3 for 48 hours. Once the 
cells were lysed in Trizol and the RNA purified, PCR was performed using Agilent’s
Brilliant III ultrafast QRTPCR kit. We then analyzed gene expression and determined statistical significance using Graphpad Prism.

We examined inflammatory genes and found that there is no change in *Il6* expression, however *TNFα*, *IL1β*, and *Cox2* show an increase in gene expression (Figure 4b). These changes in expression were not seen in the analysis of M0 expression and this increase in expression could indicate that BP3 is increasing the inflammatory response of phenotypic M1 macrophages in way that M0 cells are not, indicating that LPS is activating an immune response.

Based on these interesting trends in cytokine RNA expression, we collected conditioned media off of M1 cells treated with BP3 and examined the level of secreted proteins using a Mesoscale Discovery pro-inflammatory protein analysis panel (MSD).

There is high IL6 expression in both treatment groups, although BP3 appears to decrease IL6 expression after 48 hours and less so after 72 hours (Figure 5c). IL10 is less expressed in the M1 conditioned media compared to the M0 conditioned media and it appears that BP3 may slightly decrease IL10 expression at 48 hours, however there is no change at 72 hours (Figure 5d). There is no change in IL12p70 expression at 48 and 72 hours (Figure 5d). Additionally we found that, there is high TNFα expression in both treatment groups, although BP3 appears to increase TNFα expression after 72 hours however this is not apparent at 48 hours (Figure 5c). This may indicate that the cells changed their protein secretion after being exposed to BP3 for an additional 24 hours.

These results indicate that BP3 could be upregulating TNFα, IL1β, and Cox2. This change was not observed in IL6 gene expression. When looking at secreted protein expression via MSD it is evident that BP3 increases TNFα protein expression at 72 hours
(Figure 5c). As expected from the lack of change in IL6 gene expression there was also no change in secreted protein expression of IL6. Interestingly, the protein expression of IL1β was not upregulated as was seen in the RNA expression (Figure 5c).

**TAMs**

We sought to determine whether BP3 treatment of RAW 264.7 altered polarization when co-cultured with 4T1 cells. We found that BP3 altered the polarization of RAW macrophages towards TAMs after co-incubation with tumor cells from the 4T1 cell line. TAMs have been shown to support tumor growth and to promote angiogenesis (Majidpoor & Mortezaee, 2021). Previous studies showed TAMs upregulating CCL2 which may lead to an increase in tumor vascularization (Majidpoor & Mortezaee, 2021).

To determine the effect of BP3 on these TAM cells we analyzed their gene expression. The Raw 264.7 cells co-cultured with 4T1s were treated with DMSO (control) or 30uM BP3 for 48 hours. Once the cells were lysed in Trizol and the RNA purified, PCR was performed using Agilent's Brilliant III ultrafast QRTPCR kit. We then analyzed gene expression and determined statistical significance using Graphpad Prism.

We found that *Ccl2* and *Ccl3* gene expression slightly decreases when Raw264.7 cells are co-cultured with 4T1s for 48 hours (Figure 3a). *Ccl5* had a significant decrease in gene expression. In addition, *Ccl4* appears to increase slightly. Corresponding chemokine receptors, *Ccr1* and *Ccr4* showed a decrease in gene expression. There is no change in gene expression of *Ccr2* or *Ccr3* (Figure 3b).

In order to look at *Ccl2* gene expression over time, TAMs were cultured for 24, 48, 72, and 96 hours. At the 24, 48, and 96 hour time points, *Ccl2* demonstrated a decrease in expression which was significant at 96 hours (Figure 3c). However,
interestingly at the 48 hour time point there is a trending increase in expression which contradicts the slight decrease seen in previous qRT-PCR results.

When examining inflammatory genes, *Il6* and *Il10* gene expression increases slightly, while CD163 expression decreases (Figure 3d). In addition, *Mrc1* and *ARG1* gene expression decreases and there is no change in *iNos* expression (Figure 3e). *iNos* is an important gene that enables nitric oxide production and can lead to tumor promotion or death depending on its origin (Prerrota et al. 2018).

We then wanted to examine whether TAM conditioned media with DMSO or BP3 would impact 4T1 anchorage independent growth because TAMs have been shown to support tumor growth and BP3 may alter these effects. We plated 4T1 cells at a low density on low attachment cell culture plates and looked for growth of primary and secondary colonies. 4T1s were plated as single cells and cultured for 1 week. After 1 week the cells, now in colonies, were resuspended and re-plated in the same treatment groups for another week. After week 2 colonies were measured and any colony above 40um was quantified in each treatment group. The anchorage independent assay showed an increase in secondary colonies (Figure 3f).

Based on these interesting trend in cytokine RNA expression, we collected conditioned media off of TAM cells treated with BP3 and examined the level of secreted proteins using a Mesoscale Discovery pro-inflammatory protein analysis panel (MSD).

We found that There is a decrease in *IL6* expression although it is not significant. There is high KC/Gro expression in both treatment groups although there is no BP3 dependent effect of KC/Gro protein expression in TAMs conditioned media (Figure 3g).
Additionally, when looking at inflammatory protein expression we found that there is no BP3 dependent effect on IL10, IL12p70, or TNFα expression (Figure 3e and 3f).

These results are indicative that TAMs exposed to BP3 may be stimulating 4T1 anchorage independent growth. BP3 could also be inhibiting TAM upregulation of CCL2, CCL3, and CCL5. The lack of protein changes shows that BP3 is not triggering an immune response that alters the protein levels of IL10, IL12p70, or TNFα, but may be triggering an immune response through IL6.

**2.4 Question: Does exposure to BP3 affect the recovery of RAW264.7 macrophages following a pro-inflammatory signal?**

The recovery phase of Raw 264.7 is important to consider because BP3 is excreted regularly in humans and it has the potential to cause changes to macrophage behavior even when it is no longer present. We explored the recovery period M1 macrophages by treating them with DMSO or BP3 for 48 hours and then replacing the media with fresh untreated RPMI media for varying time periods. As a control we measured proliferation after the 48 hour time period and saw no change which is consistent with previous data. Interestingly, during the 24 hour period of M1 recovery proliferation increases at 0.3uM and more so at 30uM. At 3uM there is a slight decrease in proliferation. When looking further at the difference between live and dead cells at 24 hours of recovery there is no change, however at the 72 hour period there is no change in the number of live cells but there is a significant increase in the number of dead cells in the BP3 treated group.

Following this, we examined gene expression by isolating and purifying the RNA and PCR was performed using Agilent’s Brilliant III ultrafast QTTPCR kit. We then
analyzed gene expression and determined statistical significance using Graphpad Prism. When examining the recovery phase of M1, the gene expression analysis showed that BP3 was found to reduce IL1β, IL6 and TNFα during LPS activation (Figure 5d). It does not appear to affect the resolution of the protein levels except for TNFα which appears to be elevated for longer. This suggests that BP3 may affect the metabolic activity. In particular this may relate to mitochondrial succinate dehydrogenase.

2.5 Question: Does indirect BP3 exposure with secretion factors from 4T1s affect cytokine gene expression?

We were interested in how Raw 264.7 would respond to exposure of BP3 through 4T1 conditioned media. This would lead to an indirect exposure and would answer whether the secreted factors of 4T1s exposed to BP3 could alter Raw 264.7 gene expression.

Raw cells were treated with 4T1 conditioned media DMSO (vehicle) or BP3 for 48 hours. We examined gene expression by isolating and purifying the RNA and PCR was performed using Agilent’s Brilliant III ultrafast QRT PCR kit. We then analyzed gene expression and determined statistical significance using Graphpad Prism.

We found that CCL2 expression decreased in the Raw264.7 cells exposed to BP3 and 4T1 secreted factors. Additionally CCL3 and iNOS appear to have an increase in expression. We found no change in IL6 expression. These changes is chemokine expression indicate that indirect exposure to BP3 through 4T1 conditioned media could be altering the immune response of Raw 264.7 cells (Figure 6).

2.6 Question: Does BP3 affect the release of proteins from RAW264.7 which can impact CD4+ T cell activation?
We were interested in whether BP3 affected the release of proteins from Raw 264.7 cells which in turn could impact CD4+ T cell activation. Macrophage and T cell interactions are essential to coordinating appropriate immune responses. In particular these CD4+ T cells can be divided into TH1 and TH2 cells. We wanted to determine whether Raw 264.7 secreted factors exposed to BP3 altered the activation of CD4+ T cells.

T cells were treated with direct DMSO or 7.5uM BP3 for 48 hours or Raw (M0) conditioned media for 48 hours. The control direct treatment of DMSO or 30uM BP3 showed a significant decrease in total cells and a decrease in live cells. This is consistent with unpublished data from Stephanie Morin. The T cells treated with Raw CM had a decrease in total cells in the BP3 group but no change in live cells. Interestingly, both directly treated T cells and T cells treated with Raw CM show BP3 significantly decreasing the number of dead cells.

Following this, gene expression was quantified by by isolating and purifying the RNA and PCR was performed using Agilent’s Brilliant III ultrafast QRTPCR kit. We then analyzed gene expression and determined statistical significance using Graphpad Prism. In the control group there was an increase in gene expression of IL4 and IFNγ, Additionally, we saw a significant decrease in TGFβ1 and IL2 expression.

We also exposed T cells to TAMs conditioned media and gene expression analysis showed a decrease in IL4 expression and no change in IFNγ gene expression. The T cells treated with TAMs CM had no change in gene expression of TGFβ1 or IL2 expression between TAMs CM DMSO and TAMs CM BP3 (Figure 7f).
We also wanted to look at protein expression. We collected conditioned media off of the T cells and examined the level of secreted proteins using a Mesoscale Discovery pro-inflammatory protein analysis panel (MSD). This analysis showed significant decreases in IFNγ, TNFα, IL-4, IL-5, IL6, IL-10, KC/GRO, IL12p70, and IL1β (Figure 7d).

We found that there is a general decrease in cell proliferation following exposure to conditioned media from BP3 treated RAW but no change in viability (Figure 7a,b,c). There is also a significant decrease in cytokine secretion. It is unclear whether this was due to a direct effect or an indirect effect. These results indicate that T cells treated with M0 conditioned media showed a rescue in cell proliferation but not in cytokine release.

**Conclusion**

Several studies regarding oxybenzone have shown this chemical to have the potential of causing adverse immune effects. Although the mechanism of action is unclear, our research shows that oxybenzone appears to enhance the pro-inflammatory state of RAW264.7 cells and may result in release of unidentified factors that can impact 4T1 cell anchorage independent cell growth in these pro-inflammatory conditions. BP3 may impact the metabolic activity of recovering RAW264.7 cells following LPS induced activity, but future work is required. BP3 may impact the release of factors from macrophages that control T cell activation induced proliferation.
Figure 1. The effect of 30uM BP3 on 4T1 cell gene and protein expression. (a) Chemokine gene expression. (b) Chemokine receptor gene expression. (c) Anchorage independent growth of 4T1s directly and indirectly treated with BP3. (d) Gene expression of immune markers TGFβ1, IL1β, Cox2, and IL6. (e) Gene expression of stemness markers CDH1, Sox4, and Prom1. (f) Proliferation of 4T1s treated with varying concentrations of BP3 (0.3uM, 1uM, 3uM, 10uM, and 30uM). (g) Migration analysis using a scratch wound assay, at 10uM and 30uM BP3 and microscopy measurements taken at 8hr and 24hr. (h) Migration analysis using a scratch wound assay. 4T1s were pretreated with BP3 at 10uM and 30uM for 1 hour and then scratch was made and microscopy measurements taken at 8hr and 24hr. (i) Protein expression of interleukins KC/Gro and IFNG.
Figure 2. The effect of 30uM BP3 on M0 cells (Raw 264.7) gene and protein expression. (a) Chemokine gene expression. (b) Chemokine receptor gene expression. (c) Gene expression of immune markers ARG1, Mrcl, IL10, and IL6. (d) Gene expression of immune markers IL1β and TNFα. (e) Protein expression of interleukins IL10 and IL12p70. (f) Protein expression of interleukins TNFα and IL6. (g) Anchorage independent growth analysis of secondary colonies above 40um in 4T1 cells indirectly treated with BP3 through M0 conditioned media.
Figure 3. The effect of 30μM BP3 on TAMs (Raw 264.7 & 4T1 co-culture) gene and protein expression. (a) Chemokine gene expression. (b) Chemokine receptor gene expression. (c) Gene expression of CCL2 over time (24, 48, 72, and 96 hours). (d) Gene expression of immune markers. (e) Gene expression of immune markers. (f) Anchorage independent growth analysis of secondary colonies above 40μm in 4T1 cells indirectly treated with BP3 through TAMs conditioned media. (g) Protein expression measured via ELISA of KC/GRO. (h) Protein expression measured via ELISA of IL6 and TNFα. (i) Protein expression measured via ELISA of IL-10, IL12p70, IL1β.
Figure 4. The effect of 30μM BP3 on Raw 264.7 cells stimulated with LPS towards M1 phenotype. (a) Proliferation of M1 cells treated with varying doses of BP3 including, 0.3μM, 3μM, and 30μM. (b) Gene expression of immune markers IL6, TNFα, IL1β, and Cox2.
Figure 5. The effect of 30uM BP3 on recovery of RAW264.7 macrophages following a pro-inflammatory signal. (a) Proliferation of M1 recovering cells at varying doses of BP3 including 0.3uM, 3uM, and 30uM measured at 0hr and 24hr via MTS assay. (b) Total number of live or dead cells during recovery at 0hr, 24hr, and 72hr. (c) Protein expression of immune markers IL6 and TNFα after 48 or 72 hours of recovery. (d) Protein expression of immune markers IL-10 and IL12p70 after 48 or 72 hours of recovery.
Figure 6. The effect of 30uM BP3 on gene expression of Raw264.7 cells treated with 4T1 conditioned media.
Figure 7. The effect of 30uM BP3 on T cells directly treated with 30uM BP3 and indirectly through M0 conditioned media. (a) Total cells after 48hours of direct or indirect treatment of BP3. (b) Total live cells after 48hours of direct or indirect treatment of BP3. (c) Total dead cells after 48hours of direct or indirect treatment of BP3. (d) Protein expression of immune markers IFNγ, TNFα, IL-4, IL-5, IL6, and IL-10. (e) Protein expression of immune markers KC/Gro, IL12p70, and IL1β. (f) Gene expression of T cells directly treated with 7.5uM BP3 and indirectly treated with BP3 via TAMs conditioned media.
CHAPTER 3

IMMUNE EFFECT OF BP3 ON A p53-/ TUMOR MODEL

Introduction

The tumor model design consists of Tp53-/ epithelial cells transplanted into mammary fat pads of BALB/c mice where females were exposed to either 0 (control) or 3000 µg/kg/day oxybenzone throughout pregnancy and lactation. Klara Matouskova surveilled these mice until they reached 52 weeks of age to investigate tumor latency. Figure 8 illustrates a schematic created by Klara Matouskova with the study design. The tumors were taken for analysis of gene expression of several immune related markers. We found these markers especially interesting because unpublished work has shown that in mice exposed to BP3 until harvest, these genes were significantly altered.

We chose INos and IL6 to determine whether the tumors showed M1 polarized macrophages. On the other hand we also explored TGFβ, IL10, and IDO1, as they are generally immunosuppressive cytokines found in association with Tregs, M2c macrophages, tolerogenic dendritic cells and MDSC. ARG1 is an important marker for wound healing and Aldh contributes to the production of retinoic acid, which are necessary for Tregs. NRP1 is a marker for Tregs. IFNγ and Cox2 are important inflammatory mediators frequently associated with the upregulation of IDO1. CCL2 is released by many cell types, including macrophages, and is important for chemoattracting other CCR2 + cells.

Results

3.1 Question: Does exposure to BP3 during pregnancy and lactation impact the gene expression related to immune endpoints in a p53-/ tumor model.
Gene expression from a panel of genes were quantified and included CCL2, CCL4, NRP-1, ARG1, TGFβ1, TGFβ3, IL4, IL10, ALDH1a1, ALDH1a2, IFNγ, IDO1, IL6, Cox2, INOS, and IL1β. The results were particularly interesting in regards to IDO gene expression and GR-1 protein expression. In the following section I will explain how IDO is involved in the immune system (Figure 9).

3.2 Question: Does exposure to BP3 during pregnancy and lactation impact the gene expression of IDO1 in a p53-/- tumor model.

Indoleamine 2,3-dioxygenase 1 (IDO1) is a cystolic, monomeric, heme-containing enzyme that catalyzes the initial rate-limiting step in the degradation of an amino acid, L-tryptophan using the kynurenine pathway. In addition to this function, IDO1 can act as a mediator of a signaling pathway that can change the functional phenotype of specific immune cells, which can be associated with distinct protein conformations (Pallota et al. 2021). IDO1 is a physiologic checkpoint that can guarantee homeostasis by modulating immune responses orchestrated by dendritic cells and macrophages. IDO1 plays a key role in supporting immune privilege including protecting the fetus from the maternal immune system (Pallota et al. 2021).

There is a strong correlation between IDO1 and immune cell populations including dendritic cells and T cells (Feng et al. 2020). IDO1 functions as an immune regulator that has highly regulated catalytic activity as well as nonenzymic functions that can reprogram the expression profile of immune cells towards a highly immunoregulatory phenotype (Pallota et al. 2021). There is evidence that IDO1 can tilt the immune system towards supporting tumors (Prendergast, 2014). IDO1 is an inducible
enzyme and can be induced by IFNγ (Pallota et al. 2021). Interestingly, TNFα, IL6, and IL-10 can synergize with each other to increase IDO1 expression (Pallota et al. 2021).

IDO1 resides in the cytosol and can have distinct intra and extracellular topology depending on the cell microenvironment (Pallota et al. 2021). There is conflicting data regarding whether elevated IDO1 expression is associated with a better and poor cancer prognosis (Feng et al. 2020). This may be due to IDO1 expression in both tumor cells and tumor infiltrating cells which can lead to changes in the tumor microenvironment (TME) and varying outcomes of tumor prognosis (Feng et al. 2020). IDO1 is expressed in non-tumor cells in the TME including, dendritic cells, fibroblasts, endothelial cells, eosinophils, and macrophages. This correlation between IDO1 and antigen presenting cells (APCs), including macrophages suggests that APCs could mediate immune responses via IDO1 (Feng et al. 2020). Additionally, IDO1 can be secreted in extracellular vesicles, including exosomes (Pallota et al. 2021).

IDO1 has been established as a central driver of malignant development and progression. It is able to suppress T cells, activate T regulatory cells and myeloid derived suppressor cells, and further the promotion of tumor angiogenesis (Prendergast, 2014). It has been implicated in numerous disease including, cancer, chronic viral infection, allergy, autoimmune, and inflammatory diseases that are categorized by disordered immune control. It can act on multiple levels to create a favorable environment for tumor development and metastatic progression (Prendergast, 2014).

Typically, it is activated in the skin and tumor draining lymph nodes as part of the chronic inflammation required for tumor emergence (Prendergast, 2014). IDO1 is significantly correlated with tumor mutation burden in breast invasive carcinoma.
(BRCA) and cervical squamous cell carcinoma (CESC) (Feng et al. 2020). IDO1 participates in anti-tumor immune process and is correlated with mutation burden (Feng et al. 2020). In addition IDO1 drives tumor angiogenesis and metastasis and is capable of causing angiogenic defect in lungs even in the absence of tumors (Prendergast, 2014).

Studies have shown elevated IDO1 expression on the mRNA and protein level in BrCa (breast cancer) cells (Heng et al. 2020). IDO1 gene expression in BrCa tissue is upregulated in triple negative and HER2-enriched BrCa subtype when compared to luminal BrCa, hormone-receptor positive breast cancers, and healthy control tissues (Heng et al. 2020). IDO1 was highly expressed in triple negative and HER-2 enriched BrCa subtypes but was absent in luminal BrCa (Heng et al. 2020).

Our results show that BP3 causes an increase in IDO expression. No other genes showed a significant BP3 dependent effect on expression (Figure 9f)). Following gene expression analysis our interesting results with IDO expression led us to wonder how protein markers for cellular subsets may be affected. If so, this might suggest an activity associated with IDO1 such as angiogenesis, immune suppression involving, Tregs, tolerogenic DC,M2 macrophages or changes in MDSC/neutrophils. Protein expression that was quantified via IHC and included GR-1, CD-31, CD25, ARG1, FoxP3 (Figure 10).

3.3 Does exposure to BP3 during pregnancy and lactation impact the population of CD31 expressing cells (labeling vascular cells) in a p53/- tumor model?

Endothelial cells play a major role in the control of coagulation, thrombolysis, vascular tone, permeability, inflammation, tissue repair, and angiogenesis (Pusztaszeri et al. 2006). Interestingly, endothelial cell molecular characteristics vary in different blood
vessels and from organ to organ (Pusztaszeri et al. 2006). An important marker for endothelial cells and angiogenesis is CD31. CD31 is a 130kDa platelet-endothelial cell adhesion molecule. It also triggers downstream inhibitory signaling upon transhomophilic CD31 engagement during cell to cell interactions. CD31 signaling is a part of leucocyte detachment, T-cell activation, platelet activation, and angiogenesis (Liu & Shi, 2012). CD31 is a constituent of the endothelial intercellular junction, which promote cell-to-cell adhesion and are required for the organization of blood vessels during embryo development and tissue proliferation (Pusztaszeri et al. 2006; Dejana et al. 2015).

CD31 plays a major role in the adhesion cascade between endothelial cells during inflammation and angiogenesis (Pusztaszeri et al. 2006). It is important to note that CD31 expression is not restricted to blood vessels (Pusztaszeri et al. 2006). CD31 is present on the surface of platelets, monocytes, macrophages, and neutrophils (Pusztaszeri et al. 2006). CD31 loses its expression at intercellular junctions after endothelial cells are exposed to inflammatory cytokines (Liu & Shi, 2012).

Our results show a decrease in CD31 protein expression suggesting that BP3 decreases the number of vascular cells in the tumor (Figure 10a).

3.4 Does exposure to BP3 during pregnancy alter the number of FoxP3 expressing cells in tumors of mice implanted with P53 ko epithelium?

The transcription factor forkhead box P3 (FoxP3) is a transcription factor and a marker for Tregs as an important part of Treg development and function (Zhao et al. 2019). Tregs that are FoxP3+ are suppressors of antitumor responses and maintain immunological tolerance in host tissues. In general high expression of FoxP3 is thought to indicate poor prognosis (Shang et al. 2015). Some studies have reported that both
FoxP3+ T cells and FoxP3+ tumor cells also can promote tumor progression by fostering angiogenesis. However, there is conflicting evidence indicating that FoxP3+ T cells and tumor cells could improve survival. It is important to note that FoxP3+ cells are associated with ER+ cancers (Qian et al. 2017). In addition FoxP3 is also a regulator in CD4+CD25+ regulatory T cell development and function (Fontenot, 2003).

Our results show no change in FoxP3 protein expression in the tumors exposed to BP3 (Figure 10b).

3.5 Does exposure to BP3 during pregnancy and lactation impact the population of ARG1 expressing cells (M2 polarized macrophages) in a p53-/− tumor model?

Increased expression of ARG1 suggests that macrophages are exhibiting M2-like characteristics. In M2 mouse macrophages arginine is converted to ARG1. Tumor-associated macrophages (TAMs) are also thought to share similar behaviors as the M2 phenotype. Additionally, lactic acid may play a role in polarizing TAMs to a tumor promoting phenotype that is shown through increased ARG1 expression (Saha et al. 2017).

Our results show no change in ARG1 expression between the vehicle and BP3 exposed mouse tumors (Figure 10c).

3.6 Does exposure to BP3 during pregnancy and lactation impact the population of GR-1 expressing cells (neutrophils or myeloid derived suppressor cells) in a p53-/− tumor model?

GR-1 positive myeloid suppressor cells are thought to suppress the immune system and promote angiogenesis (Magdalena et al., 2011). They are actively recruited by various chemokines and are able to produce angiogenic factors including VEGF,
MMPs, and interleukins (Magdalena et al., 2011). As a result, neovascularization occurs around tumors and is necessary for tumor growth because of the supply of nutrients and oxygen (Schmid & Varner, 2010). These myeloid cells are then able to enhance cancer cell survival. Inflammatory monocytes that are positive for GR-1 have been shown to be recruited towards pulmonary metastases. GR-1 expression may also be correlated with CCL2 expression. In tumor cells CCL2 may recruit GR-1+ monocytes to further the extravasation of the tumor cells (Qian et al. 2012).

Our results show a decrease in GR-1 expression, suggesting that BP3 is decreasing the number of myeloid derived suppressor cells in the tumor. This could mean that there is a decrease in MDSC or neutrophils (Figure 10d).

3.7 Does exposure to BP3 during pregnancy and lactation impact the population of CD25 expressing cells (T cells and myeloid tolerogenic cells) in a p53-/- tumor model?

CD25+ regulatory T cells are important in maintaining immune tolerance and immune homeostasis including maintaining the TH1/TH2 balance. (Zhang et al. 2020). They are also important in the prevention of autoimmunity. CD25 is a subunit of the IL2 receptor and is transiently upregulated during activation of T cells. This in turn activates IL2 signaling (Shatrova et al. 2016). Infiltrating cells in the tumor have high CD25 expression is associated with poor prognosis. In human cancers CD25 is mainly expressed in CD4+FoxP3+ T regulatory cells (Song, 2021). CD25+ cells can promote the secretion of Th2 cytokines and inhibit the release of Th1 cytokines (Zhang et al. 2020).

When mice were exposed to BPA one week before tumor infection, they found that there was not only an increase in IL4 and IFNγ expression but there was a decrease
in CD4+CD25+ cells. This shows BPA promoting the development of TH2 cells (Yan et al. 2008).

Our results show no change in CD25 expression between the vehicle and BP3 exposed mouse tumors (Figure 10e).

**Conclusion**

Exposure during pregnancy and lactation results in tumors with higher IDO1 expression. This did not appear to correlate with increases gene expression changes of immunosuppression (IL10 TGFβ, NRP, ALDH or bio markers of cell subsets capable of suppression (CD25, GR1, FOXP3) however it was associated with significant changes in angiogenesis. There was a significant elevation of large vessels in these tumors and a non-significant decrease in micro vessels (CD31). When looking at gene expression of cytokines and inflammatory factors known to induce IDO1 (IFNγ IL6 and COX2), we found no associated elevation suggesting a different route of expression. Future work is needed to understand the contributing factors and it impact on tumorigenesis.
Figure 8. The p53/- mouse model exposed to oxybenzone during pregnancy and lactation. At 3 weeks of age, female mice were transplanted with Trp53/- mammary epithelium. From there they were split into 3 treatment groups by 6 weeks of age. These included parous oxybenzone, parous controls, and virgin controls. For this thesis, we will focus only on the parous treatment groups. During pregnancy and lactation the mice received either 0 (control) or 3000 µg/kg/day oxybenzone orally. The mice were surveilled throughout adulthood and involution where at 52 weeks the mice were euthanized and tissue was collected for analysis.
Figure 9. Gene expression of immune markers in a p53-/- mouse model. (a) Gene expression of CCL2 and CCL4. (b) Gene expression of NRP1 and ARG1. (c) Gene expression of TGFβ1 and TGFβ3. (d) Gene expression of IL-4 and IL-10. (e) Gene expression of Aldh1a1 and Aldh1a2. (f) Gene expression of IFNγ and IDO-1. (g) Gene expression of IL6 and Cox2. (h) Gene expression of iNOS and IL1β.
Figure 10. Protein expression of immune and angiogenic markers in p53-/- mouse model exposed to 3000 µg/kg/day BP3 during pregnancy and lactation. (a) CD-31 (b) FoxP3 (c) ARG1 (d) Gr-1 (e) CD25
Chapter 4

DISCUSSION AND CONCLUSION

Discussion

In this study we sought to determine if BP3 induced changes in myeloid cell interactions in the immune system through ER mediated mechanisms. We focused on answering two main questions:

1. Does BP3 alter macrophage polarization, cytokine/chemokine secretion, viability in vitro?
2. Does exposure to BP3 in vivo during pregnancy/lactation affect the RNA expression of cytokines and immunosuppressant factors associated with myeloid population?

Previous data has shown that BP3 can increase the cytokine CCL18. This cytokine is present in dendritic cells and is an M2 marker in human breast tissue. This led us to investigate whether BP3 exposure pushes macrophages towards either an M1 or M2 phenotype when there is an absence of other inflammatory signals. Numerous EDCs including BP3 have been demonstrated to affect chemokine expression. They are important for dictating which populations of cells arrive at the tumor location. Our results indicate an elevation of CCL2 by BP3 under certain circumstances, MO not TAM. This suggests that BP3 might have a different effect when there is cancer present versus no cancer. This also might suggest a reason for why the Schwartz lab observed a protective effect of BP3 on one diet and not on the other.

We chose to study the 4T1 triple negative cell line as well as the Raw 264.7 macrophage cell line because of their extensive use in previous research. The 4T1 tumor cell line was important in establishing whether BP3 could have an effect even when no
receptors were present. Additionally, the Raw 264.7 cell line has been shown to polarize towards M1 in previous literature and this is important throughout my thesis in order to create a baseline when creating the M1 or TAM phenotype.

Our results show that when inflammatory signals are absent BP3 alone does not push M0 cells towards the M1 or M2 phenotype. When analyzing gene expression of the M1 cytokines including IL6, TNFα, and IL1β there was no change. Following gene expression analysis, protein analysis using the MSD confirmed that there was no change in the secreted protein levels either. Additionally, there was no statistically significant change in M2 biomarker expression. There was a general increasing trend in chemokine RNA expression and chemokine receptor expression, but this did not reach significance.

In cells expressing the M1 phenotype, BP3 showed an increasing trend in TNFα, IL1β, and Cox2 expression. This change was not observed in IL6 gene expression. As expected from the lack of change in IL6 gene expression there was also no change in secreted protein expression of IL6. Interestingly, the protein expression of IL1β was not upregulated as was seen in the RNA expression.

When looking at secreted protein expression via MSD in the resolution macrophages, it is evident that BP3 increases TNFα protein expression at 72 hours. This is especially important when placed in the context of resolution. If proper resolution does not occur in the body, this can lead to a chronic low level inflammation. Diseases that could follow this unresolved inflammatory response includes diabetes, cardiovascular disease, cancer, arthritis, and chronic obstructive pulmonary disease (Minihane et al. 2015). Our results showed no change in total cells but an increase in viable cells, determined by MTS. This assay targets an enzyme in the mitochondria and is a sign of metabolic activity. Future
work should be done in order to determine if there is prolonged SDH, a marker of metabolic activity, following BP3 exposure.

BP3 altered the polarization of RAW macrophages towards TAMs after co-incubation with tumor cells from the 4T1 cell line. When examining the recovery phase of M1, BP3 was found to reduce IL1β, IL6 and TNFα during LPS activation. It does not appear to affect the resolution of the protein levels except for TNFα which appears to be elevated for longer. This suggests that BP3 may affect the metabolic activity. In particular this may relate to mitochondrial succinate dehydrogenase. 4T1s treated with M0 conditioned media showed a significant increase in secondary colonies.

We expected BP3 to have a stronger effect on macrophage polarization. In RAW 264.7 cells, stimulated with LPS, E2 treatment suppressed its ability to polarize towards the pro-inflammatory phenotype (Kim, 2003; Tomaszewska, 2003). While BPA has been shown to stimulate pro-inflammatory pathways (Yamashita, 2015). Based on this we expected a result similar to BPA and it is interesting that BP3 did not appear to have this effect in our research.

We sought to determine whether BP3 treatment of RAW264 cells affected the release of any protein which affects activation induced T cell proliferation or gene expression. We found that there is a general decrease in cell proliferation following exposure to conditioned media from BP3 treated RAW but no change in viability. There is also a significant decrease in cytokine secretion. It is unclear whether this was due to a direct effect or an indirect effect.

The p53 model causes several subtypes of breast cancer. Klara Matouskova was able to identify different tumor types in her study and this could show how the impact of BP3
on the tumor directly or the immune population may be dictated by these subtypes. A limitation of the protein analysis is that within the vehicle and BP3 exposed mice, there are several different tumor types and tumor grades. This could be associated with the variability seen in each treatment group between mice. Future work should be done in order to increase the number of tumor samples in each subtype in order to draw proper conclusions from the results.

In the p53-/ mouse experiment animals were exposed to 3mg/kg/day during pregnancy and lactation. This dosage was also used by the Joe Jerry lab, where they found BP3 causes permanent damage to ductal density in mice (Jerry et al. 2018). By using the p53-/ mouse tumors we found that exposure to 3mg/kg/day BP3 during pregnancy and lactation did not result in a residual effect on most markers of macrophage or dendritic cell activity in tumors a year later. However, interestingly there was a change in IDO1 expression showing an increased expression of IDO1. IDO1 RNA expression was not associated with markers of immunosuppressive cell types, but did appear to correlate with increased large vessels in tumors and a decrease in micro vessels (CD31). As an angiogenesis marker that is associated with immunosuppressed environments such as Tregs or MDSC populations, CD31 was expected to increase based on an increase in blood vessels analyzed through H&E in these tumors. Our results show a decrease in CD31 expression suggesting that the CD31 marker may be more sensitive to staining all blood vessels which may not be quantifiable via IHC.

GR-1 is expressed in neutrophils and myeloid derived suppressor cells and we found a slight decrease in protein expression in the oxybenzone exposed group. Additionally, by
using the FoxP3 marker we saw no change in Tregs between both groups. The CD25 marker which also marks Tregs and tolerogenic dendritic cells had no change as well.

**Conclusion**

Oxybenzone is a ubiquitous chemical that is going to continue to be used to protect humans from UV exposure as well as in consumer products. Therefore, it is important to consider its effects as it is constantly absorbed by the body. Although oxybenzone is not as thoroughly researched as other endocrine disrupting chemicals, such as BPA, does not mean that it is not causing harmful effects to the body, particularly in the immune system.

We observed an interesting impact on anchorage independent growth of 4T1. It is important to consider that anchorage independent growth is cell growth without adhesion and this is a marker for stem like abilities. Our results show that there is a protein that could be elevated in M0 following BP3 exposure. Further exploration is necessary in order to determine the mechanism involved in the changes we observed.

We also observed significant decreases in the activation induced T cell cytokines secretion in the presence of conditioned media from BP3 treated M0. In the future it is necessary to extend this research in order to determine whether the changes seen were due to a BP3 dependent effect or due to the secreted proteins from macrophages.

Potential future direction to continue this project includes, using the p53/- mouse model to stain more tissue samples and analyze RNA from other tumors that were not included in this thesis. The original experiment had a larger group of mice in the vehicle and BP3 treatment groups and adding more samples would increase the validity of the
results and may help to identify if there is a correlation in tumor type and protein expression of the immune and angiogenic marker.
Appendix

Methods

Chemicals

DMSO was used as the control.

30uM BP3 was used as the standard exposure.

Conditioned Media

Once cells were cultured for 48 hours or 72 hours, the supernatant from the 6-well plates was centrifuged at 1000g, collected, and aliquoted into microcentrifuge tubes and stored in the 4 degree fridge.

Cell Culture

- **4T1 Direct Treatment**

Using 10cm plates, 4T1s were cultured in … media and once they reached 75% confluency they were plated into 6-well plates where n=3 for DMSO and 30uM BP3 exposure. DMSO and BP3 was added directly to the media at 1:1000.

- **4T1 Indirect Treatment**

Using 10cm plates, 4T1s were cultured in … media and once they reached 75% confluency they were plated into 6-well plates where n=3 for DMSO conditioned media and n=3 for BP3 conditioned media

- **Raw 264.7 (M0)**

Using 10cm plates, Raw 264.7 were cultured in RPMI media and once they reached 75% confluency they were plated into 6-well plates where n=3 for DMSO and 30uM BP3 exposure. DMSO and BP3 was added directly to the media at 1:1000.

- **M1**
Using 10cm plates, Raw 264.7 were cultured in RPMI media and once they reached 75% confluency they were plated into 6-well plates where n=3 for DMSO and 30uM BP3 exposure. DMSO and BP3 was added directly to the media at 1:1000. Additionally Lipopolysaccharide (LPS) was added to each well at a concentration of 10nM.

- **Co-culture of 4T1s and Raw 264.7 (TAMs)**

Cells were treated in triplicate with DMSO control or 30uM BP3 for 48 hours using 6-well plates.

- **M1 recovery**

Raw 264.7 cells were stimulated with LPS and treated with DMSO or 30uM BP3 for 48 hours and then replacing the media with fresh untreated RPMI media for varying time periods.

- **Primary T cells**

After being euthanized with CO2, spleens from Balb/C mice were dissociated and using the … kit T cells were cultured and treated with DMSO or 30uM BP3.

**Cell viability/proliferation (MTS Assay)**

*After cell culture in their respective treatment paradigm, 10uL of cell titer 96 Aqueous one solution was added to each well. The cells incubated at 37 degrees Celsius for 4 hours and absorbance was measured at 490nm using a 96-well plate reader. The values were then recorded and analyzed using the statistical analysis method described below.*

**Cell migration**

We examined the direct exposure on migration using a scratch wound assay.

Using 10cm plates, 4T1s were cultured in … media and once they reached 75% confluency they were plated into 6-well plates in triplicate and treated with DMSO or
30uM BP3. Using a sterile pipette tip, a vertical scratch was made in the middle of each well. The length of the scratch wound was measured at 0hr, 8hr, and 24hr to determine the rate of migration and pictures were taken using Nikon Eclipse TE2000-Uusing Metaview™ software.

**Anchorage Independent Growth**

We plated 4T1 cells at a low density on low attachment cell culture plates and looked for growth of primary and secondary colonies. 4T1s were plated as single cells and cultured for 1 week. After 1 week the cells, now in colonies, were resuspended and re-plated in the same treatment groups for another week. After week 2 colonies were measured and any colony above 40um was quantified in each treatment group. Images of mammosphere formation were captured with a Nikon Eclipse TE2000-Uusing Metaview™ software.

**Gene expression and analysis**

Total RNA from all cell lines was extracted in triplicate for each treatment using an acid–phenol extraction procedure, according to the manufacturer’s instructions (TRIzol, Invitrogen, Carlsbad, CA, USA). Relative expression levels of mRNA were determined by quantitative real-time PCR using the Mx3005P real-time PCR system (Agilent, Santa Clara, CA, USA) and all values were normalized to the amplification of an appropriate normalizer gene. The assays were performed using the 1-Step Brilliant SYBR Green III QRT-PCR Master Mix Kit (Agilent) containing 200 nM forward primer, 200 nM reverse primer and 10 ng total RNA. The conditions for complementary DNA synthesis and target mRNA amplification were as follows: 1 cycle of 50°C for 30 min; 1 cycle of 95°C for 10 min and 35 cycles each of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s.
Nontemplate controls were included to control for primer dimers and no reverse
transcriptase controls were included to control for genomic DNA amplification.
Quantitative analysis was carried out as follows: Ct values were assigned for each sample
using an automatic threshold level determined by the Mx3000P software. Delta Ct (dCt)
values were determined according to the following formula: dCt(target gene) = Ct(target
gene) – Ct(housekeeping gene). Changes in gene expression are shown as RQ values and
calculated using the following formula: RQ=2^{−ddCt}, where ddCt values were calculated as
ddCt = dCt(sample) – dCt(reference sample) (Gregory et al. 2020).

Protein Expression

- Immunohistochemistry

Immunohistochemistry (IHC) was performed on a DakoCytomation autostainer using the
Envision HRP Detection system (Dako, Carpinteria, CA). Each mammary tissue block
was sectioned at 4 μm on a graded slide, deparaffinized in xylene, rehydrated in graded
ethanols, and rinsed in Tris-phosphate-buffered saline (TBS). Heat induced antigen
retrieval was performed in a microwave at 98°C in 0.01 M citrate buffer. After cooling
for 20 minutes, sections were rinsed in TBS and subjected to the primary rabbit
polyclonal antibody for 45 minutes. Immunoreactivity was visualized by incubation with
chromogen diaminobenzidine (DAB) for 5 minutes. Tissue sections were counterstained
with hematoxylin, dehydrated through graded ethanols and xylene, and cover-slipped.
Images were captured with an Olympus BX41 light microscope using SPOTSOFTWARE
(Gauger, 2012).

- ELISA
Following cell culture and collection of conditioned media (detailed above), V-Plex pro inflammatory panel 1 mouse from Meso scale discovery and it was run according to manufacturer’s instructions. The analysis was run on discovery workbench.

Statistical Analysis

Group means were compared using Student’s *t*-tests and overall patient responses were analyzed using a Wilcoxon signed-rank test using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). A *P* value of <0.05 was considered significant (Gregory et al. 2020).
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