Influences of Antroquinonol and 4-Acetylantroquinonol B on Inflammatory Tumorigenesis in the MCF-7 Breast Cancer Cell Line with or without TNF-α Stimulation

Ting-Chun Lin
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INFLUENCES OF ANTROQUINONOL AND 4-ACETYLANTRROQUINONOL B ON INFLAMMATORY TUMORIGENESIS IN THE MCF-7 BREAST CANCER CELL LINE WITH OR WITHOUT TNF-α STIMULATION

A Thesis Presented

By

TING-CHUN LIN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

September 2018

Department of Nutrition
INFLUENCES OF ANTROQUINONOL AND 4-ACETYLANTROQUINONOL B ON INFLAMMATORY TUMORIGENESIS IN THE MCF-7 BREAST CANCER CELL LINE WITH OR WITHOUT TNF-α STIMULATION

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ACKNOWLEDGMENTS

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ABSTRACT

INFLUENCES OF ANTROQUINONOL AND 4-ACETYLANTROQUINONOL B ON INFLAMMATORY TUMORIGENESIS IN THE MCF-7 BREAST CANCER CELL LINE WITH OR WITHOUT TNF-α STIMULATION

SEPTEMBER 2018

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Directed by: Dr. Zhenhua Liu

Breast cancer (BC) is one of the most common cancers among women worldwide that ~25% of new cancer cases diagnosed every year would be BC; moreover, ~15% of cancer deaths per year caused by BC makes it the leading cause of cancer death among women worldwide. To date, though the cause of a large proportion of BC are still unclear, recent studies have revealed that a supportive breast tissue microenvironment is critical for the development and progression of BC, especially the communication with immune cells within breast tissue. Therefore, breast inflammatory microenvironment is currently received a substantial attention in the prevention and treatment of BC. Research on breast cancer immunology suggests that inflammatory mediators, estrogen and several
inflammation-related tumorigenic pathways are potentially contributors for inflammatory breast tumorigenesis. It is evidenced that elevated levels of inflammatory mediators, such as cytokines, chemokines, prostaglandins, and enhanced estrogen production while suffering from chronic inflammation is responsible for not only activating oncogenic pathways, for example NF-κB, STAT3 and Wnt signaling pathways, but also reducing the efficacy of cancer-specific immunity against tumor cells. Accordingly, targeting the chronic inflammatory status in breast tissue has become a promising strategy for breast cancer therapy. Recently, due to the annoying side effects accompanying by traditionally anticancer drugs, there is an increased interest in finding out natural sources to treat BC. Herein, we report that antroquinonol (AQ) and/or 4-acetylantroquinonol B (4-AAQB) isolated from Antrodia Camphorata were able to modulate the expression of several inflammatory mediators, IL-6 and IFN-γ in particular, and downregulate the aromatase expression and Wnt signaling responses induced by inflammatory status. Taken together, the present findings provide new insights into the role of AQ and 4-AAQB in inflammatory breast tumors and also suggest them as promising candidates for breast cancer immunotherapy.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2.1 Breast Cancer</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2.2 Inflammatory Microenvironment and Breast Cancer</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2.2.1 Inflammatory Cytokines</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2.2.2 Chemokines</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2.2.3 Cyclooxygenase-2 and Prostaglandins</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2.2.4 Immune Checkpoints</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2.3 Chronic Inflammation and the Estrogen Mechanisms</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>2.3.1 Pro-tumor Immunomodulatory Effects of Estrogen</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2.3.2 Aromatase as a Mediator Between Inflammation and Breast Tumorigenesis</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>2.4 Inflammation and Breast Cancer: The Wnt-Signaling Pathway Connection</td>
<td>23</td>
</tr>
</tbody>
</table>

ACKNOWLEDGMENTS ........................................................................................................ iv

ABSTRACT .......................................................................................................................... v

LIST OF TABLES ................................................................................................................ x

LIST OF FIGURES ............................................................................................................... xi
6. DISCUSSION .................................................................................................................................66

APPENDICES

A. TABLE OF PERFORMED ASSAYS AND EXPERIMENTAL DESIGN .......... 80
B. TABLE OF PRIMERS USED FOR REAL-TIME PCR ANALYSIS ................. 82

REFERENCES ........................................................................................................................................84
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Experimental design</td>
<td>81</td>
</tr>
<tr>
<td>2. Primers of targeted genes and GAPDH</td>
<td>83</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Breast tumor microenvironment</td>
<td>7</td>
</tr>
<tr>
<td>2. Chemokines &amp; metastatic breast cancer</td>
<td>14</td>
</tr>
<tr>
<td>3. CD47 signaling &amp; reduced T cell immunity</td>
<td>18</td>
</tr>
<tr>
<td>4. Inflammatory mediators &amp; aromatase expression</td>
<td>23</td>
</tr>
<tr>
<td>5. Nutritional value of mushrooms</td>
<td>33</td>
</tr>
<tr>
<td>6. AQ, 4-AAQB and TNF-α reduce cell viability of MCF-7 cells</td>
<td>53</td>
</tr>
<tr>
<td>7. Heatmap and relative gene expression level of targeted inflammatory mediators in MCF-7 cells with or without 24 h AQ treatment</td>
<td>56</td>
</tr>
<tr>
<td>8. Heatmap and relative gene expression level of targeted inflammatory mediators in MCF-7 cells with or without 24 h 4-AAQB treatment</td>
<td>57</td>
</tr>
<tr>
<td>9. Gene expression levels of targeted inflammatory mediators in MCF-7 cells after TNF-α stimulation, and gene expression levels of IL6, IFNγ and CCL2 after TNF-α and AQ/4-AAQB cotreatment</td>
<td>60</td>
</tr>
<tr>
<td>10. Gene expression levels of aromatase in MCF-7 cells</td>
<td>62</td>
</tr>
<tr>
<td>11. Gene expression levels of CD47 and Wnt targeted genes in MCF-7 cells</td>
<td>65</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Recent studies have indicated that within a tumor microenvironment (TME), tolerant immune responses involved in innate and adaptive immune systems play key roles in cancer progression (Wang et al., 2004). Prolonged and enhanced inflammatory activity caused by different conditions, such as microbial infections, chemicals exposures, autoimmune diseases and obesity, would lead to chronic, “smoldering” and low-grade inflammation (Balkwill et al., 2005; Mantovani et al., 2008; Divella et al., 2016). It is now evidenced that several immunosuppressive cells that are responsible for tolerant immunity including regulatory T (T_{reg}) cells and myeloid-derived suppressor cells (MDSCs) are often found in breast tumors (Jiang et al., 2014). These cells along with soluble molecules could induce an immunosuppressive microenvironment predisposing breast tumor development.

Recently, due to the various side effects of anti-cancer drugs, research on complementary and alternative medicine (CAM), which is used to help patients lessen side effects and discomfort following conventional medicine, has risen in scientific community. According to 2012 national health interview survey, 33.2% of US adults used CAMs, and the most common CAM for cancer treatment in United States was the use of natural products (17.7%
of adults), some of which possess a variety of phytonutrients with potential benefits to human health (NHIS, 2012). Among these natural products, mushrooms have been considered as one of the most powerful functional foods for preventing and treating cancer via their effective anti-cancer and immunomodulatory effects (Valverde et al., 2015).

*Antrodia Camphorata* (AC) as one of the medicinal mushrooms used in traditional Taiwanese medicine has been reported to possess diverse health-promoting effects in modern medicine, such as anti-microbial, anti-hepatitis B virus, anti-oxidant and especially anti-cancer and immunomodulatory effects, attributed to the various nutraceuticals found in AC (Geethangili et al., 2011; Lien et al., 2014). Two recently isolated compounds, antroquinonol (AQ) and 4-acetylantr-oquinonol B (4AAQB), from AC mushrooms emerge as promising anti-cancer drug candidates. Experiments conducted in various cancers demonstrated the roles of AQ and 4-AAQB in diverse anti-cancer properties such as anti-proliferation (Kumar et al., 2011; Lin et al., 2011), pro-apoptosis (Yu et al., 2012), anti-invasion and metastasis (Thiyagarajan et al., 2015; Lee et al., 2015) and anti-cancer stemness (Chang et al., 2015; Lin et al., 2017). However, the effects of these compounds on anti-tumor immunity in breast cancer (BC) remain unclear. Our present study aims to understand how AQ and 4-AAQB modulates mammary inflammatory microenvironment,
aromatase, an enzyme responsible for a key step in the biosynthesis of estrogens in women, as well as tumorigenic Wnt pathway related genes in MCF-7 BC cells.
CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

BC is the most common cancer in women worldwide. Nearly 1.7 million new BC cases were diagnosed in 2012, representing about 25% of all types of cancers in women (Ferlay et al., 2015). BC, with a standardized mortality rate of 12.9 per 100,000, is the second highest cause of cancer death (Ghoncheh et al., 2016). In the United States, about 12% of women, that is 1 in 8 women, will be diagnosed with invasive BC during their lifetime. Despite the incidence and death rates of BC in the US are both gradually decreasing for the past two decades, BC death rates are still higher than any other cancer, besides lung cancer (DeSantis et al., 2014).

In general, based on molecular subtypes, BC can be divided into mainly three categories: hormone receptor-positive BC, HER2 protein-positive BC and triple-negative BC (TNBC). Hormone receptor-positive BC is the major type among these three, and includes estrogen receptors (ER)-positive and/or progesterone receptors (PR)-positive BC, accounting for ~70% of human BC (Spears et al., 2009). TNBC, which is ER-negative, PR-negative and HER2-negative, is the most complex one, since it is not the hormones that facilitate cancer
cell growth, and currently, there is no targeted therapy specifically for its treatment (Reddy et al., 2011). Since BC in women is prevalent worldwide, particularly in the industrialized countries, and current treatments, including surgery combined with chemotherapy, radiation and hormone therapy, have significant side effects, it is highly attractive to develop dietary strategies, e.g. identifying natural medicinal compounds that have preventive or chemopreventive capability for reducing BC risk with less adverse effects.

The cause of BC is complex and may attributes to several risk factors related to age, family history of breast and other cancers, endogenous and exogenous hormone exposure, and environmental and lifestyle changes. About 5 to 10% of BCs are associated with hereditary gene mutations, and among them more than 50% of the inherited cases can be directly linked to BRCA1 and/or BRCA2 gene mutations (Ford et al., 1998; Slavin et al., 2017). Other factors, such as sex hormone, exposures to environmental chemicals and pollutants, obesity, dietary factors, alcohol and tobacco are responsible for the majority of BC (Chen, 2008; Hiatt et al., 2018). By understanding the causation between these risk factors and BC, researchers and health promotion practitioners can develop effective strategies or policy against BC in both prevention and therapy levels.

Overview the history of BC therapy strategies, researchers tend to focus their studies on
tumor cell-intrinsic factors such as genetic, epigenetic properties and biology of tumor cell per se. However, recent development of cancer intervention has shifted the topics toward the whole TME (Williams et al., 2016). In fact, in 1889 Stephen Paget had already proposed a “Seed and soil” theory for cancer development suggesting that tumors can only be formed by neoplastic cells with a favorable and supportive environment (Paget, 1989). In general, breast TME is composed of tumor cells, stromal cells, adipocytes, immune cells, as well as various extracellular molecules, such as cytokines, chemokines, and growth factors, secreted by tumor cells and surrounding cells (Place et al., 2011; Huang et al., 2017) (Figure 1). Within a breast TME, immune cells, especially macrophages, play critical roles in the development and progression of BC (Ward et al., 2015). Therefore, breast inflammatory microenvironment is currently received substantial attention in the prevention and treatment of BC.
2.2 Inflammatory Microenvironment and Breast Cancer

The first connection between inflammation and cancers can be traced back to 19th century, Rudolf Virchow noted that infiltrated leukocytes present within tumor tissue, and hypothesized that “lymphoreticular infiltrate reflected the origin of cancer at sites of chronic inflammation” (Balkwill et al., 2001). Indeed, though this idea has been undervalued more than a century, the shift of recent cancer research from tumor cells to the TME uncovers myriad solid evidence that describe the interaction between immune cells and tumor cells in the microenvironment.
Evidence has clearly indicated that chronic inflammation with elevated serum levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) caused by obesity or other inflammatory conditions are associated with increased BC risk, and may play an important role in tumorigenesis (Agnoli et al., 2017). Additional evidence is the use of anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs), e.g. COX2 inhibitors, aspirin, ibuprofen and other NSAIDs, steroidal anti-inflammatory drugs, e.g. corticosteroids, and anti-cytokine and chemokine drugs, e.g. anti-IL-6, anti-TNF-α, and anti-CXCR4 drugs in cancer therapy, and these drugs have been shown to be beneficial for reducing cancer incidence, improving therapy, and decreasing mortality from cancers, including BC (Harris et al., 2003; Rayburn et al., 2009; Grivennikov et al., 2010). These findings supported Virchow’s hypothesis, reemphasized the importance of inflammation toward cancer progression, and significantly encouraged the research involved in cancer immunology and its implications for cancer prevention and therapy.

The key factors contributing to the adverse effects of inflammation on breast cancer are transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3), and related
inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Mantovani et al., 2008). In the inflammatory microenvironment, neoplastic cells, adipocytes, stromal cells and immune cells are all under an inflammatory status through a positive feedback loop between transcription factors and their targeted inflammatory mediators. Therefore, the influence of the various inflammatory molecules that secreted from all types of cells in the microenvironment play critical roles in breast tumorigenesis and tumor progression.

2.2.1 Inflammatory Cytokines

**TNF-α:** In an inflammatory microenvironment, the inflammatory cytokines, particularly TNF-α, IL-1β and IL-6 are most extensively studied. TNF-α was originally identified to be a cytokine inducing cell lysis, especially tumor cells (Locksley et al., 2001). Later research discovered a wide range of effects of chronic, low dose TNF-α on tumor cells in the aspects of cell proliferation, survival, differentiation, anti-apoptosis, angiogenesis, epithelial–mesenchymal transition (EMT) as well as inflammatory responses (Dempsey et al., 2003; Balkwill et al., 2009), which are mainly through the activation of NF-κB inflammatory pathway (Wang et al., 2008; Hoesel et al., 2013). Eftekhari et al. (2017) indicated that in a breast TME, TNF-α expression level is gradually increasing and parallel with the breast tumor stage. To date, several studies have demonstrated the role of TNF-α in inflammation-
related cancers. Moore et al. (1999) showed that TNF-α knockout mice is resistant to skin carcinogenesis caused by inflammation. In addition, Oshima et al. (2005) pointed out that the inhibition of TNF-α related inflammation is capable of suppressing gastric tumor progression in transgenic mice induced by cyclooxygenase-2 (COX2) and microsomal prostaglandin E synthase-1 overexpression. The pro-tumor effects of TNF-α and TNF-α induced other cytokines, chemokines and prostaglandins (PGs) may attribute to their acts on different cell types in the whole TME that eventually skew it to a tumor friendly environment (Balkwill, 2009).

**IL-1β:** Similar with TNF-α, IL-1β is capable to activate NF-κB and AP-1 by binding with IL-1 receptors and/or toll-like receptors, promoting tumor cell survival, proliferation, metastasis, tumor angiogenesis (Divella et al., 2016). IL-1β in a TME is shown to be responsible for the recruitment of COX-2-expressing leukocytes and the promotion of inflammatory angiogenesis caused by upregulating angiogenic factors such as prostaglandin E2 (PGE₂), vascular endothelial growth factor, CXC chemokines, and matrix metalloproteinases (MMPs) (Nakao et al., 2005). Escobar et al. (2015) also indicated that IL-1β highly expressed by metastatic ER negative BC cell, MDA-MB-231, is able to stimulate the secretion of chemokines related to aggressive metastasis by mesenchymal
stem cells in a co-culture cell model.

**IL-6:** IL-6 is a pro-inflammatory cytokine that is regulated by NF-κB pathway and is also a key modulator in cancer-related inflammation (Divella et al., 2016). IL-6 is the major activator of STAT3 transcription factor, which is responsible for the expression of genes related to pro-proliferation, anti-apoptosis, tumor metastasis, angiogenesis, and immunosuppression (Yu et al., 2009). Moreover, the activation of STAT3 in various immune cells is able to promote the maturation of immunosuppressive cells such as tumor-associated macrophages (TAMs), MDSCs, T<sub>reg</sub> cells and T helper 17 (T<sub>H</sub>17) cells, whereas reduce the stimulation of tumor cytotoxic cells such as T<sub>H</sub>1 cells and cluster of differentiation (CD) 8<sup>+</sup> T cells (Yu et al., 2009). Recent studies of IL-6 in BC have point out its pro-tumor effects and association with poor patient outcomes (Salgado et al., 2003; Won et al., 2013).

**Other inflammatory cytokines:** Other proinflammatory cytokines, such as interferon-γ (IFN-γ), IL-10 and transforming growth factor-β (TGF-β), is also widely studied in cancer immunology field. 1) IFN-γ is regulated by transcription factor NF-κB, and would stimulate JAK/STAT1 signaling pathway. The role of IFN-γ in cancer-related inflammation is a two-faced factor in terms of pro- and anti-tumor effects. In the tumor initiation stage,
IFN-γ can protect normal cells from damage, whereas during this process, it may lead to the accumulation of oncogenic mutations, resulting in potent cancer cell transformation (Zaidi et al., 2011). 2) IL-10 in immunology is generally known as an anti-inflammatory cytokine, but its effects in the pathogenesis of cancer is still controversial. Evidence has suggested that IL-10 signaling alone or co-stimulation with other cytokines such as IL-2 or IL-4 can activate either STAT3 or STAT1 according to in vitro or in vivo studies (Mannino et al., 2015). Since STAT3 and STAT1 activation is responsible for immunosuppression and immunostimulation respectively, IL-10 may serve as pro-tumor or anti-tumor factor depending on the existence microenvironment. 3) TGF-β also has two-faced property toward tumor development. In normal cells, TGF-β serving as a tumor suppressor protein can protect cells from tumor cell transformation through cell cycle inhibition, induction of apoptosis and inhibition of cell immortalization (Lebrun, 2012). However, elevated TGF-β secreted from tumor cells, stromal cells and immune cells in a TME can promote tumor cell progression and metastasis. In addition, TGF-β in a TME can positively contribute to tumor immunosuppression by directly or indirectly weaken function of cytotoxic T and B lymphocytes against tumor cells, allowing tumors to evade from immunosurveillance (Yu et al., 2009; Lebrun, 2012).
2.2.2 Chemokines

Chemokines are another group of molecules that are elevated in an inflammatory environment, particularly obesity-associated inflammation. Chemokines and chemokine receptors also play important roles in terms of their signaling greatly regulating tumor cell migration and invasion and immune cell infiltration (Figure 2). Given the phenomenon of immune cell infiltration in a TME, various inflammatory mediators upregulate the expression of chemokines and chemokine receptors in immune cells as well as tumor cells. Müller et al. (2001) found that in breast tumor cells, C-C motif chemokine receptor 7 (CCR7) and C-X-C motif chemokine receptor 4 (CXCR4) are highly expressed compared to normal mammary epithelial cells in vitro and in vivo. Similar with the mechanisms involved in lymphocyte trafficking, chemokine signaling also triggers actin polymerization in breast tumor cells in order to form pseudopod and move the cells forward, resulting in tumor cell migration and invasion to distal metastatic niches with high levels of chemokine ligands C-C motif chemokine ligand 21 (CCL21) and C-X-C motif chemokine ligand 12 (CXCL12). On the other hand, chemokines acting as chemoattractants can attract immune cell infiltrating to tumor site, such as CCL2 (monocyte chemoattractant protein 1, MCP-1)-mediated macrophage recruitment in BC, and through the cytokines and chemokines
interaction between macrophages and tumor cells, it can further promote the maturation of tumor-associate macrophages (TAMs) and metastatic tumor cells favorable for tumor immune evasion, tumor angiogenesis and metastasis (Stewart et al., 2012; Tariq et al., 2017).

Figure 2. Elevated expression of chemokines & chemokine receptors promote metastatic breast cancer

2.2.3 Cyclooxygenase-2 and Prostaglandins

The links between cyclooxygenase (COX; also known as prostaglandin-endoperoxide synthase, PTGS) and inflammation can be traced back to the first discovery of NSAIDs in
the early 1970. They were shown to be able to mitigate inflammation by inhibiting PGs synthesis mediated by enzyme COX. Moreover, the later research on COX revealed that its increased activity is correlated to the inflammatory status, and could be induced by inflammatory cytokines and endotoxins (Seibert et al., 1994). There are two COX isozymes found in human body, including COX-1 and COX-2. COX-1 is normally existed and constitutively activated in various tissue to maintain physiological functions, for example gastrointestinal, renal, endothelium protection and platelet aggregation, whereas, COX-2 is almost silent in normal tissue and is inducible only in the context of inflammatory stage, including cancer-related inflammation. Recently, studies have pointed out that COX-2 is upregulated or overexpressed in various types of cancers, such as oral, gastric, bile duct, colorectal, head and neck, skin, prostate, breast, liver, pancreatic, bladder, lung, ovarian, and cervical cancers (Koki et al., 2002).

The main function of COX-2 pathways is to produce prostanoids, including thromboxane and PGs, from enzymatically conversion of arachidonic acids (Smyth et al., 2009). PGs involve in various physiological functions in human body such as vasodilation, bronchodilation, muscle contraction, platelet aggregation; moreover, they can greatly contribute to advanced inflammation and tumor progression, especially PGE₂. Several
molecular pathway involved in inflammation and tumor development directly target COX-2 promoter, including NF-κB, STAT3 and MAPK pathways (Xiong et al., 2014; Desai et al., 2018), and by upregulated COX-2 expression and increased production of PGE₂, a positive feedback loop can be generated between these pathways and COX-2 pathway, further exacerbating the inflammation and tumor progression (Lin et al., 2015; Aoki, 2015). In general, constitutive COX-2 activation and PGE₂ production can potentially promote both the initiation of breast tumorigenesis and later progression through a variety of pathways (Harris et al., 2014; Galván et al., 2017).

2.2.4 Immune Checkpoints

Recently, research on the interaction between cancer cells and immune cells in terms of effects on escaping from immunosurveillance is quite promising for cancer immunotherapy. During inflammation, immune checkpoints, serving as regulators of various immune cell activation, are key factors for maintaining self-tolerance, which represents the ability for immune system to correctly identify and target foreign antigens instead of autoantigens, avoiding autoimmune responses and tissue damage. Generally, anti-tumor immunity can be referred to a cancer-immunity cycle with several steps, including a) releasing of cancer cell antigens, being captured by antigen-presenting cells (APCs), and presenting cancer
antigen to T cells by APCs; b) activating effector T cells (T_{eff}) against cancer cells, T_{eff}
trafficking to tumor site, and infiltrating into tumor; c) recognizing cancer cells by binding
cancer antigen with T cell receptor, killing cancer cells, and releasing additional cancer
antigens (Chen et al., 2013). In the process of cancer-immunity cycle, the activation of T_{eff}
would be the key step to initiate effective tumoricidal function of anti-tumor immunity. T
cell activation is not merely regulated by interaction between cancer-associated antigens
and T cell receptor, but also may co-regulated with other ligands and receptor proteins
presented on the surface of APCs and tumor cells. These proteins can be divided into two
categories, positive and negative regulators, responsible for co-stimulating or co-inhibiting
T_{eff} function respectively (Topalian et al., 2015), and between these two membrane proteins,
negative regulators are referred to immune checkpoints.

However, this host defense mechanism is dysfunction within a TME, resulting in evasion
of tumor immunosurveillance (Pardoll, 2012). For instance, recent findings suggest that
CD47 as a pro-phagocytic signal is commonly upregulated in various tumor cells including
breast tumor (Manna et al., 2004; Chao et al., 2011), possibly through dysregulated NF-κB
and HIF-1 pathways (Lo et al., 2015; Zhang et al., 2015; Betancur et al., 2017), and
increased expression of CD47 could specifically engage with its receptor signal regulatory
protein α (SIRPα), generating “don’t eat me“ signals to macrophages (McCracken et al., 2015) (Figure 3). By blocking CD47-SIRPα signal with CD47 antibody or CD47 knockdown, macrophage-mediated phagocytosis of tumor cells is retrieved (Bener et al., 2016; Liu et al., 2017).

**Figure 3.** Activated CD47 signaling toward macrophage disrupts macrophage phagocytosis of breast cancer and the following T cell immunity activation

2.3 Chronic Inflammation and the Estrogen Mechanisms

Hormone receptor-positive cancer is the major type of BC, with approximately 70% of
human BCs depending on hormones and the expression of their receptors (Spears et al., 2009). Reproductive issues such as ages at menstruating, first birth and menopause as well as the use of hormone replacement therapy for menopause symptoms and oral contraceptives are all established factors for BC via hormonal mechanisms (Chen, 2008). Although it is still in its infancy, researches have demonstrated the interplay between inflammation and hormones. The sex hormones appear to have important, but complex effects on the body’s inflammatory response. For example, many observers have wondered if the increase in inflammatory diseases that coincide with menopause, such as arthritis, might be related to shifts in the balance of progesterone and estrogens, and thereby hormones may contribute to breast tumorigenesis via the inflammatory response. However, estrogens, by themselves, directly promotes tumorigenesis via through immunomodulatory effects and chronic inflammation, in fact may also contribute to the development of BC via hormonal pathways (Amadou et al., 2013; Quigley et al., 2017).

2.3.1 Pro-tumor Immunomodulatory Effects of Estrogen

It is the fact that the susceptible of infection and related mortality for women are generally lower, whereas the susceptible of autoimmune disease is higher, than men, and one of the underlying factor contributing to this difference is suggested to be female sex steroid
hormone, which is capable of interacting with immune system (Grossman, 1985). The immunomodulatory effects of estrogen are two-faced, including immunosuppression and immunostimulation, and its effects on immune function seem to be extensively dependent on estrogen concentration, composition of estrogens and targeted cell types (Straub, 2007). In a TME, various cell types, such as tumor cells, stromal cells as well as immune cells, secret aromatase and estrogen, and via autocrine and/or paracrine estrogen pathways, these hormones may promote tumor progression by weakening anti-tumor immune responses (Rothenberger et al., 2018). Estrogen is suggested to have the ability to induce tumoricidal M1 macrophage polarization toward immunosuppressive M2 in a TME (Gilliver, 2010; Svensson et al., 2015). Moreover, evidence indicates that estrogen can increase the accumulation of MDSCs in tumors and enhance their immunosuppressive activities by estrogen receptor-mediated upregulation of STAT3 signaling (Svoronos et al., 2017); influence CD4+ helper T cell differentiation, APCs functions and chemokines production, by which would lead to increased Th2 cytokines production such as IL-4 and IL-10, decreased cell-mediated Th1 immune response and impaired T cell activation (Salem, 2004); stimulate Treg activation (Tai et al., 2008); increase T cell anergy (Polanczyk et al., 2007; Yang et al., 2017); inhibit cytotoxic T lymphocyte- and NK cell-mediated apoptosis against tumor cells (Jiang et al., 2006). Above-mentioned immunosuppressive effects of
estrogen on both innate and adaptive immune system could converge to pro-tumor immunity, creating a tumor friendly microenvironment and promoting cancer progression.

2.3.2 Aromatase as a Mediator Between Inflammation and Breast Tumorigenesis

Since epidemiological studies have indicated that obesity or adiposity and its underlying chronic inflammatory status are associated with BC risk in post-menopausal women (Rose et al., 2010), the interaction and related molecular mechanisms among obesity, inflammation and BC have been intensively investigated, and the increased pro-inflammatory mediators within mammary adipose tissue are proposed as critical cellular mechanisms (Rose et al., 2015). In an obese state, large number of macrophages infiltrate into adipose tissue, typically being around individual adipocytes to form a “crown-like structure” (CLS), which is a representative feature of adipose tissue inflammation and simultaneously serve as a major contribution to proinflammatory cytokines such as TNF-α, IL-6 and chemokine MCP-1. The mediators secreted from CLS can further recruit additional monocytes and lymphocytes, in turn deteriorating the inflammatory process (Rose et al., 2015; Cowen et al., 2015).

In addition to direct effects of inflammatory cytokines in the modulation of tumorigenic pathways as described in previous sections of this review, an
The obesity→inflammation→aromatase axis is present in the breast tissue of most overweight and obese women (Morris et al., 2011; Rose et al., 2015; Cowen et al., 2015). In postmenopausal women, estrogen production is mainly from enzymatic conversion of androgen (androstenedione) into estrogen (estrone) in the adipose tissue instead of the ovaries, which are the main sources of estrogen production in premenopausal women, and the responsible key enzyme is called aromatase (cytochrome P450 19A1, CYP19A1). In an obese state, the increased numbers of CLS, enhanced activation of the NF-κB transcription factor (Hursting, 2011; Zahid et al., 2016), and increased secretion of inflammatory mediators, such as IL-6 (Reed et al., 1992), TNF-α (Zhao et al., 1996), IL-11, leukemia inhibitory factor, oncostatin M, insulin-like growth factor 1 (IGF-1) (Zhao et al., 1995) and PGE2 (Richards et al., 2003), are directly associated with elevated aromatase levels and activity in the mammary glands (Figure 4). The inflammation-aromatase axis indicates a hormonal mechanism between inflammation and breast tumorigenesis.
Figure 4. Elevated levels of inflammatory mediators during chronic inflammation stimulate aromatase expression and activity.

2.4 Inflammation and Breast Cancer: The Wnt-Signaling Pathway Connection

While chronic inflammation plays a critical role in breast tumorigenesis, inflammatory mediators-induced oncogenes expression also greatly involves in this causality, for instance, the secretion of Wnt proteins and triggered signalings are one of the major oncogenic pathways.

2.4.1 Wnt-Signaling Pathway and Breast Cancer

The discovery of Wnt protein is first proposed with the term “Int-I” (integration 1) by Roel
Nusse and his colleagues (1984) in the light of a research on mouse mammary tumour virus (MMTV) infected mice and the oncogene correlated to following breast tumorigenesis. However, the identification of this gene was actually characterized as *swaying* and *wingless* (*Wg*) previously in both mouse and drosophila models respectively with the functions involved in embryonic development such as cell proliferation, differentiation, and polarity (Nusse, 2005; Klaus et al., 2008). Due to the multiple names of this gene and its related genes, researchers renamed these gene family as *Wnt* family standing for the blend of *Wg* and *Int* (Klaus et al., 2008). Similar with other molecular mechanisms involved in embryonic development, mutations appeared in these pathways or their inhibitors would lead to dysregulation of cell growth and motility control, resulting in tumorigenesis. Recent studies have indicated that *Wnt* family in human comprises 19 proteins, and as ligands there are three signaling pathways, one canonical and two non-canonical pathways, could be activated by binding Wnt proteins to corresponding receptors and co-receptors, frizzled receptor family and low-density lipoprotein-related protein 5/6, respectively (Komiya et al., 2008).

The canonical pathway, also known as Wnt/β-catenin pathway, is thought to be the major signaling contributing to oncogenesis. Due to the receptors binding of Wnt protein,
increased β-catenin accumulates in the cytoplasm, eventually translocating into nucleus and acting as a coactivator of T-cell factor/lymphoid enhancer factor transcription factors for upregulating Wnt targeted genes expression (Nusse, 2005), such as c-myc, axin-2, and cyclin D1, some of whom are responsible for regulating critical cell functions, cell survival, stem cell renewal and organogenesis for example (Komiya et al., 2008). The other two signalings are β-catenin independent pathways, including planar cell polarity pathway and the Wnt/Ca\textsuperscript{2+} pathway, of which’s role is primarily in regulating cell motility and polarity and Ca\textsuperscript{2+}-dependent cellular signaling via the activation of protein kinase C (PKC) and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) (Seifert et al., 2007; De, 2011).

Furthermore, recent studies show that Wnt5a might serve as a tumor suppressor gene via the stimulation of Wnt/Ca\textsuperscript{2+} pathway to block Wnt/β-catenin pathway through CaMKII-mediated and calcium-sensing receptor-mediated β-catenin phosphorylation and increasing β-catenin degradation in thyroid and colon carcinoma, respectively (Kremenevskaja et al., 2005; MacLeod et al., 2007).

Wnt/β-catenin pathway activation is commonly found in various cancer types, and most of the dysregulations of this pathway can be attributed to genetic mutations on signaling-related genes, posttranslational modification of key regulators as well as the
downregulation of Wnt antagonists such as secreted frizzled-related protein and dickkopf protein. The first connection between BC and Wnt/β-catenin pathway was built based on the discovery of Int-1, later known as Wnt1 (Nusse et al., 1984), and evidence showed that the activation of int-1 gene transcription is associated with mammary gland hyperplasia and following breast tumorigenesis (Tsukamoto et al., 1988). Subsequently research also suggested that similar with the effects of Wnt1 on breast tumorigenesis, Wnt3 and Wnt10b are able to induce breast tumor development (Roelink et al., 1990; Lane et al., 1997). In addition, stabilized β-catenin has been demonstrated as the major characteristic contributing to tumorigenic Wnt signaling (Incassati et al., 2010); moreover, the activation of β-catenin signaling is responsible for increased invasion and metastasis capacity for breast tumor progression via enhanced EMT (Klauzinska et al., 2012). Despite the deregulated Wnt/β-catenin signaling and nucleus β-catenin accumulation is widely involved in BC development, the somatic mutations related to Wnt/β-catenin signaling, such as APC, CTNNB1 and Axin, in BC is rare (Yu et al., 2016); whereas, the overexpression of Wnt corresponding receptors and epigenetic silencing of Wnt antagonists play roles in the elevated activation of Wnt pathways (Zhan et al., 2017).

2.4.2 Inflammation Associated Tumorigenesis in Wnt-Signaling Pathway
Although the chronic inflammation status is responsible for a tumor-favorable microenvironment, it possesses synergistic effects with other tumorigenic pathways as well; for example, Wnt/β-catenin signaling as one of the classical collaborative pathways is under extensively research. Since obesity play a role in breast tumorigenesis, Wnt-signaling is evidenced to involve in tumor progression promoted by diet-induced obesity in C57BL/6 mice model implanted with MMTV-Wnt1 cells from transgenic mice; however, in contrast to obese mice, lean mice showed a reduced tumor growth (Nunez et al., 2008). To establish the causality between obesity and breast tumor, Zheng et al. (2011) found that leptin is essential for MMTV-Wnt1 tumor growth in transplanted mice and also involves in the development of cancer stem cells; whereas, Wang et al. (2006) indicated that contrary to the pro-tumor effects of leptin in Wnt-signaling, adiponectin acts as an anti-tumor factor for BC cell line MDA-MB-231 by decreasing phosphorylation of Akt and GSK-3β, leading to decreased accumulation of β-catenin in cytoplasm and nucleus and downregulation of cyclin D1 (CCND1) expression. In the context of obesity-related chronic inflammation, leptin and adiponectin act as pro- and anti-inflammatory mediators, respectively; hence their balance in mammary tissue profoundly modulates the inflammation and tumor progression, on the other hand, both leptin and adiponectin are the intermediaries between chronic inflammation and tumorigenic Wnt-signaling pathway.
In the process of inflammation-related tumorigenesis, the crosstalk between tumor cells and stromal cells as well as the secreted molecules in the TME greatly promotes tumor progression, and among the interactions, Wnt-signaling also plays a role. TAM accumulation in BC as the major link between inflammation and cancer progression is associated with BC progression and poor patient outcomes (Obeid et al., 2013). Evidence has suggested that Wnt-signaling is positively incorporated in the crosstalk between tumor cells and macrophages as well as cell derived inflammatory cytokines: Kaler et al. (2009a,b) and Oguma et al. (2008) demonstrated that macrophage-derived IL-1β and TNF-α are able to induce the phosphorylation of GSK-3β through NF-κB-dependent and -independent pathway, leading to elevated tumorigenic β-catenin signaling in colorectal and gastric cancers, respectively; Liu et al. (2012) proposed that the elevated TNF-α expression from diet-induced obesity in C57BL/6 mice is correlated to the increased phosphorylation of GSK-3β and β-catenin stabilization; our previous study on obesity-related breast tumorigenesis also found that the TNF-α recombinant protein is capable of increasing the expression of active β-catenin, whereas the anti-TNF-α antibody could attenuate β-catenin accumulation (Roubert et al., 2017); TGF-β (Wu et al., 2017) and CXCL12 (Shan et al., 2015) are shown to be stimulators for active Wnt/β-catenin signaling, leading to enhanced invasion and metastasis of BC via the upregulation of EMT.
In addition to the involvement of canonical pathway in inflammation-associated tumorigenesis, non-canonical pathway also directly or indirectly contributes to tumor progression. Proinflammatory cytokines such as IFN-γ (Newman et al., 2012) and TNF-α (Shao et al., 2016) or lipopolysaccharide (LPS) (Newman et al., 2012; Shao et al., 2016) are able to stimulate the expression of Wnt5a in macrophages, of which’s signaling is generally participating in non-canonical pathways such as CaMKII, PKC and JNK (Shao et al., 2016). Pukrop et al. (2006) showed that in a coculture cell model, the increased secretion of Wnt5a by macrophage could stimulate the expression of Wnt targeted gene MMP-7 and its regulated TNF-α secretion, leading to upregulation of MMP-2,3,9 expression within macrophages by autocrine mechanism; moreover, Wnt5a would go through paracrine mechanism to stimulate the activation of AP-1/c-Jun signaling in MCF-7. Taken together, Wnt5a possesses the ability to promote the invasive capacity of BC cells. Besides, macrophage-derived Wnt5a is evidenced to be endowed with proangiogenic effects by promoting the maturation of proangiogenic macrophages and the proliferation and migration of endothelial cells, and enhancing the production of pro-inflammatory cytokines as well as pro-angiogenic factors in macrophages by autocrine and paracrine mechanisms (Newman et al., 2012). Other pro-tumor effects of non-canonical pathway include the induction of proinflammatory mediators mediated by the activation of CaMKII,
PKC, NF-κB, and MAPK signaling, and induced insulin resistance (Shao et al., 2016).

As the evidence outline in the previous session, progressive chronic inflammation is responsible for the elevated estrogen concentration in both local and systemic levels. It is noteworthy that estrogen could influence Wnt-signaling pathway. Several studies have reported that beta-estradiol treated MCF-7 would upregulate the expression of frizzled-10 (Saitoh et al., 2002), WNT2 (Katoh, 2001) and WNT5B (Saitoh et al., 2002), resulting in the raised activation of Wnt/β-catenin signaling. In summary, Wnt-signaling pathway is not only involved in tumorigenesis triggered by mutations or epigenetic alteration in tumor cells, but the chronic inflammation caused upregulation of proinflammatory mediators and estrogen level as well as the crosstalk between tumor cells and stromal cells in the whole TME profoundly influence the activation of this pathway.

2.5 The Prevention of Breast Cancer: With A Focus on Mushrooms

Phytochemicals and bioactive compounds in plant-based foods are widely accepted as plant nutrients with some specific biological functions that are beneficial to human health and possess positive effects on various human disease. Among these foods, mushrooms are considered as flavorful delicacies in a part of the human diet for many years across the global. Mushrooms, technically, are classified as fungi and generally regarded as
vegetables because of their nutritious properties. Around the world, more than 12,000 species of mushrooms exist in nature, however, only ~20-30 species are widely accepted as cultivated edible mushrooms (Feeney et al., 2014; Valverde et al., 2015; Rathore et al., 2017).

2.5.1 Market Trends of Mushrooms

In many cultures, the extensively use of mushrooms in cuisine has been an indispensable part for centuries, even some of the Asian cultures believe them to have medicinal value and apply them into traditional medicine to treat various diseases (Khan et al., 2013). The global production of mushrooms had dramatically grown from less than 1 million metric tons in 1970 to 10.7 million metric tons in 2016 (FAOSTAT, 2018). In 2016, China, Italy, United States of America, Netherlands and Poland were the top 5 countries for the production of mushrooms and truffles, and they were reported to produce 72%, 6%, 4%, 3% and 2% of global mushrooms and truffles (FAOSTAT, 2018). The production and sales of mushrooms in the USA is gradually increased from $0.8 to $1.2 billion during 1997-2017 (National Agricultural Statistics Service, USDA, 2018). The cash receipt for mushrooms in the USA in 2016 is ranked 28th among all agricultural commodities, but ranked 4th among vegetables (Economic Research Service, USDA, 2018). However, the
mushroom production in the USA is predominantly supplied for domestic utilization, and only less than 5% of mushroom supplies exported annually (Feeney et al., 2014). In addition to the economic value, the cultivation of mushrooms also is environmental friendly that the required land and water for the growth of cultivated mushrooms are relatively less than other crops, moreover, spent mushroom substrate is an excellent source as a soil amendment for crops or a garden (Feeney et al., 2014).

2.5.2 Nutritional Value of Mushrooms

Long honored as a superfood with high nutritional and functional value, mushrooms provide high protein, high fiber, abundant sources of vitamins and minerals, but low fat contents (Mattila et al., 2001; Wang et al., 2014). Carbohydrates found in mushrooms mainly consist of mannitol and high proportion of non-digestible polysaccharides such as β-glucans, chitin and mannans (Cheung, 2010). One of the significant differences between mushrooms and most vegetables is their protein content, mushrooms contain higher proportion of protein, of which’s nutritional quality is higher than that of other plant protein, providing all the essential amino acids for human, even some of the species possessing the comparable protein composition with that of hen’s egg (Wang et al., 2014). In addition, the low amount of fat is composed of high proportion of unsaturated fatty acids, such as linoleic
and oleic acid (Valverde et al., 2015). Mushrooms are also a good source of several vitamins and minerals, including riboflavin (vitamin B$_2$), niacin, folate, vitamin B$_{12}$, D, E, potassium, phosphorus, magnesium, zinc, copper and selenium (Cheung, 2010; Feeney et al., 2014; Valverde et al., 2015). Beyond the nutritional value of mushrooms, they are able to synthesize a bunch of biologically active components, including some bioactive carbohydrates, proteins, lipids and secondary metabolites (Valverde et al., 2015), that possess diverse positive effects against various human diseases. Nowadays, mushrooms are recognized as a functional food and are getting worldwide attention on their nutraceutical attributes and pharmacological characteristics.

![Carbohydrate](image)
- High fiber
- Bioactive polysaccharides

![Protein](image)
- High quantity and quality
- Bioactive protein

![Fat](image)
- Low quantity
- High proportion of PUFA

![Vitamins](image)
- Vit B$_2$, B$_{12}$, D, E, niacin and folate

![Secondary metabolites](image)
- Phenolic compounds and Triterpenes

![Minerals](image)
- K, P, Mg, Zn, Co and Se

**Figure 5.** Nutritional value of mushrooms

### 2.5.3 The Health Beneficial Properties of Mushrooms
The health benefits of mushrooms for human, including improved cognition, autoimmune disease, chronic disease such as asthma, obesity, diabetes, cardiovascular diseases and cancers, are mainly discovered on the basis of previous *in vitro* experiments and *in vivo* animal trials, and these health-promoting effects are evidenced mostly attributing to the biological activities of mushrooms on the aspects of immunomodulation, interaction with gut microbiota, and anti-cancer effects (Roupas et al., 2012).

**Immunomodulation.** One of the most well-known health-promoting effects of mushrooms is their anti-inflammatory and immunomodulatory including both immunosuppressive and immunostimulatory properties. These properties are attributed to the wide variety of bioactive compounds in mushrooms such as bioactive saccharides, proteins, fatty acids, vitamins and minerals, secondary metabolites including phenolic compounds, indole compounds and terpenoids (Muszyńska et al., 2018).

*Macronutrients:* β-glucans are the most abound polysaccharides exist in mushrooms, and researches on β-glucans indicate they possess anti-inflammatory and immunostimulatory effects by inhibiting NF-κB, COX-2 and inducible nitric oxide synthase (iNOS) signaling in RAW 264.7 macrophages (Ma et al., 2013) and enhancing the proliferation and stimulation of immune cells such as macrophages and NK cells (Akramiene et al., 2007),
respectively. Proteins such as mushroom lectins and fungal immunomodulatory proteins are characterized as immunoregulatory factors directly targeting immune cells for activating both innate and adaptive immunity (Wang et al., 1996; Sze et al., 2004; Xu et al., 2011). Polyunsaturated fatty acids, especially α-linolenic acid (ALA), are generally considered as anti-inflammatory factors. Indeed, study conducted by Grzywacz et al. (2016) showed that high ALA content found in *Imleria badia* play a role in the inhibition of LPS-induced inflammatory responses in RAW 264.7 macrophages.

*Micronutrients*: antioxidative vitamins such as ascorbic acid, tocopherols as well as carotenoids are important not only for their role in free radical scavenging, but also in preventing inflammatory cytokines production (Grimble, 1997). Ergosterol (vitamin D precursor) and ergocalciferol (vitamin D$_2$) found in mushrooms are important food sources of vitamin D, which possesses a board beneficial effects for human. Babu et al. (2014) demonstrated that 10 weeks feeding of ultraviolet-B light (UVB)-exposed white button (WB) mushrooms powder for Sprague-Dawley rats significantly increased the plasma total 25-hydroxyvitamin D levels, promoted innate immunity as well as improved anti-inflammatory effects in LPS-challenged rats compared to rats feeding without VitD enrichment (UVB exposure). Another experiment conducted by Drori et al. (2016)
demonstrated that in an immune-mediated hepatitis mice model, after the feeding of UVB-exposed *Lentinula edodes* (shiitake mushroom) powder for C57B1/6 mice treated with concanavalin A, the liver damage and plasma IFN-γ was alleviated compared to nonenriched mushroom group. In addition, the authors also pointed out that vitamin D may have synergistic effects with other bioactive compounds in mushroom on anti-inflammatory activities.

*Other bioactive compounds:* phenolic compounds in mushrooms exerting anti-inflammatory effects is partially attributed to their role as antioxidants. Caffeic acid, for example, has been shown to attenuate vascular inflammation by inhibiting NF-κB signaling and the expression of cell adhesion molecules, chemoattractants such as MCP-1 and IL-8 in the TNF-α-induced human umbilical vein endothelial cells (Moon et al., 2009). Indole compounds such as melatonin and serotonin possess immunoregulatory effects including immunostimulation and/or anti-inflammation by targeting various immune cells (Carrillo-Vico et al., 2013; Herr et al., 2017). Triterpenes derived from *Ganoderma lucidum* are endowed with immunomodulatory and anti-inflammatory effects by inhibiting NF-κB signal transduction or other transduction pathways such as AP-1/ nuclear factor of activated T-cells (NF-AT) and STAT3 signaling (Rios, 2010; Muszyńska et al., 2018).
**Gut microbiota.** The microbiota inhabiting in human gastrointestinal (GI) tract is comprised of more than $10^{14}$ microorganisms (10 times more than the number of human cells), with over 1000 species of known bacteria encompassing more than 3 million genes (150 times more than human genome) (Jayachandran et al., 2017). The complexity makes gut microbiota a dynamic ecosystem that is profoundly influenced by multiple factors through the lifetime including genotype, the mode of delivery at birth and infant feeding, diet, lifestyle, stress and environmental exposures. A healthy and balanced gut microbiota could contribute many benefits to the host such as strengthening gut barrier (Natividad et al., 2013), protecting against pathogens (Bäumler et al., 2016), xenobiotic metabolism (Claus et al., 2016), regulating host energy and immune homeostasis (Wu et al., 2012; Rosenbaum et al., 2015); whereas, a disrupted gut microbiota would lead to various diseases.

One of the major role of mushrooms in improving health is by providing prebiotics such as non-digestible oligosaccharides and polysaccharides to ameliorate and maintain the host’s gut microbiota. Recent study has shown that C57BL/6 mice feeding with 1% WB mushrooms (1 g/100 g diet) for 2-4 weeks displayed an increased diversity of gut microflora by increasing the *Bacteroidetes* phyla and decreasing the *Firmicutes* phyla.
including pathogenic bacteria *Clostridia*, compared to mice feeding with control diet (Varshney et al., 2013). Research also points out that the ratio between *Firmicutes* and *Bacteroidetes* is intimately associated with obesity. Chang et al. (2015) demonstrated that the consumption of the water extract of *Ganoderma lucidum* mycelium in C57BL/6 mice feeding with high-fat diet (HFD) for 2 months showed a significant reduced weight gain and fat accumulation by reversing HFD-induced gut dysbiosis characterized by an increased *Firmicutes* to *Bacteroidetes* ratio.

The fact that the growth of specific gut bacteria is favored by distinct polysaccharides and the intervention of certain polysaccharides or oligosaccharides could alter the composition of human gut microbiota to a healthier and balanced condition (Koropatkin et al., 2012) gives rise to a prosperous research on the field of gut microbiota and prebiotics. Mushrooms as a potential source of prebiotic polysaccharides and oligosaccharides are now under an extensively exploration. For example, polysaccharides derived from *Lentinula edodes, Phellinus linteus, Trametes versicolor* and *Hericium erinaceus* are proved to render an alteration of gut microflora that could promote host’s health (Jayachandran et al., 2017).

**Anti-cancer effects.** The anti-cancer effects of mushrooms are largely due to the above-
mentioned two characteristics that are responsible for the regulation of both innate and adaptive immune systems, leading to effective anti-cancer immunosurveillance. On the other hand, various bioactive compounds found in mushrooms also possess diverse anti-tumor activities such as pro-apoptosis, anti-proliferation, anti-invasion, anti-metastasis, and anti-angiogenesis, which are dependent on the suppression of several tumorigenesis signaling such as phosphoinositide 3-kinase (PI3K)/AKT, Wnt/β-Catenin, and NF-κB signaling pathways (Joseph et al., 2017).

2.5.4 Mushrooms and Breast Cancer Prevention

The benefits of mushrooms on the prevention of BC are generally demonstrated by *in vitro* or *in vivo* studies, as well as a limited number of epidemiological studies. Clinical intervention trails related to oral administrations of mushrooms conducted in humans are rare. Epidemiological studies suggested that per 1 g/day increased mushroom intake is associated with 0.97 BC relative risk, and this correlation exists in both pre- and post-menopausal women (Li et al., 2014). In addition, adjuvant treatments of BC with dietary supplementation with mushrooms or mushroom extracts have shown to improve the quality of life and survival rates of patients (Novaes et al., 2011; Eliza et al., 2012).

Myriad pre-clinical researches on biologically active substances derived from various
mushrooms have revealed that the anti-cancer activities of mushrooms are directly attributed to the diverse bioactive components found in mushrooms. The major bioactive components in mushrooms are polysaccharides, and their anti-tumor activities are extensively addressed. For example, Lu et al. (2011) revealed that polysaccharide Krestin extracted from *Trametes versicolor* are capable of inducing dendritic cell (DC)-mediated $T_{H1}$ immune response and stimulating cytotoxic T cells and NK cells against BC growth in C57BL/6 mice. Jeong et al. (2012) indicated that polysaccharides extracted from WB mushrooms are able to activate J774A.1 macrophages toward tumoricidal ones and possess cytotoxicity against MCF-7 BC cell line. A study conducted by Shi et al. (2013) showed that polysaccharides isolated from *Pleurotus abalonus* could induce ROS-mediated cell apoptosis in MCF-7 cells. In addition, Alonso et al. (2013) demonstrated that polysaccharide complexes derived from Maitake mushroom are responsible for the anti-tumor effects, such as pro-apoptosis, anti-proliferation, anti-metastasis and improving drug sensitivity, in MCF-7 cells.

Other bioactive compounds of mushrooms that possess anti-BC effects include lectins (Savanur et al., 2014), terpenoids such as ganoderic acids (Jiang et al., 2008) and ganodermanontriol (Jiang et al., 2011), and secondary metabolite panepoxydone (Arora et
al., 2014). The anti-BC functions of these bioactive compounds mainly relates to the induction of apoptosis and suppression of proliferation and invasion, which are preventive functions generally for almost all types of cancer. Specifically for BC, several previous studies pointed out that phytochemicals extracted from WB mushrooms would block ER$^+$ BC proliferation in \textit{in vitro} and \textit{in vivo} by suppressing aromatase activity (Grube et al., 2001; Chen et al., 2006).

\textbf{2.5.5 Antrodia Camphorata}

AC is a rare medicinal fungus parasitic on the \textit{Cinnamomum kanehirae Hayata} (bull camphor tree), which only grows in Taiwan. AC, also known as “niu-chang-chih” in Chinese name, is commonly used in Taiwanese traditional medicine for alcohol intoxication, diarrhea, abdominal pain, hypertension, fatigue, viral infection and liver disease (Geethangili et al., 2011).

As other mushrooms, AC also possesses anti-inflammatory and immunomodulatory effects. In LPS-stimulated macrophages, AC extracts were reported to suppress the enhanced production of nitric oxide, TNF-$\alpha$, IL-1$\beta$, IL-12, PGE2 as well as iNOS and COX-2 protein expression via inhibiting NF-$\kappa$B signaling pathway (Hseu et al., 2005; Rao et al., 2007) and STAT3 signaling (Lin et al., 2017b). AC extracts and bioactive compounds found in
AC such as zhankuic acids and antcin K also could inhibit the production of inflammatory mediator ROS in activated neutrophils or mononuclear cells (Shen et al., 2004a; b). In addition, Kuo et al. (2008) demonstrated that AC extracts could enhance innate immune response by upregulation TNF-α and IL-6 expression and polymorphonuclear neutrophils (PMN)- and monocytes -mediated phagocytosis in diluted peripheral blood culture. Although according to previous studies AC has been proven to have effective immunomodulatory properties, its role in inflammatory tumorigenesis within a TME remains unclear.

A series in vitro and in vivo experiments for identifying the anti-BC effects of fermented culture broth of AC have suggested that it could induce tumor-specific ROS-mediated apoptosis and cell cycle arrest against hormone receptor-positive and -negative and HER2/neu-overexpressing BC (Yang et al., 2006; Hseu et al., 2007; Hseu et al., 2008; Lee et al., 2012). In addition to the selective cytotoxic effects, it is able to suppress COX-2 and HER2/neu expression and inhibit PI3K/AKT and β-catenin signaling, of which’s upregulations are involved in cancer invasion and metastasis (Hseu et al., 2007; Lee et al., 2012).

Recently, researchers have isolated a new group of bioactive compounds, ubiquinon
derivatives, including AQ and 4AAQB, from the AC mushrooms. These compounds are shown to exhibit inhibitory effects against BC growth and metastases (Lee et al., 2015) as well as anti-inflammatory effects (Chang et al., 2018). Their anti-tumor effects also have been reported in hepatomas (Lin et al., 2011), brain cancer (Thiyagarajan et al., 2015), pancreatic cancer (Yu et al., 2012), lung cancer (Kumar et al., 2011), colorectal cancer (Chang et al., 2015; Lin et al., 2017a) and ovarian cancer (Liu et al., 2017). However, due to limited studies, the immunomodulatory efficacy of these compounds is still not clear, not to mention their influence on the interaction between inflammation and BC progression.

2.6 Conclusions and Perspectives

In the light of the concept of TME, the links between inflammation and BC progression are well-established. Within a TME, inflammatory mediators such as cytokines, chemokines and PGs play critical roles in promoting breast tumor growth and metastases by suppressing anti-tumor immunity and triggering multiple oncogenic signaling pathways, including NF-κB, STAT3 and Wnt/β-catenin signaling. On the other hand, these mediators could also stimulate the activation of aromatase, leading to enhanced estrogen production and contributing to BC promotion and repression of tumor immunosurveillance. AC, as a traditional Chinese medicine in Taiwan, has been of interest in CAM research in recent
decades. Although there is a quite amount of evidence from pre-clinical experiments that suggests meaningful association between AC and cancer risk, it remains unclear which food components actually account for protection against cancer, and which cellular processes are critically involved. Recently, new bioactive components, AQ and 4-AAQB were isolated from AC, and have been regarded as promising bioactive compounds in treating various cancer, including BC. A greater understanding of specific molecular targets for AQ and 4-AAQB is fundamental to establish them as complementary and alternative strategies for reducing cancer in humans.
CHAPTER 3

PURPOSE OF THE STUDY

Numerous studies have indicated that chronic inflammation acts as a risk factor as well as tumor-promoting factor for BC development. Inflammatory mediators such as cytokines, chemokines and PGs produced by various cells including cancer cells are proposed to be the key regulators in BC progression through an autocrine/paracrine mechanism. Multiple studies also indicated that these mediators could further interfere with estrogen production, oncogene expression, and tumor immunosurveillance within a TME, leading to advanced BC. Recently, two novel bioactive components, AQ and 4-AAQB have been isolated from AC mushroom and are recognized as potential anti-cancer and anti-inflammatory natural compounds; however, their effect on immunomodulation in BC is still unknown. We therefore propose to characterize the effects of AC and 4-AAQB on the expression of BC-related inflammatory mediators and examine their effects on the aromatase activity as well as Wnt-signaling responses with or without the stimulation of TNF-α, one of the most critical inflammatory cytokines in the breast TME.

Specific Aim #1: To characterize the influences of AQ and 4-AAQB on the production of inflammatory mediators in the MCF-7 BC cell line with or without TNF-α
stimulation. Our working hypothesis is that TNF-α stimulation on MCF-7 BC cells would deteriorate the gene expression profile of inflammatory mediators and treatment with AQ and 4-AAQB to MCF-7 cells could improve the gene expression profile of inflammatory mediators before and after TNF-α stimulation.

Specific Aim #2: To examine the impact of AQ and 4-AAQB on the expression of aromatase in the MCF-7 BC cell line. Our working hypothesis is that TNF-α stimulation on MCF-7 BC cells would upregulate the gene expression of aromatase and treatment with AQ and 4-AAQB to MCF-7 cells could suppress the gene expression of aromatase before and after TNF-α stimulation.

Specific Aim #3: To examine the influences of AQ and 4-AAQB on immune-checkpoint CD47 and tumorigenic Wnt-signaling downstream genes. Our working hypothesis is that TNF-α stimulation on MCF-7 BC cells would upregulate the gene expression of immune-checkpoint CD47 and Wnt-signaling responses and treatment with AQ and 4-AAQB to MCF-7 cells could inhibit the gene expression of CD47 and Wnt-signaling responses.
CHAPTER 4

MATERIALS AND METHODS

Our experiment aimed to identify the role of AQ and 4-AAQB in the modulation of inflammatory mediators as well as their associated gene expression in MCF-7 BC cells with or without TNF-α stimulation. The appropriate doses for AQ/4-AAQB and TNF-α treatment was first evaluated by using MTT assays. The gene expression of inflammatory mediators, aromatase and Wnt-signaling downstream genes was measured in the treated MCF-7 cells by using quantitative real-time PCR to determine the effects of TNF-α stimulation and AQ/4-AAQB treatment with or without TNF-α stimulation in MCF-7 cells.

4.1 Experimental Design

See Table 1. in the Appendix

4.2 Reagents and Chemicals

The compounds antroquinonol (AQ, > 99% purity) and 4-acetylanthroquinonol B (4-AAQB, > 99% purity) were obtained from New Bellus Enterprises Co., Ltd. (Tainan, Taiwan). AQ and 4-AAQB were dissolved in dimethyl sulfoxide (DMSO, Santa Cruz Biotechnology Inc., Dallas, TX) to make 25mM and 50mM stock solutions, respectively. The stock
solutions were filter-sterilized and stored at -20°C in aliquots. Dulbecco's modified Eagle medium (1X) (DMEM, Gibco™), heat-inactivated fetal bovine serum (FBS, Gibco™), phosphate buffered saline (1X) (PBS, Gibco™), 0.25% trypsin-EDTA (1X) (Gibco™), penicillin-streptomycin (10,000 U/mL) (Gibco™), TRIzol® reagent (Invitrogen™), high-capacity cDNA reverse transcription kit (Applied Biosystems™) and PowerUp™ SYBR™ green master mix (Applied Biosystems™) were purchased from Thermo Fisher Scientific Co. (Waltham, MA). DEPC-treated water was purchased from Santa Cruz Biotechnology (Dallas, TX). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT, Calbiochem®) was purchased from MilliporeSigma (Burlington, MA). MTT were dissolved in PBS to make a 5 mg/ml stock solution. The MTT stock solution were filter-sterilized and stored at -20°C in aliquots. Recombinant human TNF-α was purchased from PeproTech Inc. (Rocky Hill, NJ). rhTNF-α was reconstituted in sterile double distilled water containing 0.1% bovine serum albumin (BSA, Cell Signaling Technology Inc., Danvers, MA) to make a 100ng/μL stock solution and was stored at -20°C in aliquots.

4.3 Cell Lines and Cell Culture

Breast cancer cell line MCF-7 was obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF7 cells were maintained in DMEM supplemented with 10%
FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and 1 mM sodium pyruvate at 37 °C in a humidified incubator containing 5% CO₂. Cells were sub-cultured at 80% confluency, and culture medium was replaced every 72 hours.

4.4 Cell Viability Assays

MCF-7 cells were seeded in 96-well plates with 2×10⁴ cells per well and incubated for 48 hours. After cell growth reached 80% confluency, cells were subsequently starved in DMEM containing 0.5% FBS overnight. After starvation, cells were either treated with DMEM containing 0.5% FBS and AQ/4-AAQB (0~50 μM with DMSO<0.01%) or control medium containing 0.5% FBS for 24 hours and 48 hours or treated with medium containing 0.5% FBS and rhTNF-α (0~10 ng/mL) or rhTNF-α (0~10 ng/mL) combining with AQ/4-AAQB (0.4 μM with DMSO<0.01%) for 24 hours. Blanks were incubated with culture medium without seeding cells. Cell viability was determined by incubation with MTT (0.5 mg/mL) for 1 hour. Formazan crystals were dissolved in DMSO, and the absorbance was measured by a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA) at 570 nm wavelength. The average value obtained from blanks was subtracted from average values obtained from treatment and control groups. Cell viability was expressed as a percentage compared to control. Experiments were performed in sextuplicate.
4.5 Quantitative Real Time PCR Analyses

RNA extract and cDNA preparation: MCF-7 cells were seeded in 6-well plates with $6 \times 10^5$ cells per well and incubated for 48 hours. After cell growth reached 80% confluency, cells were subsequently starved in DMEM containing 0.5% FBS overnight. After starvation, cells were either treated with DMEM containing 0.5% FBS and AQ/4-AAQB (0.4 µM with DMSO<0.01%) or control medium containing 0.5% FBS for 24 hours or treated with DMEM containing 0.5% FBS and rhTNF-α (0.2 ng/mL) or rhTNF-α (0.2 ng/mL) combining with AQ/4-AAQB (0.4 µM with DMSO<0.01%) or control medium containing 0.5% FBS for 0.5, 1, 2, 3, 4 hours. Total RNAs from MCF-7 cells were extracted by using TRIzol reagent (Invitrogen™) according to the manufacturer's instructions. cDNA was synthesized from RNA samples by using high-capacity cDNA reverse transcription kit (Applied Biosystems™).

Gene expression analysis: The expression of target genes was measured by ViiA™ 7 Real-Time PCR System (Applied Biosystems®). Relative gene expression levels were calculated by $\Delta\Delta\text{Ct}$ method with GAPDH acting as the reference gene. Statistical analyses were based on $\Delta\text{Ct}$. $\Delta\text{Ct}$ is defined as $\text{Ct}_{\text{(target gene)}} - \text{Ct}_{\text{(ref. gene)}}$, and $\Delta\Delta\text{Ct}$ is defined as $\Delta\text{Ct}_{\text{(treatment)}} - \Delta\text{Ct}_{\text{(control)}}$. The design of DNA primers for target genes ($IL1\beta$, $IL6$, $IL10$, $IFN\gamma$),
TNF, TGFβ1, PTGS2, CCL2, CSF1, CYP19A1, CD47, CCND1, C-MYC, AXIN2 and GAPDH) were available from PrimerBank (https://pga.mgh.harvard.edu/primerbank/). All primers were ordered from Thermo Fisher Scientific (Invitrogen™ Custom DNA Oligos). A list of the primers used can be found in Table 2. in the Appendix.

4.6 Statistical Analysis

All statistical analyses were performed in Excel and SAS. Statistical significances were evaluated by unpaired t test, and time-dependent associations were assessed by Cochran-Armitage test for trend. P-value < 0.05 was considered to be statistically significant.
CHAPTER 5

RESULTS

5.1 Cell Viability Assay of AQ and 4-AAQB Treatment in MCF-7 Cells

In order to determine the appropriate treatment dosage for AQ and 4-AAQB, we treated MCF-7 cells with AQ and 4-AAQB. After 48 hours of treatment with either of these compounds at different concentrations (0-1.6 μM), the cell viability was measured by MTT assay. The results showed that the reduction of cell number was in parallel with the elevated concentration of AQ and 4-AAQB compared to untreated control (Figure 6A). Specifically, the cell growth inhibition in 4-AAQB treatment against MCF-7 cells was greater than in AQ treatment. Both treatment showed a modest cytotoxic response (~24%) toward MCF-7 cells while the concentration below 0.4 μM; therefore, we decided to use 0.4 μM as our treatment dosage for AQ and 4-AAQB in the following experiments.

5.2 Cell Viability Assay of TNF-α Treatment in MCF-7 Cells

Appropriate treatment concentration for TNF-α is also determined by MTT assay. MCF-7 cells were treated with TNF-α at different concentrations (0-10 ng/mL) or treated with TNF-α combined with 0.4 μM AQ or 4-AAQB. After 24 hours of treatment, results showed
that TNF-α exhibited a growth inhibitory effect on MCF-7 cells; whereas, the combination treatment with AQ or 4-AAQB partially abrogated the inhibitory effects of TNF-α (Figure 6B). TNF-α showed a moderate cytotoxic response (~40%) at 0.2 ng/mL, while the combination treatments exhibited a less effective cytotoxicity (~20%) with 0.2 ng/mL TNF-α. Hence, we decided to use 0.2 ng/mL TNF-α as our treatment dosage for the following experiments.

**Figure 6.** AQ, 4-AAQB and TNF-α reduce cell viability of MCF-7 cells. (A) Growth inhibitory effects of AQ and 4-AAQB against MCF-7 cells after 48 h treatment. (B) Growth inhibitory effects of TNF-α against MCF-7 cells after 24 h treatment. Cell viability is measured by MTT assay and is expressed as the percentage viability relative to the untreated control group. All experiments were performed in sixplicate and data are presented as mean ± SEM. *P < 0.05, as compared to TNF-α treatment.
5.3 AQ and 4-AAQB Alter the Inflammatory Mediators Profile of MCF-7 Cells

To evaluate the effects of AQ and 4-AAQB on the modulation of inflammatory status in MCF-7 cells, gene expression levels of 9 inflammatory mediators (IL6, IL10, IL1β, IFNγ, TNF, TGFβ1, PTGS2, CCL2 and CSF1) were measured using quantitative real-time PCR analyses (Applied Biosystems®). Unpaired t tests were calculated to evaluate the differences between control group and treatment group.

The heatmap of AQ treatment showed that the expression levels of PTGS2, TNF, IL10, CCL2, IL6 and TGFβ1 is lower in AQ treatment group than in control group (Figure 7A). Significantly inhibition by AQ treatment compared to control were found in the expression levels of IL10 (44.0%) and PTGS2 (42.2%) (p-values were 0.026 and 0.040, respectively; Figure 7B). The expression of TNF, TGFβ1, CCL2 and IL6 in AQ treatment group exhibited a decreased trend compared to control (35.7%, 19.3%, 32.7%, respectively; Figure 7B). Similar to the effect of AQ treatment, 4-AAQB lower down the expression levels of TNF, PTGS2, IL10, IL6, IL1β, CCL2 and TGFβ1 as well but with a stronger suppressive effect (Figure 8A). Compared to untreated control, the expression of TNF, PTGS2 and IL10 were significantly inhibited in 4-AAQB treatment by 46.2%, 48.4%, 53.4%, respectively (p-values were 0.002, 0.013 and 0.017, respectively; Figure 8B). The expression of IL6 and
*IL1β* exhibited a decreased trend in 4-AAQB treatment compared to control (50.3% and 21.6%, respectively; Figure 8B). However, the expression level of *CSF1* was significantly increased 19.0% after 4-AAQB treatment, which was not a case in AQ treatment group (Figure 8B).
Figure 7. Heatmap (A) and relative gene expression level (B) of targeted inflammatory mediators in MCF-7 cells with or without 24 h AQ treatment. Relative expression was calculated by $\Delta \Delta Ct$ method, and all statistical analyses were based on $\Delta Ct$ values. Unpaired $t$ test was applied to identify the difference between control and treatment groups. All experiments were performed in tetraplicate and data are presented as mean ± SEM.
Figure 8. Heatmap (A) and relative gene expression level (B) of targeted inflammatory mediators in MCF-7 cells with or without 24 h 4-AAQB treatment. Relative expression was calculated by ΔΔCt method, and all statistical analyses were based on ΔCt values. Unpaired t test was applied to identify the difference between control and treatment groups. All experiments were performed in tetraplicate and data are presented as mean ± SEM.
5.4 AQ and 4-AAQB Suppress IL6 Upregulation but Strengthen IFNγ Upregulation

Induced by TNF-α Stimulation

TNF-α as one of the most common cytokines majorly secreted by macrophages in a breast TME was reported to be a strong inducer for the activation of inflammatory response pathway, leading to the upregulation of a variety of inflammatory mediators. To evaluate the inhibitory effect of AQ and 4-AAQB on the inflammatory status induced by TNF-α in MCF-7 cells, gene expression levels of IL6, IL10, IFNγ, PTGS2 and CCL2 were measured. Cochran-Armitage test for trend were performed to analyze the relationship between time and expression levels; unpaired t tests were calculated to evaluate the differences between control group and treatment group.

By adding rhTNF-α to stimulate MCF-7 cells, gene expression of inflammatory mediators, including IL10, PTGS2, IL6, IFNγ, and CCL2, showed a time-dependent increase (p for trend values were 0.005, <0.0001, <0.001, 0.035 and <0.0001, respectively; Figure 9A~E). However, 4 hours after AQ/4-AAQB and TNF-α cotreatment, only the induced upregulation of IL6 was significantly suppressed up to 51.4% and 35.9% by AQ and 4-AAQB, respectively (p-values were 0.0002 and 0.009, respectively; Figure 9F). In contrast, the expression level of IFNγ was further upregulated 182.5% and 113.6% while cotreating
with 4-AAQB rather than with AQ for 3 and 4 hours, respectively (p-values for 3 h was 0.033, for 4 h was 0.034; Figure 9G). Moreover, AQ showed a stronger inhibitory effect (~29.4%) on the upregulation of CCL2 induced by TNF-α but did not achieve statistical significance (p-value for 3 h was 0.062; Figure 9H).
Figure 9. Gene expression levels of targeted inflammatory mediators in MCF-7 cells after TNF-α stimulation (A–E) Gene expression levels of IL6 (F), IFNγ (G) and CCL2 (H) after TNF-α and AQ/4-AAQB cotreatment. Relative expression was calculated by ΔΔCt method, and all statistical analyses were based on ΔCt values. Cochran-Armitage test for trend and unpaired t test was applied to identify the time-dependent manner of TNF-α stimulation and difference among control and treatment groups, respectively. All experiments were performed in triplicate and data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
5.5  AQ and 4-AAQB Inhibit the Expression of Aromatase in MCF-7 Cells

Aim to evaluate the potential effect of AQ and 4-AAQB on the production of estrogen in breast cancer, we measured the gene expression level of aromatase (CYP19A1) in MCF-7 cells before and after treatment. Aromatase expression was significantly suppressed by up to 50% in both AQ and 4-AAQB treatment groups (p-values were 0.044 and 0.036, respectively; Figure 10A). Since TNF-α in a TME is positively correlated to the aromatase activity (Zhao et al., 1996), our data also showed that the expression of aromatase in MCF-7 cells was time-dependently increased after 0.2 ng/mL TNF-α induction (p for trend value was < 0.001; Figure 10B). However, after 3 and 4 hours cotreatment with 0.4 μM 4-AAQB, the elevated expression of aromatase induced by TNF-α was significantly inhibited by 37.6% and 47.9%, respectively (p-values were 0.040 in 3 h treatment and 0.031 in 4 h treatment; Figure 10C); moreover, after 4 hours cotreatment, the inhibitory effect against TNF-α induction in 4-AAQB group was even significantly stronger than in AQ group (p-value was 0.044; Figure 10C), which also was a similar case exhibited while only AQ and 4-AAQB treatment in MCF-7 cells (mean expression level was 0.525 and 0.471, respectively; Figure 10A).
Figure 10. Gene expression levels of aromatase (CYP19A1) in MCF-7 cells after either AQ/4-AAQB treatment (A) or TNF-α treatment (B) or TNF-α and AQ/4-AAQB cotreatment (C). Relative expression was calculated by ΔΔCt method, and all statistical analyses were based on ΔCt values. Cochran-Armitage test for trend and unpaired t test was applied to identify the time-dependent manner of TNF-α stimulation and difference among control and treatment groups, respectively. All experiments were performed in triplicate and data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
5.6 Effects of AQ and 4-AAQB on the Expression of CD47 and Wnt Targeted Genes in MCF-7 Cells

To investigate the influence of AQ and 4-AAQB treatment on the expression of immune checkpoint and Wnt signaling response in MCF-7 cells, the expression levels of CD47 and Wnt targeted genes C-MYC, CCND1 and AXIN2 were measured before and after treatment. The gene expression level of CD47 was significantly suppressed by 17.3% and 27.4% after AQ and 4-AAQB treatment, respectively (p-values were 0.044 and 0.007, respectively; Figure 11A); whereas, CD47 expression was not influenced by TNF-α stimulation, either the inhibitory effect of AQ and 4-AAQB on CD47 expression was abrogated after TNF-α stimulation (data not shown). The expression level of C-MYC was suppressed by 28.6% and 40.3% after AQ and 4-AAQB treatment, however, data analysis only showed a statistical significant in 4-AAQB treatment group compared to control (p-value was 0.001; Figure 11A). CCND1 and AXIN2 expression showed no difference after AQ and 4-AAQB treatment relative to control group (data not shown). On the contrary to the upregulation of expression of inflammatory mediators and aromatase induced by TNF-α stimulation, C-MYC expression levels was time-dependently suppressed by TNF-α (p for trend value was <0.001; Figure 11B), and CCND1 expression also showed a decreased trend paralleled to
treatment time period (p for trend value was 0.067; Figure 11B). After 3 and 4 hours of TNF-α and AQ/4-AAQB cotreatment, only C-MYC expression among the three Wnt targeted genes was further suppressed by 32.9% and 24%, respectively, after combination treatment of TNF-α and AQ (p-values were 0.006 and 0.008, respectively; Figure 11C), while the expression levels after combination treatment of TNF-α and 4-AAQB for 3 and 4 hours showed no difference compared to TNF-α treatment group (Figure 11C).
Figure 11. Gene expression levels of CD47 and Wnt targeted genes in MCF-7 cells after either AQ/4-AAQB treatment (A) or TNF-α treatment (B) or TNF-α and AQ/4-AAQB cotreatment (C). Relative expression was calculated by △△Ct method, and all statistical analyses were based on △Ct values. Cochran-Armitage test for trend and unpaired t test was applied to identify the time-dependent manner of TNF-α stimulation and difference among control and treatment groups, respectively. All experiments were performed in triplicate and data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
Breast cancer as one of the leading diseases among women globally has long been focused on in the biomedical area. However, in most of the cases, surgery still remains the primary treatment nowadays. Although several medical treatments including hormone therapy, chemotherapy, targeted therapy and radiotherapy are used clinically against breast cancers, any one of these therapies solely is not enough to treat breast cancer effectively; hence a combination treatment strategy is recommended. Since the important role of well-functioned immune system in anti-tumor strategy is widely studied over the past decades, cancer immunotherapy becomes a promising adjuvant treatment for cancer therapy that could potentially boost human immune system against cancers, including breast cancers.

As previous reviewed, a TME is under a chronic inflammatory status, and the relationship between inflammation and cancer can be involved in two pathways, intrinsic and extrinsic pathway (Mantovani et al., 2008). Genetic mutations of cancer-related oncogenes, tumor suppressor genes and inflammation-related genes caused by randomly occurring or environmental factors through the intrinsic pathways are capable of activating related transcription factors and upregulating the expression of pro-inflammatory cytokines,
chemokines and some mediators such as growth factors and prostaglandins, leading to the recruitment of immune cells to neoplastic tissue (Colotta et al., 2009). Infiltrated leukocytes and myeloid cells further deteriorate the status by dramatically increasing the secretion of inflammatory mediators, resulting cancer-related inflammation. On the other hand, inflammatory status caused by obesity, infection or other disease through the extrinsic pathway can also create an inflammatory microenvironment favorable for cancer progression. Recent studies have pointed out inflammation greatly contributes to tumor cell growth, angiogenesis, invasion, metastasis, as well as the immunosuppressive effects responsible for reduced anti-tumor immunity (Mantovani et al., 2008; Allen et al., 2015).

*A. camphorata* is a medical mushroom with several evidenced biological activities including anti-inflammatory and anti-cancer effects (Geethangili et al., 2011). AQ and its derivative, 4-AAQB, have been reported as potent bioactive compounds providing these health-promoting effects in AC mushroom. The aim of present study is to investigate the inflammatory status of breast cancer and the potential role of AQ and 4-AAQB as candidates of CAMs for using in immunotherapy in a MCF-7 breast cancer cell model. In addition to evaluate the inflammatory mediators related to breast cancer progression, we included immune checkpoint CD47, aromatase and Wnt signaling in our research targets.
In our present study, results showed that AQ possessed mild cytotoxicity against MCF-7 breast cancer cells (Figure 6A), which was similar to the effect obtained in the study conducted by Lee et al. (2015). Furthermore, 4-AAQB treatment exhibited a stronger growth inhibitory effect compared to AQ treatment against MCF-7 cells (Figure 6A), and this phenomenon was also evidenced by Chang et al. (2015) that 4-AAQB showed a more potent growth inhibitory effect against aggressive human colorectal cancer cells. Although we did not determine the cytotoxicity of AQ and 4-AAQB in normal breast cells. Wang et al. (2014) indicated that antroquinonol D showed no cytotoxic effect toward MCF-10A normal mammary gland cells. However, due to the aim of present study was to investigate the immunomodulatory effects of AQ and 4-AAQB in MCF-7 cells, we thus decided to use a low concentration of AQ and 4-AAQB possessing relative low cytotoxicity against MCF-7 cells as our treatment dosage. On the other hand, since higher levels of TNF-α can be found in advanced breast cancer patients, the level of TNF-α may also positively correlated to poor patient outcome (Anderson et al., 2004). However, treatment of TNF-α could potentially inhibit MCF-7 cell growth even at low concentration, of which’s effect is totally contrary to the pro-tumor properties mentioned previously (Figure 6B). Specifically to MCF-7 cells, previous studies had pointed out that TNF-α is responsible for reduced cell growth by induction of cell apoptosis and inhibition of cell proliferation (Burow et al., 1998;
Rozen et al., 1998; Lee et al., 2008); however, the inflammatory NF-κB pathway in MCF-7 cells after TNF-α treatment remains activated (Machuca et al., 2006). Our results showed that despite AQ and 4-AAQB could partly abolish the cytotoxic effect of TNF-α (Figure 6B), the inflammatory response induced by TNF-α was still suppressed by cotreatment with AQ or 4-AAQB (Figure 9F).

Since tumorigenic pathways such as NF-κB and STAT3 are often involved in inflammatory responses, tumor cells per se are capable of secreting inflammatory mediators. By directly treatment of AQ or 4-AAQB to MCF-7 cells, expression of IL10 and COX2 (PTGS2) were significantly suppressed, and TNF expression was also significantly inhibited by the treatment of 4-AAQB (Figure 7 & 8). Moreover, comparing the inhibitory effects on the expression of targeted inflammatory mediators between the treatment of AQ and 4-AAQB, 4-AAQB showed a much stronger influence (Figure 7 & 8). Among the suppressed inflammatory mediators, IL-10 is generally considered as an anti-inflammatory cytokine that is responsible for the resolution of host inflammatory response and its secretion is intimately related to inflammatory responses for the purpose of keeping immune homeostasis (Iyer et al., 2012). However, depending on the context of a microenvironment, IL-10 could potentially serve as an immunosuppressive factor, especially in a TME.
Despite the anergy of T cells by IL-10 is profoundly affected in certain condition (Groux et al., 1996), its crucial effects on M2-like macrophage polarization, reduced antigen presenting capacity on both macrophages and tumor cells, and inhibited macrophage-associated Th1 immune response may indirectly contribute to the escape of tumor immunosurveillance (Fiorentino et al., 1991; Moore et al., 2001; Shiratori et al., 2017). However, in the context of TNF-α induced upregulation of IL10, AQ or 4-AAQB failed to suppress the elevated expression of IL10 (data not shown). This situation may due to the complexity of IL10 regulation induced by stimuli (Saraiva et al., 2010), and it is likewise the same reason for the inconsistent results in COX2 expression after TNF-α stimulation (data not shown).

In the process of cancer associated inflammation, immune cell infiltration represents the hallmark in a TME, and macrophages are the most prevalent immune cells among them. Evidence suggest that in breast tumor tissue, macrophages can occupy more than half of tumor mass (Obeid et al., 2013). The macrophage recruitment provides the major source of the secretion of inflammatory mediators. Macrophage colony-stimulating factor (M-CSF, also known as CSF-1) along with CCL2 are the key molecules triggering macrophage recruitment to tumor site and activating M2-like macrophage polarization, which is
responsible for tumor cell growth, enhanced invasion, metastasis and tumor angiogenesis as well as tumor immunosuppressive capacity (Stewart et al., 2012; Tariq et al., 2017). However, in our results, data showed that the reduction of the expression of CCL2 after treatment of AQ or 4-AAQB was failed to achieve statistical significance (Figure 7 & 8), either in both of the TNF-α and AQ/4-AAQB cotreatment groups compared to TNF-α treatment group (Figure 9H), and even the expression of CSF1 was significantly increased up to about 20% after 4-AAQB treatment (Figure 8B). These evidences suggested that the upregulation of CCL2 is not merely attributed to certain pathway activation, in particular NF-κB pathway in present study, same situation was involved in the regulation of CSF1 expression. Therefore, it is possible that AQ or 4-AAQB treatment is not able to inhibit the recruitment of monocytes and macrophages into a breast TME; however, by altering the inflammatory mediator profile of tumor cells and even the other immune cells and stromal cells, the recruited macrophages are still likely to maintain their anti-tumor activity (Wang et al., 2017).

Among the gene expression of 5 inflammatory mediators we aimed to detect in cotreatment experiment, including IL6, IL10, IFNγ, CCL2, and COX2, only TNF-α induced IL6 upregulation was significantly suppressed by cotreatment of AQ or 4-AAQB (Figure 9F).
Although treatment of AQ or 4-AAQB to MCF-7 cells failed to significantly reduce the expression of \( IL6 \), the inhibited upregulation of \( IL6 \) stimulated by TNF-\( \alpha \) treatment in AQ/4-AAQB cotreatment groups demonstrated that AQ and 4-AAQB are potentially responsible for the inhibition of TNF-\( \alpha \)-induced \( IL6 \) upregulation, which was mainly through the suppression of NF-\( \kappa B \) pathway activation. In contrast, the expression level of \( IFN\gamma \) was further significantly elevated up to 2~3 fold in 4-AAQB and TNF-\( \alpha \) cotreatment group compared to TNF-\( \alpha \) treatment group, but not in AQ and TNF-\( \alpha \) cotreatment group (Figure 9G). This \( IFN\gamma \) enhancement phenomenon is also possibly attributed to the modulatory effect of 4-AAQB on NF-\( \kappa B \) pathway. It is evidenced that NF-\( \kappa B \) is involved not only in the production of pro-inflammatory mediators but also in the regulation of anti-tumor immune response (Yu et al., 2009). NF-\( \kappa B \) family consists of five proteins, including RelA (p65), RelB, c-Rel, p50 and p52, and among them RelA-p50 heterodimer is considered as prototype NF-\( \kappa B \) transcription factor, which is mainly involved in the regulation of genes encoding oncogenes and pro-inflammatory mediators, such as IL-6, IL-1\( \beta \) and COX2; whereas, the other heterodimer c-Rel-p50 is crucial for the expression of genes related to T\( \h \)1 immune response, such as IL-12, IFN-\( \gamma \), CD40 and CD80. Although a constitutive NF-\( \kappa B \) activation is commonly found in cancer cells, a majority of the active form is RelA-p50. Furthermore, it is strongly suggested that the activation of NF-\( \kappa B \) partly
crosstalk with other transcription factors such as STAT3, by which’s activation could persistently activate RelA-p50 but reduce c-Rel-p50 (Yu et al., 2009). Nevertheless, the clear interactions between NF-κB and other signaling still remain to be fully studied. Therefore, in our results, the reinforced IFNγ expression after TNF-α and 4-AAQB cotreatment may potentially be via the property of 4-AAQB to interfere with the crosstalk between NF-κB and the other activated signaling.

As breast cancer can be categorized into ER+ and ER− types, ER+ breast cancer is profoundly and directly influenced by estrogen, which is able to regulate the mitogenic signaling through the estrogen receptor (Quigley et al., 2017). However, the pathology of ER− breast cancer is largely different from ER+ breast cancer, and may greatly attributed to chronic low-grade inflammation (Amadou et al., 2013). In addition to the pro-tumor proliferation effect of estrogen, estrogen may still serve as an immunosuppressive factor by interfering other ER+ stromal cells or immune cells in a TME (Quigley et al., 2017). Despite estrogen in breast tumor tissue being mainly produced locally by aromatase enzyme expressed in stromal cells as well as breast cancer cells, previous studies reported that aromatase expression could be further enhanced by positive feedback from estradiol and a chronic inflammatory status (Santner et al., 1997; Kinoshita et al., 2003; Morris et
Indeed, our results showed that TNF-α treatment time-dependently increased the expression level of aromatase in MCF-7 cells (Figure 10B); whereas AQ and 4-AAQB treatment inhibited aromatase expression in transcriptional level (Figure 10A). Interestingly, only TNF-α and 4-AAQB cotreatment successfully suppressed the TNF-α-induced upregulation of aromatase expression (Figure 10C), by which meant that the mechanisms involved in the inhibitory effect of AQ and 4-AAQB on aromatase expression are distinct. Since a variety of inflammatory mediators and growth factors (Reed et al., 1992; Zhao et al., 1995; Zhao et al., 1996; Richards et al., 2003) are reported to be associated with aromatase expression, the potential molecular mechanisms could be highly complicated and still remain nuclear. Therefore, our results might only suggest that both AQ and 4-AAQB potentially are able to inhibit aromatase expression in breast cancer cells, however, only 4-AAQB is responsible for the suppression of TNF-α-induced aromatase upregulation.

Besides the connection between inflammation and estrogen mechanisms in breast cancer, previous review also points out the importance of immune checkpoint and Wnt signaling responses in an inflammatory breast TME. CD47 as an immune checkpoint is highly expressed on various tumor cells, including MCF-7 breast cancer cells, compared to
corresponding normal cells (Willingham et al., 2012; Betancur et al., 2017); however, the mechanisms responsible for the abnormally upregulation of CD47 expression in cancer cells are still poorly understood (Betancur et al., 2017). Recently, research have addressed several transcription factors potentially contributing to the regulation of CD47 expression. Lo et al. (2015) found that NF-κB could regulate the transcription of CD47 gene by directly binding to CD47 promoter, and after TNF-α stimulation, the NF-κB binding efficiency increased. Similar results also were reported by Betancur et al. (2017) that NF-κB is responsible for the activation of CD47 constituent enhancer and promoter, leading to the upregulation of CD47 in MCF-7 cells, however, the authors also mentioned that even though NF-κB is a necessary transcription factor for the initiation of CD47 transcription, only for its binding is far enough to regulate the expression of CD47. The role of NF-κB in CD47 regulation might partly explain why CD47 expression failed to be enhanced after TNF-α stimulation (data not shown). Moreover, the possibility that CD47 inhibitory effect of AQ and 4-AAQB might be counteracted by the elevated NF-κB stimulated by TNF-α also elucidated the inconsistence in our results that AQ and 4-AAQB lose their ability to inhibit CD47 expression after cotreatment with TNF-α (data not shown).

In breast cancers, research found that Wnt/β-catenin signaling is activated among ~60% of
breast carcinomas (Lin et al., 2000). While a less proportion of aberrantly activated Wnt/β-
catenin signaling in breast tumors is via somatic mutations directly involved in this
pathway (Yu et al., 2016), extracellular factors might play critical roles in regulating the
activation of this oncogenic pathway. As we mentioned previously, inflammatory
mediators are capable of increasing β-catenin accumulation, leading to advanced breast
cancer development. Specific to the effect of TNF-α on Wnt/β-catenin signaling, our
previous study reported that TNF-α level in breast tissue is positively correlated to the
expression of Wnt targeted gene, JNK1, further, TNF-α treated breast tissues compared to
nontreated control showed a 3-fold higher expression of CCND1 and marginal increased
expression of AXIN2, both of which are well-known Wnt targeted genes (Roubert et al.,
2017). In the present study, Both AQ and 4-AAQB treatment possessed the ability to
suppress C-MYC expression, which is downstream of Wnt/β-catenin signaling (p-values
were 0.052 and 0.001, respectively; Figure 11A). Chang et al. (2015) also pointed out that
after 4-AAQB treatment, the expression levels of Lgr5 and β-catenin were dose-
dependently inhibited in DLD-1 colorectal cancer cells. However, due to the property that
C-MYC is targeted by multiple signaling pathways in addition to Wnt signaling, hence we
were not able to conclude that the inhibitory effect on C-MYC expression of AQ and 4-
AAQB was via their ability to interfere Wnt signaling pathway. Next, we treated MCF-7
cells with TNF-α or TNF-α combined with AQ/4-AAQB and measured the expression levels of Wnt targeted genes, *CCND1*, *C-MYC* and *AXIN2*. Contrary to our expectation, TNF-α treatment time-dependently suppressed *C-MYC* expression and marginally suppressed *CCND1* expression (*p* for trend values were <0.001 and 0.067, respectively; Figure 11B). Since previous studies have demonstrated that TNF-α treatment to MCF-7 cells would mitigate cell proliferation and increase cell apoptosis by decreasing the expression levels of growth factor receptor and estrogen receptor, which are responsible for tumor cell growth (Rozen et al., 1998; Lee et al., 2008), it is possible that the TNF-α induced pro-tumor signaling activation, such as NF-κB and Wnt pathways, in MCF-7 cells fail to counteract or overcome the anti-proliferation and pro-apoptosis effects; therefore, *C-MYC* and *CCND1* expression, which profoundly mediate cell proliferation, are suppressed in MCF-7 cells after TNF-α treatment. Furthermore, our data showed that TNF-α and AQ cotreatment exerted stronger inhibition on *C-MYC* expression compared to 4 hours TNF-α treatment or TNF-α and 4-AAQB cotreatment (*p*-values were 0.008 and 0.0003, respectively; Figure 11C). On the other hand, *AXIN2* expression marginally increased by 1.2-fold higher after 3 hours of TNF-α treatment (*p*-value was 0.088; data not shown), and cotreatment of TNF-α and AQ successfully inhibited this elevated *AXIN2* expression (*p*-value was 0.047; data not shown). Taken together, these data suggested that
AQ compared to 4-AAQB exerted a much powerful inhibitory effect on TNF-α induced Wnt signaling responses.

Overall, our results thus do suggest that AQ and 4-AAQB derived from AC mushroom possess the function to modulate the expression of inflammatory mediators potentially responsible for generating a chronic inflammatory breast TME in MCF-7 cells. Moreover, it seems to support our hypothesis that AQ and/or 4-AAQB both are potential compounds for inhibiting aromatase and CD47 expression. However, consider to Wnt signaling responses, only AQ showed an effective inhibition on TNF-α induced upregulation of Wnt targeted genes. Compare these two compounds, 4-AAQB seems to hold a much powerful modulatory effects on the expression of inflammatory mediators, aromatase as well as CD47, whereas, AQ may be a much effective inhibitory compound for Wnt signaling responses. Several limitation should be taken into account while interpreting our results. 1) Only one stimulus, TNF-α, was included in the present study. 2) Relative higher TNF-α concentration used in treatment, since TNF-α levels in serum or breast tissue are generally in the pg/mL range. 3) The levels of secreted inflammatory mediators were not measured. 4) Activation levels of targeted pathways, including NF-κB and Wnt signaling, were not measured. 5) Experiments were only conducted in breast cancer cells, hence the effects on
stromal cells or immune cells and their interaction with cancer cells remain unclear. In
order to more clearly understand the role of AQ and 4-AAQB in treating breast cancer
through their immunomodulatory effects, further experiments should be conducted. For
instance, measuring NF-κB and Wnt signaling active levels before and after treatment to
clarify the actual mechanisms and designing a co-culture model to better explore the
influence between cancer cells and other cells. Eventually, experimenting on an animal
model to thoroughly understand their effects on breast cancer in the context of an intact
breast TME. Nevertheless, our results provided promising evidence that AQ and/or 4-
AAQB derived from AC mushroom may influence the development of breast cancer via
the modulation of inflammatory mediators and inflammation-driven upregulation of
aromatase as well as Wnt signaling.
APPENDIX A

TABLE OF PERFORMED ASSAYS AND EXPERIMENTAL DESIGN
### Table 1. Experimental design

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line</th>
<th>Design</th>
<th>Dosage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT Assay 1</td>
<td>MCF-7</td>
<td>Control group (non-treatment)</td>
<td>-</td>
<td>24 &amp; 48 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experimental group</td>
<td>AQ treatment</td>
<td>0~50 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-AAQB treatment</td>
<td>0~50 μM</td>
</tr>
<tr>
<td>MTT Assay 2</td>
<td>MCF-7</td>
<td>Control group (non-treatment)</td>
<td>-</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experimental group</td>
<td>TNF-α treatment</td>
<td>0~10 ng/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNF-α + AQ treatment</td>
<td>TNF-α: 0~10 ng/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNF-α + 4-AAQB treatment</td>
<td>TNF-α: 0~10 ng/mL</td>
</tr>
<tr>
<td>Gene Expression</td>
<td>MCF-7</td>
<td>Control group (non-treatment)</td>
<td>-</td>
<td>24 hours</td>
</tr>
<tr>
<td>Analysis</td>
<td></td>
<td>Experimental group</td>
<td>AQ treatment</td>
<td>Value based on MTT assay 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-AAQB treatment</td>
<td>Value based on MTT assay 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control group (non-treatment)</td>
<td>-</td>
<td>0.5, 1, 2, 3, 4 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experimental group</td>
<td>TNF-α treatment</td>
<td>Value based on MTT assay 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNF-α + AQ treatment</td>
<td>TNF-α: value based on MTT assay 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNF-α + 4-AAQB treatment</td>
<td>TNF-α: value based on MTT assay 2</td>
</tr>
</tbody>
</table>
APPENDIX B

TABLE OF PRIMERS USED FOR REAL-TIME PCR ANALYSIS
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Genes</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer-related inflammation</td>
<td><strong>IL6</strong></td>
<td>ACTCACCTCTTCAGAAACGAATTG</td>
<td>CCATCTTTGGAAGGTTTCAAGTTT</td>
</tr>
<tr>
<td></td>
<td><strong>IL10</strong></td>
<td>GACTTTAAGGGTTACCTGGGGTTG</td>
<td>TCACATGCGCCTTGATGTCTG</td>
</tr>
<tr>
<td></td>
<td><strong>IL1β</strong></td>
<td>ATGATGGCTTATTACAGTGGCAA</td>
<td>GTCGGAGATTCGTAGCTGGA</td>
</tr>
<tr>
<td></td>
<td><strong>IFNγ</strong></td>
<td>TCGTAACTGAATTTGAATGTCCA</td>
<td>TCGCTTCCCTGTTTTAGCTGC</td>
</tr>
<tr>
<td></td>
<td><strong>TNF</strong></td>
<td>CCTCTCTCTAATCAGCCTCTCTG</td>
<td>GAGGACCTGGGAGTAGATGAG</td>
</tr>
<tr>
<td></td>
<td><strong>TGFβ1</strong></td>
<td>GGCCAGATCCTGTCCAAGC</td>
<td>GTGGGTTTCCACCATTAGCAC</td>
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<tr>
<td></td>
<td><strong>PTGS2</strong></td>
<td>CTGGCGCTCAGGCCATACAG</td>
<td>CGCACTTTAATCTGGTCAAAATCC</td>
</tr>
<tr>
<td></td>
<td><strong>CCL2</strong></td>
<td>CAGCCAGATGCAATCAATGCC</td>
<td>TGGAATCCTGAACCCACTTCT</td>
</tr>
<tr>
<td></td>
<td><strong>CSF1</strong></td>
<td>TGGCGAGCAGGAGATATCA</td>
<td>AGGTCTCCATCTGTACGTCAAT</td>
</tr>
<tr>
<td>Wnt-signaling pathway</td>
<td><strong>CCND1</strong></td>
<td>GCTGCGAAGTGGAACCATC</td>
<td>CCTCCTCTGCAACCATTTGA</td>
</tr>
<tr>
<td></td>
<td><strong>C-MYC</strong></td>
<td>GGCTCCTGGCAAAAGGTCA</td>
<td>CTGCGTAGTTTGTGCTGATGT</td>
</tr>
<tr>
<td></td>
<td><strong>AXIN2</strong></td>
<td>CAACACCAGGCAGGACAA</td>
<td>GCCCAATAAGGAGTGAAGGACT</td>
</tr>
<tr>
<td>Immune checkpoint</td>
<td><strong>CD47</strong></td>
<td>AGAAGGTGAAACGATCGACGAC</td>
<td>CTGATCCGTACCAGGGATCT</td>
</tr>
<tr>
<td>Aromatase</td>
<td><strong>CYP19A1</strong></td>
<td>TGGAAATGCTGAACCCCATAC</td>
<td>AATTCCCATGCTAGCAGGAG</td>
</tr>
<tr>
<td></td>
<td><strong>GAPDH</strong></td>
<td>GGAGCGAGATCCCTCACAAT</td>
<td>GGCTGTTGTCACTTCTCATTG</td>
</tr>
</tbody>
</table>

Table 2. Primers of targeted genes and GAPDH
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