THE FLAVONOID APIGENIN INHIBITS INDUCIBLE PROGRAMMED DEATH LIGAND 1 EXPRESSION BY BREAST CANCER CELLS

by

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Abstract

Programmed death ligand 1 (PD-L1) is a T cell inhibitory molecule expressed by activated T cells and antigen presenting cells, as well as by various tumor types. PD-L1 expression is believed to contribute to immune evasion by breast cancer cells. The goal of this investigation was to determine the effect of the phytochemical apigenin on PD-L1 expression by several breast cancer cell lines. Apigenin inhibited both IFN-γ- and IFN-β-induced upregulation of PD-L1 by various breast cancer cell lines but did not affect constitutive PD-L1 expression. Apigenin also inhibited IFN-γ-induced STAT1 phosphorylation. Apigenin-mediated reduction of IFN-γ-induced PD-L1 expression by breast cancer cells increased Jurkat T cell proliferation in the presence of breast cancer cells. These data show that apigenin inhibits IFN-induced PD-L1 expression by breast cancer cells. Therefore, apigenin may act as an immunomodulator that increases the vulnerability of breast cancer cells to anti-tumor immune responses.

List of Abbreviations and Symbols Used

5-FU	5-fluorouracil
[^{3}H]TdR	tritiated thymidine
°C	degrees celsius
Ab	antibody
ADCC	antibody-dependent cellular cytotoxicity
AICD	activation induced cell death
AIDS	acquired immunodeficiency syndrome
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ANOVA	analysis of variance
AP-1	activator protein 1
APC	antigen presenting cell
APM	antigen processing or presentation machinery
APS	ammonium persulfate
BCG	bacilli Calmette-Guérin
Bcl	B cell lymphoma
BCR	B cell receptor
BSA	bovine serum albumin
CD	cluster of differentiation
CDK	cyclin dependent kinase
cDMEM	complete DMEM
CHOP	cyclophosphamide, adriamycin, vincristine, and prednisone
CML	chronic myelogenous leukemia

CO_2	carbon dioxide
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cell
ddH_2O	double-distilled water
DLI	donor leukocyte infusion
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechin-3-gallate
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
FAK	focal adhesion kinase
FBS	fetal bovine serum
FoxP3	forkhead box P3
g	gravity

GI	gastrointestinal
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HIF-1α	hypoxia-inducible factor 1α
HMEC	human mammary epithelial cell
HRP	horseradish peroxidase
IDO	indoleamine-pyrrole 2,3-dioxygenase
IFN	interferon
IFN-γR	IFN-γ receptor
IKK	IκB kinase
IL	interleukin
IP	intraperitoneal
ITIM	immunoreceptor tyrosine-based inhibition motif
ITSM	immunoreceptor tyrosine-based switch motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LPA	lysophosphatidic acid
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MEBM	mammary epithelial basal medium
MFI	median fluorescence intensity
MIF	migration inhibitory factor
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid

mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAG	non-steroidal anti-inflammatory drug-activated gene
NF-κB	nuclear factor κB
NFATc1	nuclear factor of activated T cells
NK	natural killer
NSCLC	non-small cell lung cancer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PD-1	programmed death 1
PD-L	programmed death ligand
PE	phycoerythrin
PFA	paraformaldehyde
PI	propidium iodide
PI3K	phosphatidylinositol 3 kinase
PLCγ2	phospholipase C - γ2
PMSF	phenymethylsulfonyl fluoride
PR	progesterone receptor
rh	recombinant human
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute

SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Ser	serine
sf	serum free
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
STAT	signal transducer and activator of transcription
TAA	tumor-associated antigen
TAM	tumor associated macrophage
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TGF-β	transforming growth factor β
TIL	tumor infiltrating lymphocyte
TIM3	T-cell immunoglobulin domain and mucin domain 3
TLR	toll-like receptor
TNF-α	tumor necrosis factor α
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Treg	regulatory T cell
TSA	tumor-specific antigen
Tyr	tyrosine
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
β-ME	β-mercaptoethanol

μL	microlitre
μM	micromolar

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CHAPTER 1

INTRODUCTION

1.1 Cancer

The term cancer encompasses a wide array of diseases characterized by dysregulated proliferation of mutated cells that affects a variety of cells and tissues. Despite the progress that has been made in cancer research, cancer is a growing health problem in Canada. The Canadian Cancer Society estimates that in 2012 approximately 186,400 people will be diagnosed with cancer and 75,700 people will die from cancer (1). The number of new cases is estimated to increase from 2011 by 8,600 cases. This increase is largely due to Canada's aging population. Despite this increase in new cases of cancer, the overall mortality rate has significantly decreased in the past fourteen years (1). In 2001, Hanahan and Weinberg described six qualifications essential to the development of cancer. These qualifications are [1] sustaining proliferative signaling, [2] evading growth suppressors, [3] resisting cell death, [4] enabling replicative immortality, [5] inducing angiogenesis, and [6] activating invasion and metastasis (2). In 2011, Hannahan and Weinberg described two emerging qualifications: [7] reprogramming of energy metabolism and [8] evading detection and elimination by the immune system (3).

Great progress has been made in the screening and treatment of breast cancer, and nearly a 40% reduction in mortality has been seen since 1986. However, in women, breast cancer remains the most common cancer and has the second highest mortality rate (1). The World Health Organization has identified 18 types of breast cancer, but typically there are thought to be three main classifications: lobular carcinoma, ductal carcinoma, and inflammatory breast cancer (4). Ductal and lobular carcinomas are named for the origin of cancer development: the milk duct and lobule, repectively. Inflammatory breast cancer leads to inflammation of the breast due to blockade of lymph vessels by cancer cells and infiltration of lymphocytes (5). Each classification can be further divided based on the expression of estrogen receptor (ER), progesterone receptor (PR), and/or

overexpression of human epidermal growth factor receptor 2 (HER2) by cancer cells. Breast cancers that are negative for all three receptors are termed ‘triple negative’ (6).

The type of treatment used for a breast cancer patient is based on a variety of factors including metastatic staging (stages 0-IV), lymphovascular invasion, histologic grade, ER/PR expression, HER2 overexpression, other health concerns, age, and pre/post–menopausal status (7). The most common forms of treatment include surgery, radiation, chemotherapy, hormone therapy, and/or targeted (biologic) therapy, and depending on the factors listed above, a combination of treatments is often used to reduce recurrence and death (7,8). Hormone therapies have proven to be very effective against progesterone and estrogen-sensitive tumors, and a targeted therapy such as the anti-human HER2 monoclonal antibody (Ab), trastuzamab, is often used in conjunction with chemotherapy to target HER2-overexpressing tumors (9).

Current cancer treatments have a wide range of serious side effects. Chemotherapy causes various short- and long-term side effects that can range from fatigue and nausea to cardiac toxicity and leukemia (10). A study published in 1983 described nausea and vomiting as the two most upsetting side effects to the patient (11). Since 1983, large improvements have been made with the discovery and wide use of antiemetics such as 5HT3 antagonists, but despite prophylactic treatment, patients still describe nausea and vomiting as two of the top three most distressing side effects of chemotherapy (12,13). There is still a long way to go in reducing the side effects to cancer treatment; however, research into the use of natural products as adjuvant therapies has been shown to reduce the negative side effects of chemotherapeutic agents. Dietary anti-oxidants such as vitamin E, vitamin C, and soybean products were shown to have beneficial effects by reducing cardiotoxicity, chemotherapy-induced leukemia, and damage to the gastrointestinal (GI) mucosa, respectively (14–17).

This investigation looks at four breast cancer cell lines derived from pleural effusions: MDA-MB-468, MDA-MB-231, MCF-7, and SK-BR-3. All cell lines were isolated from glandular epithelium (18). The receptor status of the four cell lines is described in Table 1.1. The cell lines used include two triple negative lines (MDA-MB-

468 and MDA-MB-231), one ER-expressing line with basal levels of HER2 expression (MCF-7) and one HER2-overexpressing line (SK-BR-3) (19–21).

1.2 Phytochemicals and Cancer

Epidemiological studies have shown that a diet rich in fruits and vegetables positively correlates with lower risk of various diseases including cancer. Many of the disease-preventing components of fruits and vegetables are categorized as phytochemicals (22), which are natural chemical compounds derived from plants (23). The etiology of the term 'phyto' is Greek and means 'plant' (24). While the chemical structure of each phytochemical is different, all phytochemicals contain one or more hydroxylated aromatic rings. Phytochemicals were originally of interest as pharmaceuticals because of their anti-oxidant properties, and they have since been shown to play preventative or protective roles in the development of cancer, cardiovascular disease, and inflammation (23,25,26).

Typically, cancer research in this field has focused on phytochemicals that affect signaling pathways involved in promoting cancer cell death and reducing cancer cell proliferation. Some of the signaling pathways that certain phytochemicals have been shown to interrupt include the mitogen-activated protein kinase (MAPK) signaling cascade, the PI3K/Akt pathway, and cell cycle progression (27). Phytochemicals can be further classified based on their chemical structures into carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds. Most relevant to this study are the phenolics and in particular a sub-group of phenolics, frequently studied in the field of chemopreventative phytochemicals, known as the flavonoids (24). There are approximately 5000 identified flavonoids that can be found in fruits, vegetables, legumes, tea, and wine (22). Flavonoids all share a similar carbon backbone and can be further broken down into six classes based on their molecular structure: flavonols, flavones, flavanols, flavanones, anthocyanidines, and isoflavonoids (24). Some commonly studied phytochemicals and their sources are listed in Table 1.2.

Epigallocatechin-3-Gallate (EGCG)

The effects of green tea on cancer have been widely studied, and it is well accepted that the active chemopreventative components of green tea are the polyphenols. One of the most abundant and active polyphenols in green tea is EGCG (28). Studies have been performed on the effects of EGCG on a wide variety of cancers including skin, lung, gastrointestinal, colon, and liver cancer (29–33). EGCG induces cancer cell apoptosis and causes arrest in cell cycle progression (34). EGCG also interrupts cell signaling pathways involved in cell growth and proliferation, including the nuclear factor-κB (NF-κB) signaling pathway, MAPK signaling cascade, and activator protein 1 (AP-1) activation (35,36). EGCG also inhibits pro-angiogenic factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP) 2, and MMP-9 (37,38). EGCG enhances the immune response in a variety of inflammatory models. For example, EGCG enhances the anti-tumor immune response in a UVB-induced skin cancer model by decreasing IL-10 production and increasing IL-12 production (39). EGCG also inhibits IFN-γ-induced indoleamine 2,3-dioxygenase (IDO) expression in human oral cancer cells, which enhances the anti-tumor immune response (40). Clinical trials involving EGCG have shown that this green tea extract has the potential to reduce cancer risk and recurrence (41,42).

Genistein

Genistein is the predominant isoflavone found in soy products, and therefore, has been linked to low rates of breast and prostate cancer cases in populations that have diets rich in soy products such as China and Japan (43,44). Genistein is one of several phytochemicals that has a similar chemical structure to estrogen and is categorized as a phytoestrogen compound. The structural similarity allows genistein to bind estrogen receptors as an antagonist on estrogen-sensitive tumors and interrupt estrogen signaling (45). Genistein has a variety of molecular targets that are involved in promoting apoptosis and inhibiting cell cycle progression. This phytochemical causes a G_2/M cell cycle arrest in breast cancer, lung cancer, prostate cancer, and melanoma cells (46–49). Genistein can induce cancer cell apoptosis through the downregulation of B cell lymphoma (Bcl) 2 protein and HER2/neu, and the activation of caspase-3 (50,51). Genistein negatively

affects cell survival and proliferation by interrupting NF-κB, MAPK and PI3K/Akt signaling cascades (52–54). To prevent angiogenesis and metastasis, genistein inhibits a variety of molecules, including MMP-2, MMP-9, protease M, urokinase-type plasminogen activator receptor, VEGF, bone-derived growth factor, lysophosphatidic acid (LPA) receptor, transforming growth factor-β (TGF-β), thrombospondin 1, and proteinase-activated receptor-2 (53,55). The effects of genistein on the immune response have been contradictory. In an *in vivo* murine melanoma tumor model, genistein increased both cytotoxic T lymphocyte (CTL) and NK cell activity (56). In contrast, in vitro studies found genistein to inhibit NK cell activity, and showed that genistein reduced antigen specific activation of T cells (57,58). These discrepancies could be due to the high concentrations of genistein (100-200 μM) used in the *in vitro* studies (57,58). Preclinical studies show that genistein has synergistic effects when used in combination with the chemotherapeutic agents such as cisplatin, erlotinib, docetaxel, doxorubicin, and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) (59–63).

[6]-Gingerol

[6]-Gingerol is a well-studied phytochemical that is present in plants of the ginger family. This phytochemical has anti-inflammatory, antioxidant, and chemopreventative properties (64). [6]-Gingerol inhibits several proinflammatory molecules that have been linked to diseases such as Alzheimer's disease and arthritis (65). Of particular interest are the effects of [6]-gingerol on the pro-inflammatory molecule NF-κB, which is commonly associated with chronic inflammation and the development of cancer (66). Some of the main effects of [6]-gingerol is preventing p38 phosphorylation, production of tumor necrosis factor (TNF)-α, and production of interleukin (IL)-1β (67,68). The consequences of these actions include inhibition of NF-κB activation and nuclear translocation. [6]-Gingerol has anti-angiogenic properties that include preventing VEGF- and basic fibroblast growth factor-induced proliferation of endothelial cells and inducing G_1 cell cycle arrest in endothelial cells (69). [6]-Gingerol also prevents transformation of murine epidermal cells and inhibits AP-1 (70). Prostaglandins and leukotrienes are fatty acid molecules commonly involved in inflammatory processes, and [6]-Gingerol can inhibit the production of prostaglandins and leukotrienes through suppression of prostaglandin synthase or 5-lipoxygenase, enzymes involved in their synthesis (71).

Quercetin

Quercetin is found in a variety of fruits, vegetables, nuts, seeds, and red wine. The interest in quercetin as a chemopreventative natural product stems from its anti-proliferative, anti-oxidant, anti-metastatic, and pro-apoptotic properties (72). The anti-proliferative properties of quercetin can be seen in a variety of cancer cells, including breast, lung, and prostate cancer cell lines (73–75). This reduction in proliferation is caused by arrest of the cell cycle at either the G_2/M or G_1/S phase, depending on the cell type and line (73,76). Quercetin also possesses a range of pro-apoptotic properties that include triggereing both intrinsic and extrinsic pathways of apoptosis (77,78). Despite quercetin's anti-proliferative and pro-apoptotic effects on a variety of cancer types, it has been found to have no effect on the proliferation or viability of normal cells except at high concentrations (>50 μM) whereas the effects of quercetin on cancer cells are seen at very low concentrations (3.5-25 μM) (75,79,80). *In vivo* studies of the effect of quercetin on murine lung, colon, and mammary carcinoma have shown promising results. In rodent models, intraperitoneal (IP) injection of 25 mg/kg quercetin lessened lung tumor burden and increased anti-oxidant enzyme activity in mice, oral administration of quercetin reduced precancerous lesions on rat colons, and IP injection of 25 mg/kg of quercetin decreased tumor growth in a mouse mammary carcinoma model (81–83). In a murine model of asthma quercetin skewed the inflammatory respose towards a T_H1 response by increasing IFN-γ production and reducing IL-4 production (84). In patients with multiple sclerosis quercetin synergized with IFN-β treatment, causing a reduction in PBMC proliferation, TNF-α production, and MMP-9 production (85).

Resveratrol

Resveratrol can be found in grapes, berries, plums, and peanuts and has been used in both prevention and treatment of a wide range of mouse cancer models including cancers of the skin, breast, prostate, GI tract, and lung, as well as neuroblastoma and leukemia (86–93). Resveratrol has been studied extensively *in vitro* and has proven to reduce proliferation of cancer cells through a variety of mechanisms such as inducing cell cycle arrest, promoting apoptosis, inhibiting inflammation and angiogenesis, and decreasing metastasis (86). In the breast cancer cell lines MCF-7 and MDA-MB-231,

resveratrol caused apoptosis by inducing phosphorylation (activation) of p53 (94). The viability of normal peripheral blood lymphocytes was not reduced by 72 h treatment with resveratrol, suggesting that the effects of resveratrol on proliferation and viability are specific to transformed cells (95). Resveratrol reduces angiogenesis through the inhibition of hypoxia inducible factor (HIF)-1α and VEGF expression and blocks both invasion and metastasis of cancer cells by reducing MMP-2, MMP-9, and focal adhesion kinase activity (96–98). *In vivo* work in rodent models has confirmed many of these *in vitro* results. In rodent skin cancer models, topical administration of resveratrol reduced tumor burden, decreased inflammation, and increased apoptosis of tumor cells (87,99,100). In rodent mammary carcinoma models, resveratrol suppressed tumor development, decreased cancer cell proliferation, inhibited angiogenesis, and increased apoptosis of tumor cells (88,101,102). While the majority of previous research has suggested that resveratrol is anti-inflammatory through suppression of lymphocyte proliferation, IFN-γ and IL-2 production, and NF-κB activation, in a murine leukemia model resveratrol enhanced the anti-tumor immune response by increasing lymphocyte proliferation and NK cell activity (93,103). Human clinical studies have not been as extensive as *in vitro* or rodent *in vivo* studies, and have focused mainly on resveratrol bioavailability after oral ingestion. Generally, clinical studies have shown that resveratrol is quickly absorbed and metabolized after oral administration (104). A phase I clinical study looked at the therapeutic effects of a resveratrol-containing grape powder on colon cancer (105). Resveratrol was found to inhibit the Wnt pathway, which is an important pathway in the initiation of colon cancer, in normal colonic mucosa.

1.3 Apigenin and Cancer

This investigation focuses on the properties of the flavone apigenin, which is found in a variety of fruits, vegetables, and beverages including parsley, onions, grapefruit, oranges, and chamomile tea. Chamomile tea remains the most abundant and common source of apigenin with the maximum concentration of apigenin ranging from 0.8-1.2% [w/v] and over one million cups being consumed worldwide each day (106). Apigenin selectively targets cancer cells while remaining non-toxic to surrounding normal cells. Gupta and colleagues, using normal and cancerous human prostate cell

lines, showed that apigenin at low concentrations (10-40 μM) was significantly more toxic to the carcinoma cell line than the normal prostate cells (107). Rats fed relatively high amounts of pure apigenin (50 mg/kg of apigenin daily for 10 days) showed no signs of toxicity (108). Apigenin has also drawn a great deal of attention due to its anti-oxidant, anti-proliferative, anti-angiogenic, anti-mutagenic, and anti-inflammatory properties (106).

Bioavailability, absorption, and metabolism are still hurdles in the usefulness of dietary apigenin as a chemopreventative agent. Apigenin is not soluble in water; however, the natural form of apigenin is commonly a glucoside conjugate, which is slightly more water-soluble (109). In rats that ingested radioactive apigenin, 63% of the radioactivity was excreted within 10 days (110). This study also demonstrated the long half–life of apigenin (91.8 h) in comparison to other flavonoids. A study by Meyer and colleagues showed that after oral consumption of 2 g/kg of parsley by healthy volunteers, the average subject's maximum plasma concentration of apigenin was 0.13 μM at 7.2 h post-ingestion (111). The long half-life of apigenin could help to overcome the poor absorbance of this flavonoid. In an *in vitro* rat model, rat liver enzymes involved in phase I and phase II metabolism were used to determine the metabolites of apigenin (112). Oxidation of apigenin by phase I enzymes belonging to the cytochrome P450 family led to three phase I metabolites: luteolin, iso-scutellarein, and scutellarein. The main hydroxylated metabolite of apigenin luteolin has shown to have a variety of anti-tumor properties (Figure 1.1B) (113). Phase II metabolism of apigenin involved glucuronidation and sulfation reactions that led to a variety of conjugates of apigenin (112).

While it is difficult to study the dietary intake of one particular flavonoid or flavone, there have been studies examining a group of these molecules. One study looked at the average dietary intake of five flavonoids: quercetin, kaempferol, myricetin, luteolin (a metabolite of apigenin), and apigenin. Japan has the highest flavonoid intake (64 mg/day) and Finland has the lowest (6 mg/day) (114). However, these results tend to vary depending on the study and the chosen flavonoids as another study showed the Scandinavian population to have the highest consumption of flavonoids (75-81 mg/day) (115). Dietary intake of flavonoids has been associated with a reduced risk of lung, ovarian, and breast cancer occurrence in Finland, Netherlands, and Italy, respectively

(116–118). Flavonoid intake by patients with resected colorectal cancer showed a decrease in disease recurrence (119). These epidemiological studies demonstrate that flavonoids have both a preventative and therapeutic application in the treatment of cancer.

Apigenin targets a variety of molecular pathways in order to prevent the development and progression of cancer. Studies on the anti-oxidant effects of apigenin show that apigenin scavenges free radicals induced by reactive oxygen species (ROS), and during situations of oxidative stress, apigenin amplifies the intracellular concentration of the anti-oxidant molecule glutathione (120,121). The anti-inflammatory properties of apigenin centre on several molecular targets. In human T cells, apigenin induces activation induced cell death (AICD) by inhibiting phosphoinositide-3-kinase (PI3K)/Akt-mediated NF-κB activation, and therefore reduces NF-κB-induced anti-apoptotic signals (122). In a mouse model of the chronic inflammatory condition atopic dermatitis, apigenin inhibited IL-4-induced signal transducer and activator of transcription 6 (STAT) phosphorylation, which in turn reduced the level of IgE antibodies (Abs). In this same study, apigenin also reduced messenger (m)RNA expression of the pro-inflammatory cytokine interferon (IFN) γ (123). Apigenin can reduce neuroinflammation through suppression of IFN-γ-induced CD40 expression on murine microglial cells by inhibiting STAT1 phosphorylation (124). In addition to inhibiting these inflammatory pathways, apigenin also targets pathways that are critical to cancer development and progression. Apigenin reduces cancer cell proliferation by inducing cell cycle arrest and apoptosis. For example, in various breast cancer cell lines, apigenin causes a G_2/M cell cycle arrest by inducing p21 activation and stabilizing p53 or suppressing cyclin-dependent kinase (CDK) regulators and MAPK activation (125,126). Apigenin-mediated G_2/M arrest is also seen in murine leukemia cells, as well as human pancreatic and colon cancer cell lines (127–129). This is in opposition to the G_1 cell cycle arrest caused by induction of p21/WAF1 seen in human cervical carcinoma cells after treatment with apigenin (130). Apigenin induces apoptosis of both lung and breast cancer cells by a variety of mechanisms that include both extrinsic and intrinsic pathways, depending on the cell line being studied (125,131–133). A common feature among apigenin-mediated apoptosis of various cancer cell types is the dependence upon p53

stabilization (125,130,131,134). The anti-angiogenic properties of apigenin stem from the ability of apigenin to reduce VEGF and VEGF receptor (VEGFR)-2 levels (108). Apigenin also reduces migration and invasion of breast cancer cells more than other flavonoids through inhibition of the PI3K/Akt pathway and β4 integrin function (135). The wide array of effects seen on a variety of cancer cell types both *in vitro* and *in vivo* supports the role of apigenin as both a preventative and therapeutic agent. This investigation focuses on the effects of apigenin on breast cancer cell lines, but a summary of the effects of apigenin on other cancer types is outlined in Table 1.3.

Apigenin and Breast Cancer

Apigenin has anti-proliferative, pro-apoptotic, anti-angiogenic, anti-invasive and anti-metastatic effects on breast cancer cells both *in vivo* and *in vitro* (106). Another interesting characteristic of apigenin is its ability to bind the ER and act as an ER agonist, which has implications on its effectiveness as a chemopreventative for breast cancer. Phytochemicals that are comprised of a biphenolic structure are often capable of binding the ER, and phytochemicals that act as ER agonists or antagonists are categorized as phytoestrogens (136). The biphenolic structure of apigenin is depicted in Figure 1.1.

Apigenin blocks breast cancer cell proliferation through inhibition of the cell cycle at the G_2/M phase. The mechanism by which apigenin causes G_2/M arrest is cell line-dependent. In the HER2/*neu* overexpressing cell line SK-BR-3, apigenin-induced G_2/M arrest was mediated by reduced cyclin D and cyclin E expression and increased activation of the CDK inhibitor $p21^{Cip1}$ (125). Apigenin-induced G_2/M arrest in MCF-7 and MDA-MB-468 cells was caused by a decrease in CDK4, cyclin D1, and cyclin A expression. In MDA-MB-468 cells, ERK phosphorylation and activation was also inhibited (126). Mafuvadze *et al*. outlined four mechanisms by which apigenin exerts its pro-apoptotic effects on breast cancer cells: [1] induction of caspase-3, [2] induction and activation of p53, [3] upregulation of Fas-associated protein with death domain (FADD), and [4] proteasomal degradation of HER2/*neu* (137). The anti-angiogenic effects of apigenin were shown in a model of progestin-induced angiogenesis, i.e., apigenin suppressed mRNA expression, VEGF and VEGFR2 protein expression (108,137,138). This research could have major implications in women receiving hormone replacement

therapy. Apigenin also reduces the ability of the aggressive triple negative breast cancer cell line MDA-MB-231 to invade and metastasize *in vitro* and *in vivo*. Lee and colleagues demonstrated that apigenin inhibited hepatocyte growth factor-induced invasion and metastasis of MDA-MB-231 cells by blocking Akt phosphorylation and, in turn, reducing β4 integrin function (135).

The literature suggests that the effects of apigenin as a phytoestrogen on ER positive cell lines are dose dependent. In 1998, Le Bail's group showed that apigenin at low concentrations (<50 μM) causes an increase in MCF-7 proliferation but at 50 μM apigenin reduces proliferation (139). Seo and colleagues confirmed that apigenin at concentrations of 1-10 μM has stimulatory effects on ERα-positive MCF-7 and T47D breast cancer cells. However, the effects of apigenin were not as potent as those of genistein, which was probably due to the lower binding affinity of apigenin to ERα compared to genistein (140). Studies on the effect of apigenin binding to ERβ have shown apigenin to have a higher binding affinity to ERβ than ERα (136). This could explain the anti-proliferative effect of apigenin on ERβ-positive cells as binding of apigenin to ERβ has been linked to a reduction in cell growth (141). These findings suggest that if apigenin were to be used clinically for the treatment of breast cancer, the receptor status and concentration of apigenin used would need to be carefully considered.

Apigenin and Chemotherapeutics

Apigenin has synergistic effects when used in combination with a variety of commonly used chemotherapeutics (142–144). Many chemotherapeutics that are currently used in cancer treatment have a wide array of side effects that can range from mild short-term side effects such as fatigue and nausea to serious long-term side effects such as neurotoxicity and cardiac rhythm disorder. These more serious side effects have a major impact on the patient's quality of life and may lead to discontinuation of treatment (142). In hopes of reducing the dose of the chemotherapeutic agents, several groups have examined the synergistic effects of dietary supplements and natural products on chemotherapeutics. By finding non-toxic products that have synergistic effects when combined with chemotherapy drugs, the dose of chemotherapeutic agents can be reduced, which in turn reduces the severity of the side effects. Apigenin is a prime subject for this

research because it has very low toxicity to normal cells. Promising results have been seen when apigenin is combined with chemotherapy agents (paclitaxel and 5-fluorouracil [5-FU]) or radiation (142–144). Choi and Kim showed that apigenin decreased breast cancer cell resistance to 5-FU treatment, which led to increased cancer cell apoptosis through suppression of Akt activation and HER2 expression (143). *In vitro* studies show that combined apigenin and paclitaxel treatment has synergistic effects and causes an increase in apoptosis of lung, cervical, and hepatocyte carcinoma cells (142). Treatment of lung carcinoma cells with apigenin increases the radiosensitivity of these cells and leads to a decrease in proliferation and an increase in apoptosis that is Bcl-2- and WAF1/p21- dependent (144). Apigenin also increases 5-FU- or cisplatin-induced apoptosis of head and neck squamous carcinoma cells (145). These synergistic effects of apigenin with currently used cancer treatments show that apigenin has potential roles as both a chemopreventative and chemotherapeutic agent.

1.4 Cancer and Immune Evasion

Two sides of inflammation and cancer

Chronic inflammation is commonly associated with the development and progression of various tumor types (146). Therefore, chronic inflammatory diseases such as bronchitis, gingivitis, irritable bowel syndrome, Crohn's disease, ulcerative colitis, and reflux oesophagitis can lead to the development of cancer (147). Several chronic infectious diseases have also been associated with the development of cancer, including hepatitis, mononucleosis, acquired immunodeficiency syndrome (AIDS), and chronic cholecystitis (147). There are several ways that chronic inflammation can cause cancer. Some of the principal pro-tumor effects of inflammation include the production of growth and survival factors by inflammatory cells, increased angiogenesis and lymphangiogenesis, DNA damage, and remodeling of the extracellular matrix leading to an increase in tumor invasion (148–150). DNA damage to the epithelial cells within the area of inflammation is a critical step in inflammation-mediated carcinogenesis (151). This damage can be caused by reactive oxygen species (ROS) and nitrogen species produced by proliferating leukocytes, and DNA damage can be in the form of point mutations, deletions, or rearrangements (152). The production of migration inhibitory

factor (MIF) by macrophages and T cells can also be involved in cancer development and progression as MIF reduces the function of p53, which can lead to uncontrolled proliferation, an increase in DNA damage in proliferating cells, and a rise in DNA mutations (153). Tumors produce pro-inflammatory cytokines and chemokines that maintain the inflammatory response and further the growth and progression of the tumor. In particular, several tumors including melanoma, head and neck, lung, and pancreatic tumors secrete ligands to the chemokine receptor CXCR2 (148,154–156). These chemokines work in an autocrine fashion to promote tumor growth and angiogenesis. Also, chemotaxis has been implicated in tumor metastasis. Breast cancer cells express the chemokine receptor CXCR4, which causes metastasis to organs that express the CXCR4 ligand, CXCL12 (157). The induction of angiogenesis is a critical process involved in tumor invasion and metastasis. Tumor angiogenesis can be promoted by tumor associated macrophages (TAMs) as TAMS have the ability to sense hypoxic conditions and generate pro-angiogenic factors and chemokines including angiopoietin 2 and VEGF (158).

Inflammation can also suppress carcinogenesis and tumor progression, but has to be targeted specifically to the tumor. A key component to a productive anti-tumor immune response is functional tumor infiltrating lymphocytes (TIL), including $CD4^+$, $CD8^+$, and NK T cells (159–161). Although TILs receive the most attention in the study of anti-tumor immune responses, other cell types play important roles, including antigen presenting cells (APCs), NK cells, and neutrophils (162–164). In order for the immune system to launch a tumor-specific response, APCs must process and present tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs) to T cells (165). TAAs are derived from normal self-proteins that are found in abnormal quantities or locations (i.e. HER2 or p53) within tumor cells while TSAs are derived from proteins that are specific to the tumor and can represent mutated self-proteins or proteins of oncogenic viruses (166). T cell responses toward TSAs tend to be more potent and productive at eliminating the tumor, but these antigens are usually specific to a certain tumor (167). Therefore, immune therapies have been focused on targeting immune responses to TAAs that are common to a variety of tumors; however, these responses tend to be weaker (166). There are several reasons that anti-tumor responses do not effectively eliminate tumors. Often the T cell immune response is not strong enough because there is inadequate co-

stimulation to allow activated T cells to continue to expand and properly function (168,169). Also, tumor cells have developed a variety of methods to evade targeted immune responses. For example, tumor cells can reduce or completely eliminate their expression of MHC class I molecules by a mutation or a change in the regulation of the antigen processing and presentation machinery (APM) (170). Tumors are also able to inhibit activated T cells through binding of the inhibitory co-receptors cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death 1 (PD-1) found on activated T cells (171,172). These co-receptors bind to B7 family molecules expressed by the tumor cells, leading to inhibition of T cell activation, decreased IL-2 production, and T cell anergy or apoptosis. Tumors have also developed the ability to increase regulatory T cell (Treg) infiltration into the tumor microenvironment (173) and have evolved mechanisms to reduce migration of DCs to the draining lymph nodes and increase the concentration of immunosuppressive factors such as the enzyme indoleamine 2,3-dioxygenase (IDO) (174,175). Lastly, tumor cells express molecules that can lead to the inhibition of tumor-specific CTLs such as Fas ligand, programmed cell death ligand (PD-L) 1 and PD-L2; these ligands bind the corresponding receptors on activated CTLs, causing anergy or apoptosis (172,176,177). These findings demonstrate the importance of cancer immune evasion to tumor development and progression. Therefore, the pathways by which cancer evades the immune response offer promising targets for new immunotherapies.

Immunotherapy

Many current cancer treatments involve the use of immunotherapeutics (178–180). Cancer immunotherapies are developed to promote anti-tumor responses and suppress pathways that promote the tolerance of tumor cells (181). Immunotherapy used in combination with current chemotherapeutic drugs can reduce the serious side effects of these chemotherapeutic agents. Currently available immunotherapies for the treatment of cancer can be categorized into six categories: monoclonal (m) Ab, immune adjuvants, systemic administration of cytokines, supportive, prophylactic, and bone marrow transplant therapy (181). The most common and abundant type of immunotherapy currently administered for cancer treatment is mAb therapy. Presently nine mAb have been approved by the United States Food and Drug Administration for cancer therapy,

and they collectively target six proteins: CD52, CD3, CD20, epidermal growth factor receptor (EGFR), HER2/*neu*, and VEGF (182–190). The mechanisms by which mAbs exert their effects include steric inhibition, neutralization, complement activation, and activation of Ab-dependent cellular cytotoxicity (ADCC) (181). Immune adjuvant therapy works to enhance the weak anti-tumor immune response stimulated by the TAA and TSA (181). Immune adjuvant therapy is used in the treatment of bladder cancer, basal cell carcinoma, vulvar intraepithelial, and actinic keratosis (191–194). Current immune adjuvants are comprised of a toll-like receptor (TLR) 7 agonist, and a vaccination with live bacilli Calmette-Guérin (BCG) (191,192). Cytokines that are systemically delivered during cancer treatment to activate the anti-tumor immune response are IFN-α, IL-2, and TNF-α (195–197). These cytokines are effective in the treatment of renal cell carcinoma, melanoma, and soft tissue sarcoma (195–197). While systemic cytokine therapy can have serious side effects that resemble those seen during a severe inflammatory response, it does elicit immune responses in patients with tumors that are unresponsive to chemotherapy (196,198). Prophylactic immune therapies refer to vaccines that target infections associated with the development of cancer, as well as the use of anti-inflammatory drugs to reduce cancer development at sites of chronic inflammation (199,200). Supportive therapies are used in conjunction with chemotherapy and aim to reduce the toxic effects of chemotherapy on immune cells and other non-maliganant cell types, which allows for immune cells to launch an effective anti-tumor immune response (201). Lastly, bone marrow transplantation is used to treat hematologic malignancies, but it has a high risk of relapse (202). Donor leukocyte infusion (DLI) is an immune therapy used on patients that suffer from cancer relapse following bone marrow transplantation. DLI is the infusion of bone marrow donor leukocytes into bone marrow transplant recipients that have had relapse of their hematologic malignancy (203). DLI is effective in patients with relapsed acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML) and multiple myeloma (204). In addition to these current immunotherapies, a variety of immune therapies that target immune regulatory pathways are under development. Methods have been developed that regulate cells directly by interfering with regulatory molecules and signaling cascades, and indirectly by targeting regulatory cells (205–207). Many of the immunotherapies

under development seek to improve on the types of immunotherapies currently in use (181). Second generation mAbs have improved serum half-lives, Ab-mediated immune activation, ADCC response, and complement activation (208–211). Abs under development that block suppression of the immune response target TGF-β and IL-10 (212,213). A phase III clinical trial has proven that ab blockade of CTLA-4 improves survival of patients with metastatic melanoma (214). A phase I clinical trial studying an ab that blocks PD-1 signaling, BMS-936558, has shown promise in the treatment of melanoma, renal cell carcinoma, and non-small cell lung cancer (NSCLC) (215). Immune adjuvants have been improved by increasing their activity following systemic administration. Immune adjuvants being studied include TLR9 agonists and α-galactosylceramide (216,217). Other strategies for new immune therapies include targeting tumor-promoting Tregs and developing vaccines that promote the anti-tumor immune response (207,218).

1.5 PD-L1/PD-1

This investigation focuses on the ability of breast cancer cells to evade the anti-tumor immune response by upregulating cell-surface PD-L1, which binds to its receptor PD-1 on activated tumor-specific T cells causing them to enter into anergy and potentially undergo apoptosis (Figure 1.2B) (219). This interaction causes significant suppression of the anti-tumor immune response and permits the tumor to thrive (219).

Normal Function

The receptor PD-1 and its ligands PD-L1/B7-H1/CD274 and PD-L2/B7-DC/CD273 are all type I transmembrane glycoproteins (220,221). PD-1 is a member of the CD28 superfamily and is expressed by $CD4^+$ and $CD8^+$ T cells, B cells, monocytes, DCs, and macrophages (220,222–224). Resting T and B cells do not express PD-1, which is upregulated after T cell receptor (TCR) or B cell receptor (BCR) activation; PD-1 can also be slightly upregulated on monocytes, myeloid $CD11c^+$ DCs, and macrophages following activation with LPS, TLR7/8 agonists, and IFN-α, respectively (223–225). While the mechanism behind PD-1 regulation is still not completely elucidated, the transcription factor nuclear factor of activated T cells cytoplasmic 1 (NFATc1) is essential to PD-1 upregulation after T cell activation and STAT1/2 and IFN-sensitive

responsive elements are involved in constitutive and IFN-α-induced PD-1 expression on macrophages (224,226). PD-L1 is expressed on T and B cells, DCs, macrophages, bone marrow-derived mast cells, non-hematopoietic cells, including lung, vascular endothelium, liver, mesenchymal stem cells, pancreatic islets, and keratinocytes (227–232). PD-L2 expression is limited to select immune cells: activated DCs, macrophages, bone marrow-derived mast cells, and peritoneal B cells (229,233). PD-L1 expression can be induced by the cytokines IFN-α, IFN-β, and IFN-γ while PD-L2 expression is upregulated by IL-4 and GM-CSF (224,230,232,234). The anti-inflammatory cytokine IL-10 also upregulates PD-L1 expression on monocytes (235). Both PD-L1 and PD-L2 can be constitutively or inducibly expressed by a variety of tumor types (236,237).

The inhibitory mechanism behind PD-1 signaling is similar in both T and B cells (225,238). The cytoplasmic region of PD-1 contains two tyrosine residues (219). One tyrosine residue comprises an immunoreceptor tyrosine-based inhibitory motif (ITIM) while the other comprises an immunoreceptor tyrosine-based switch motif (ITSM) that is essential to the inhibitory role of PD-1 (219). Following ligation of the PD-1 receptor by PD-L1 or PD-L2, the ITIM and ITSM become phosphorylated and ITSM recruits SHP-2, which dephosphorylates TCR and BCR-proximal signaling molecules, and causes decreased stimulation of phospholipase C (PLC) γ2, PI3K, and ERK1/2 (225,238). The principal role of PD-1/PD-L1 interaction in the development and maintenance of tolerance is to restrict the activity of T cells during an inflammatory response, and in turn prevent excessive or unnecessary inflammation (Figure 1.2A) (219). Ligation of PD-1 and PD-L1 suppresses the immune response both directly through initiating T cell anergy and apoptosis and indirectly through the induction of inducible Tregs (iTregs) (219,239). PD-L1 contributes to the development of iTregs by reducing activation of the Akt-mammalian target of rapamycin (mTOR) signaling pathway, which is essential to the conversion of naïve T cells into iTregs. PD-L1 also maintains iTreg function by sustaining the expression of the transcription factor forkhead box P3 (FoxP3) (239). Although both PD-1 and CTLA-4 are T cell inhibitory molecules, they affect different stages of T cell activation and proliferation. CTLA-4 is involved in controlling the activation of naïve T cells and memory T cells while PD-1 suppresses proliferation of activated T cells (165). PD-L1 is upregulated on both inflammatory and non-

hematopoietic cells during inflammation, and the most effective pro-inflammatory cytokines responsible for PD-L1 upregulation is IFN-γ (234). The mechanism behind IFN-γ-induced upregulation of PD-L1 is poorly understood. A study looking at IFN-γ-induced PD-L1 expression on multiple myeloma cells implicated a MyD88/TRAF6/ERK-dependent pathway that induced STAT1 activation, which is commonly associated with IFN-γ signaling (Figure 1.3A) (240). In dermal fibroblasts, IFN-γ-induced upregulation of PD-L1 is dependent on activation of the PI3K/ERK pathway (Figure 1.3C) (241). In other systems, IFN-γ signals through a variety of pathways. The most common pathway is the JAK/STAT1 signaling cascade in which IFN-γ causes oligomerization of the IFN-γ receptor (R) subunits (242), leading to trans-phosphorylation of JAK1 and JAK2 followed by STAT1 recruitment and phosphorylation at tyrosine 701 (Tyr701). Phosphorylated STAT1 homodimerizes and becomes phosphorylated at the serine 727 (Ser727) location. The phosphorylated homodimer then translocates into the nucleus and initiates gene transcription (Figure 1.3B) (242). While this is the most common IFN-γ signaling pathway, alternative pathways have been characterized, several of which are independent of STAT1 activation (242). IFN-γ has been shown to also activate STAT3 and STAT5, and alternatively to the recruitment of JAK1 and JAK2, IFN-γ signaling can lead to the recruitment of Src, PI3K, and MyD88 (240,243–246). The recruitment of these alternative proteins lead to the activation of pathways involving MAPK, PI3K/Akt, CamKII, and IκB kinase (IKK) (240,246–248).

Function in Disease

Dysregulated PD-1 signaling is implicated in a broad range of diseases, including autoimmune disease, chronic viral infections, and cancer (219). Mice that are deficient in PD-1 develop lupus, dilated cardiomyopathy, type I diabetes, and experimental autoimmune encephalomyelitis (EAE) (249–252). Genetic mutations in the human PD-1 gene, resulting from small nucleotide polymorphisms, lead to the development of autoimmune diseases such as systemic lupus erythematosus (SLE), type I diabetes, progressive multiple sclerosis, rheumatoid arthritis, and ankyosing spondylitis (253–257). During infection, PD-1 signaling is responsible for promoting virus-specific $CD8^+$ T cell exhaustion, which results in the inability of T cells to proliferate and produce the pro-

inflammatory cytokines IL-2, TNF-α, and IFN-γ (258). Since reducing PD-1/PD-L1 interactions can cause autoimmunity and increasing this interaction leaves patients susceptible to infections, immunotherapy that targets this pathway must find the balance between overstimulation and complete blockade. PD-L1 is commonly upregulated in a variety of tumor types, and PD-L2 upregulation is seen mostly in lymphoid malignancies (172,259). Induction of PD-L1 on cancer cells is commonly mediated by IFN-γ, and it is this pathway that is the focus of the current investigation (172). PD-1 is expressed by the majority of tumor-infiltrating leukocytes; therefore, the upregulation of the PD-Ls suppresses the anti-tumor immune response (260). PD-L1 upregulation has been associated with poor prognosis in various cancer types including renal, esophageal, and ovarian cancers, which indicates the importance of PD-L1 interactions in regulating the anti-tumor response (177,261,262). Patients with renal cell carcinoma that express high levels of PD-L1 are 4.5 times less likely to survive than those with low levels of tumor-associated PD-L1 (263). An increase in PD-L1 expression by tumors is correlated with a decrease in tumor infiltration by $CD8^+$ T cells (264).

Pre-clinical and Clinical Trials

Many studies have shown successful inhibition of tumor growth in murine models through blocking the PD-1 or PD-L1 pathway (265). Strategies for blocking these signaling pathways have included PD-1 and PD-L1 blocking Abs, DNA vaccination with the extracellular region of PD-1, genetic elimination of the PD-1 gene, RNA interference, and expression of recombinant PD-1 and PD-L1 proteins (219). Pre-clinical studies in murine models have shown promising suppression of tumor progression by modulating PD-1/PD-L1 pathways. For example, an anti-PD-L1 blocking Ab inhibited the growth of murine multiple myeloma cells, and blocking of PD-1 in conjunction with blockade of the Th1 cell inhibitory molecule T-cell immunoglobulin domain and mucin domain 3 (TIM3) led to disease protection in animal models of AML (265,266).

There are currently five clinical trials in progress looking at the effects of various drugs that interrupt the PD-1/PD-L1 pathway on cancer progression: BMS-936558, CT-011, MK-3475, AMP-224, and MDX-1105 (267). To date, BMS-936558, a fully humanized IgG4 PD-1 blocking mAb, has been examined in depth (215). During phase I

clinical trials intravenous infusion of MDX-1106 was given to 296 patients with a variety of advanced solid tumors including NSCLC, renal cell cancer, colorectal cancer, melanoma, and prostate cancer. Of the 236 patients that were evaluated following BMS-936558 treatment, a complete or partial response was seen in 18% of patients with NSCLC, 28% of patients with melanoma, and 27% of patients with renal cell cancer (215). The humanized mAb against PD-1, CT-011, was tested in 17 patients with hematological malignancies (268). CT-011 was effective against B cell lymphoma and refractory AML, and is currently in next-generation clinical trials on patients with advanced hemotologic malignancies and a variety of solid tumors (267,268). Another humanized mAb against PD-1, MK-345, is currently in the early stages of clinical trials, and is intended to prevent ADCC and complement-mediated cytoxicity (267). AMP-224 is a recombinant protein consisting of the extracellular domain of PD-L2 fused with IgG1 (267). This protein was made to block the interaction between PD-L2 and PD-1 as well as block NK cell engagement of PD-1. Currently, Amplimmune Inc. is examining the effects of AMP-224 on patients with treatment-refractory metastatic cancers (267). Lastly, Brahmer *et al.* have recently completed phase I clinical trials studying the effects of MDX-1105, a blocking antibody specific to PD-L1, on patients with advanced solid tumors (269). The phase I clinical trial showed that intravenous injection of MDX-1105 is beneficial in the treatment of advanced NSCLC, melanoma and renal cell cancer (269). These results demonstrate that PD-1/PD-L1/PD-L2 signaling pathways are viable targets for the treatment of cancer, as well as autoimmune diseases and persistent viral infections.

1.6 Rationale and Objectives

This investigation focuses on the effects of the phytochemical apigenin on PD-L1 expression by breast cancer cells. There is a large body of research on the chemopreventative properties of apigenin; however, the immunomodulatory properties of apigenin are less well understood. Previous studies have shown that IFN-γ induces PD-L1 expression by various breast cancer cell lines, and inhibition of the immune suppressing PD-1/PD-L1 pathway boosts the anti-tumor response and can lead to tumor regression (172,265) Apigenin was chosen as a potential inhibitor of IFN-γ-induced PD-

L1 expression because STAT1 activation has been implicated in the IFN-γ-induced upregulation of PD-L1, and apigenin inhibits IFN-γ-induced STAT1 phosphorylation (124,240).

There were four objectives of this study: [1] identify a concentration of apigenin that is non-cytotoxic to a variety of breast cancer cell lines, [2] determine the effects of apigenin on PD-L1 expression by breast cancer cells and normal mammary epithelial cells, [3] examine the effects of the apigenin metabolite luteolin on PD-L1 expression by breast cancer cells, and [4] determine whether downregulation of PD-L1 on breast cancer cells leads to an increase in T cell proliferation in the presence of breast cancer cells.

Table 1.1. Receptor Status of Breast Cancer Cell Lines.

Cell Line	ER	PR	HER2
MDA-MB-231	-	-	-
MDA-MB-468	-	-	-
MCF-7	+	+	+
SK-BR-3	-	-	++

++ indicates overexpression

Table 1.2 Common Phytochemicals and Their Sources.

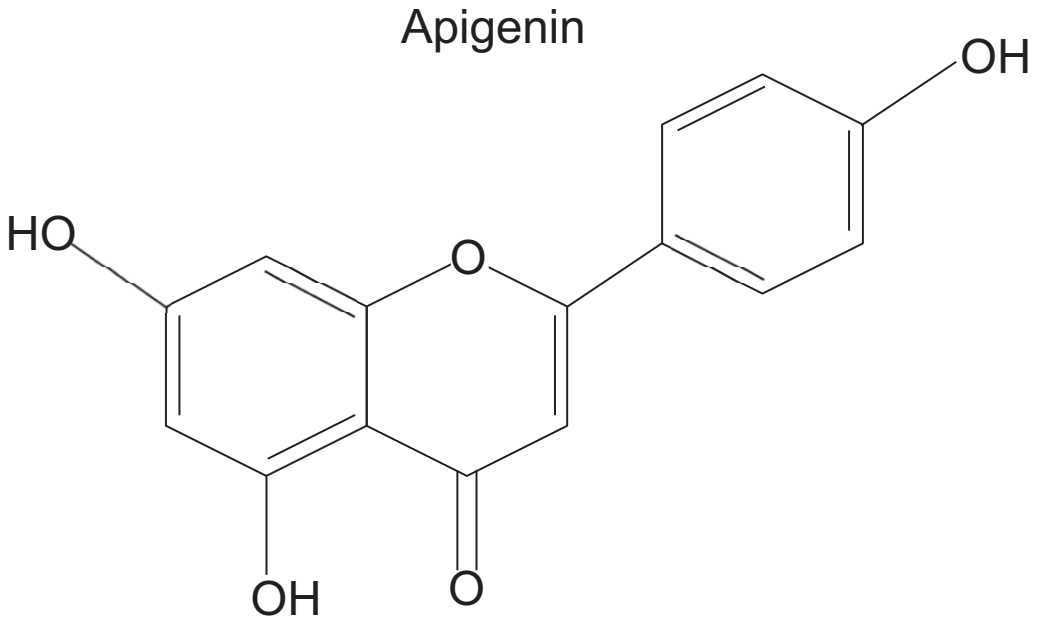
Phytochemical	**Class**	**Natural Source**
Epigallocatechin gallate	Flavanols	Green tea (28)
Genistein	Isoflavonoid	Soy products, coffee (45)
[6]-Gingerol	Vanilloid	Ginger, marjoram (64)
Quercetin	Flavonol	Onion, red apple, lettuce, broccoli, cranberry, berries, olive oil, tea, red wine (270)
Resveratrol	Stilbenoid	Grapes, berries, plums, peanuts (86)

Table 1.3 The effects of apigenin on a variety of cancer cell types.

Cancer Type	Outcome	Reference
Cervical	↑ p53-dependent apoptosis and G_0/G_1 arrest	130
	↓ motility and ↓ invasion	271
	↑ intracellular superoxide	142
	↑ TRAIL-induced apoptosis	272
Colon	G_2/M arrest	129
	↑ caspase-dependent apoptosis	273
	↑ CD26 expression	274
	↑ p53, p21, and NAG-1	275
	↓ cancer recurrence	119
	↑ ERK and p38 activation	276
Hematological	↑ intracellular ROS, ↓ telomerase activity	277
	↑ JNK, ↑ caspase, ↓ Akt activation	278
	↓ mutagenesis	279
Liver	G_2/M arrest, ↓ IL-4R, ↓ tumor growth	280
	↑ intracellular ROS and apoptosis	281
	↓ DNA repair post-radiation	282
Lung	↑ caspase-dependent apoptosis	133
	↑ DNA condensation	283
	↓ VEGF expression	284
Ovarian	↓ VEGF expression, ↓ cell growth	285
	↓ migration, ↓ invasion, ↓ FAK	286
Prostate	↓ VEGF, ↓ MMP-2, ↓ MMP-9	287
	↑ p21 (↑ apoptosis)	288
	↓ cell motility	289
	↑ p53-dependent apoptosis	290
	↓ HIF1-α, ↓ VEGF	291
	G_0/G_1 arrest	292

Acronyms used: TNF-related apoptosis-inducing ligand (TRAIL), non-steroidal anti-inflammatory drug-activated gene 1 (NAG-1), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and deoxyribonucleic acid (DNA).

A.



B.

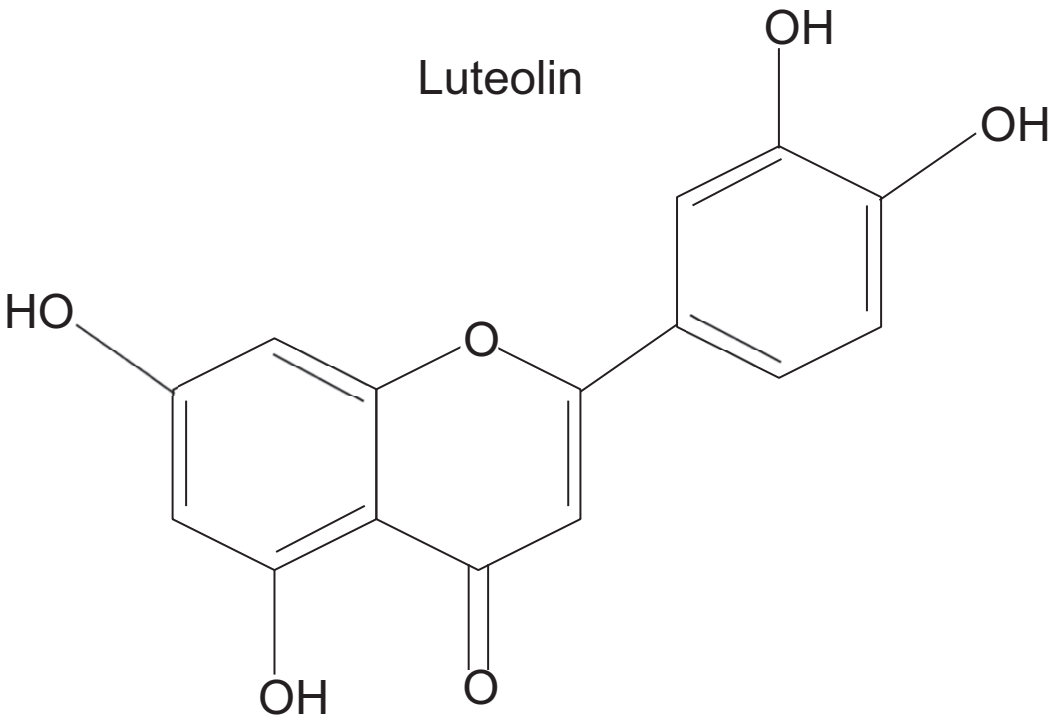


Figure 1.1. The Chemical Structure of A) Apigenin and B) Luteolin.

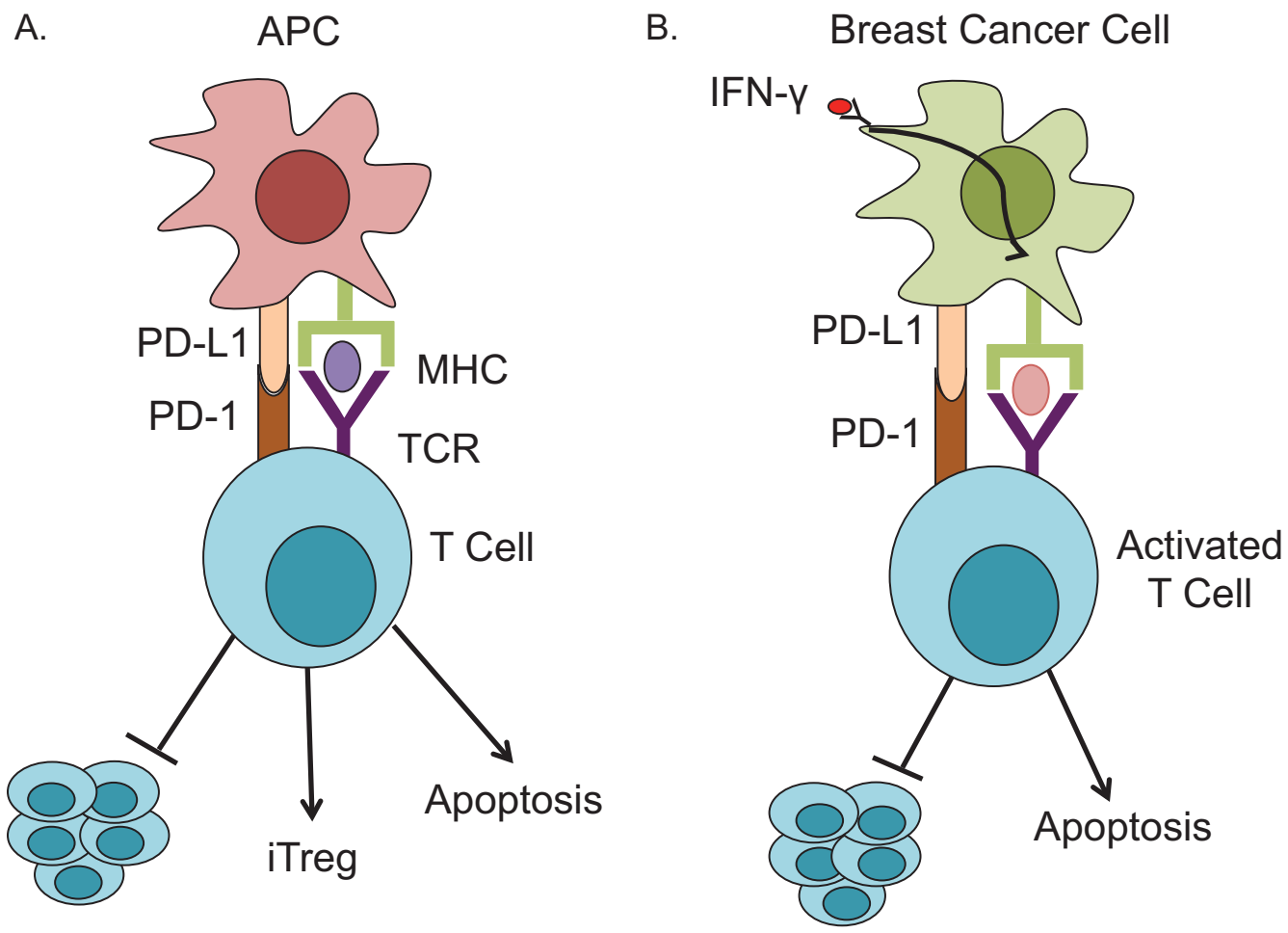


Figure 1.2. The Effects of PD-L1/PD-1 Interactions During A) a Normal Immune Response and B) Cancer Immune Evasion

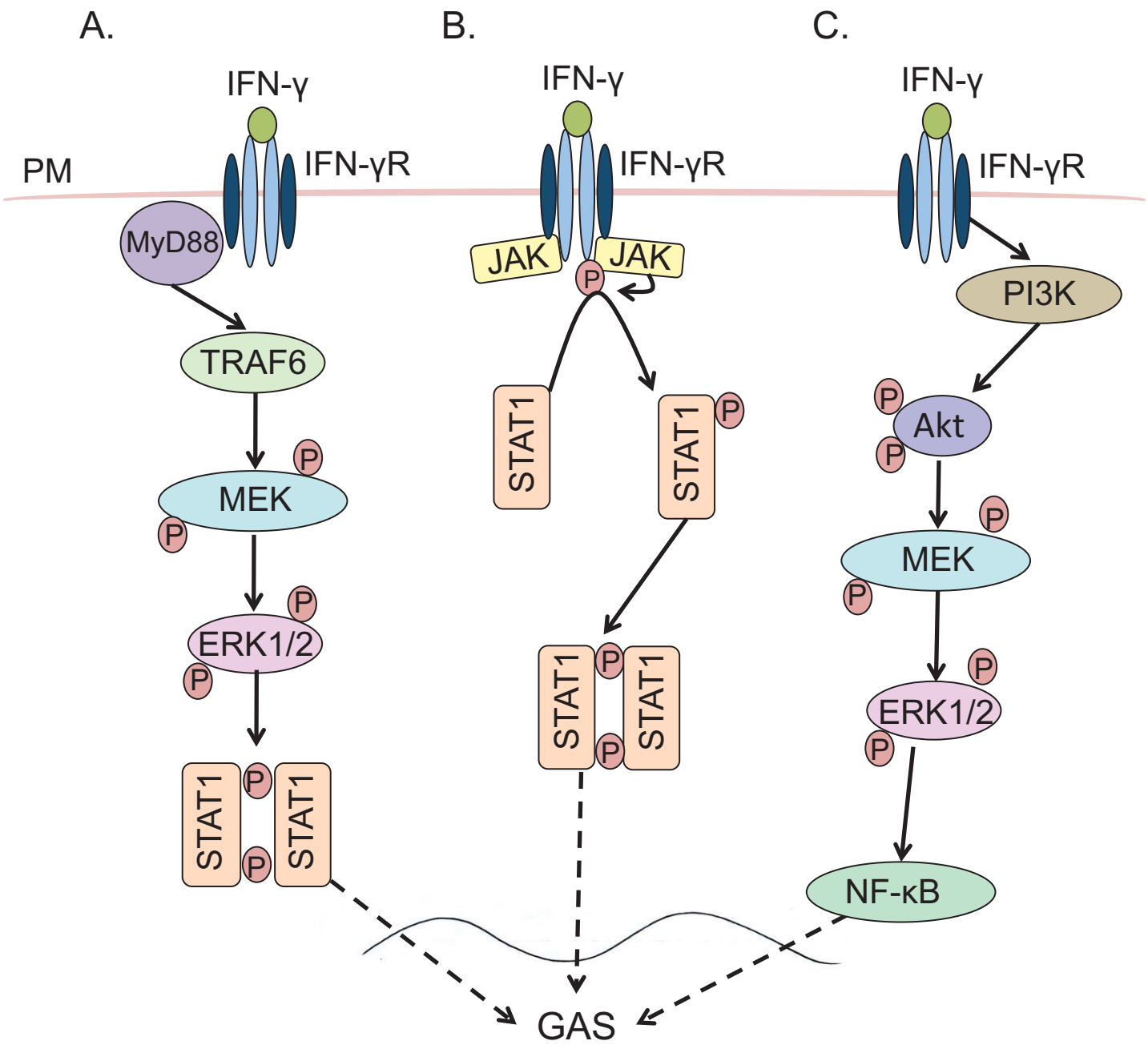


Figure 1.3. Possible Signaling Pathways Involved in IFN-γ-induced Upregulation of PD-L1 in Breast Cancer Cells. Depicted pathways have been implicated in A) IFN-γ-induced PD-L1 expression by multiple myeloma cells (234), B) normal IFN-γ signaling (236), and C) IFN-γ-induced PD-L1 expression by dermal fibroblasts (235).

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines and Normal Cells

MDA-MB-231 human breast carcinoma cells were kindly provided by Dr. S. Dover (Memorial University of Newfoundland, NL). Dr. P. Lee, Dr. K. Goralski, and Dr. G. Dellaire generously provided MDA-MB-468, MCF-7, and SK-BR-3 human breast carcinoma cells, respectively (Dalhousie University, NS). Human mammary epithelial cells (HMEC) were purchased from Lonza Inc. (Walkersville, MD). Jurkat cells, a T leukemia cell line, were purchased from American Type Culture Collection (ATCC; Manassas, VA).

2.2 Reagents

Apigenin, bovine serum albumin (BSA), Dulbecco's Modified Eagle's Medium (DMEM), dimethyl sulfoxide (DMSO), luteolin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-mercaptoethanol (β-ME), phenylmethylsulfonyl fluoride (PMSF), phosphate buffered saline (PBS), Roswell Park Memorial Institute-1640 medium (RPMI), and Triton-X-100 were purchased from Sigma-Aldrich Canada (Oakville, ON). Recombinant human interferon (rhIFN)-γ, recombinant human interleukin (rhIL)-17F, rhIL-17A, rhIL-1β, rhIL-6, and rhIFN-β were all purchased from PeproTech (Rocky Hill, NJ). Cell Trace™ Oregon Green® 488 carboxylic acid diacetate, fetal bovine serum (FBS), 200 mM L-glutamine, 10,000 U/ml penicillin/10,000 μg/ml streptomycin solution, 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, 0.4% trypan blue dye solution, TrypLE™ Express, 0.25% trypsin-EDTA, and propidium iodide (PI) were all purchased from Invitrogen Canada (Oakville, ON). Acrylamide/bis-acrylamide (29:1, 30% solution), ammonium persulfate (APS), ethylene glycol tetraacetic acid (EGTA), paraformaldehyde (PFA), sodium dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED), Tris base, and Tween-20 were purchased from Bio-Shop Canada Inc. (Burlington, ON). Mammary epithelial basal medium (MEBM) and supplements (recombinant human insulin, recombinant human epidermal growth factor, hydrocortisone, gentamicin sulphate, amphotericin, and bovine pituitary extract)

were purchased from Lonza Inc. Anhydrous ethyl alcohol (ethanol; EtOH) was purchased from Commercial Alcohols (Brampton, ON). Tritiated-thymidine ([^{3}H]TdR) was purchased from MP Biomedicals (Santa Ana, CA). Annexin-V-FLUOS was purchased from Roche Diagnostics (Laval, QC). RNase A was purchased from Qiagen Inc. (Mississauga, ON). All culture plasticware was purchased from Sarstedt Inc. (Montreal, QC). Bio-Rad Protein Assay Dye Reagent was purchased from Bio-Rad Laboratories Inc. (Mississauga, ON). Amersham^{TM} ECL^{TM} prime western blotting reagents were purchased from GE Healthcare (Baie d'Urfe, QC).

2.3 Culture Medium and Incubation Conditions

Human breast carcinoma cells were maintained at 37°C in a humidified 10% CO_2 incubator and cultured in DMEM. DMEM was supplemented with 10% heat-inactivated (56°C for 30 min) FBS, 5 mM HEPES buffer (7.4 pH), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, hereafter referred to as complete (c) DMEM. HMECs were maintained at 37°C in a humidified 5% CO_2 incubator and cultured in MEBM supplemented with a proprietary mixture of recombinant human insulin, recombinant human epidermal growth factor, hydrocortisone, gentamicin sulphate, amphotericin, and bovine pituitary extract for a maximum of six passages. Jurkat cells were maintained at 37° in a humidified 5% CO_2 incubator and cultured in RPMI 1640 supplemented with 5% heat-inactivated FBS, 5 mM HEPES buffer (7.4 pH), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, hereafter referred to as cRPMI.

2.4 Stock Solutions

Apigenin and luteolin were prepared in DMSO at stock concentrations of 20 mM and stored at -20˚C. Stock solutions of rhIFN-γ (10 μg/ml), rhIFN-β (10 μg/ml), rhIL-6 (20 μg/ml), rhIL-1β (10 μg/ml), rhIL-17A (200 μg/ml), and rhIL-17F (200 μg/ml) were prepared in 0.1% BSA in sterile double-distilled water (ddH_2O) and stored at -80˚C.

2.5 Antibodies (Abs)

Phycoerythrin (PE)-conjugated anti-human CD274 (B7-H1), anti-human CD279 (PD-1) and mouse IgG1 κ isotype control Abs were purchased from eBioscience, Inc. (San Diego, CA). Monoclonal anti-human IFN-γ R1-PE antibody (Ab) was purchased from R&D Systems, Inc. (Minneapolis, MN). Primary Abs against phospho-STAT3 (Tyr705), total STAT3, phospho-STAT1 (Ser727), phospho-STAT1 (Tyr701), and total STAT1 were purchased from Cell Signaling Technology Inc. (Beverly, MA). Primary Ab against actin, and the secondary Abs horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and HRP-conjugated bovine anti-goat IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.6 Cell Seeding Conditions

For all experiments involving apigenin or luteolin and a pro-inflammatory cytokine pretreatment indicated that cells were treated with 30 μM apigenin or luteolin for 30 min prior to treatment with pro-inflammatory cytokine. Apigenin and luteolin were not washed from cells prior to treatment with pro-inflammatory cytokine. The final volume varied depending on the type of the plasticware used for tissue culture. Experiments carried out in 96-well flat bottom plates had a final volume of 0.2 ml, 24-well flat bottom plates had a final volume of 0.5 ml, 6-well flat bottom plates had a final volume of 2 ml, and T75 flasks had a final volume of 10 ml. Adherent cells were seeded 1 d before treatment to allow them to adhere to plasticware.

2.6.1 Human Breast Carcinoma Cells

For Ab staining, cell cycle analysis, annexin-V/PI staining, and Oregon Green proliferation assays, cells were seeded in 6-well flat bottom plates at 1.5 x 10^5 cells/well or 5 x 10^4 cells/well for 24 h and 72 h treatments, respectively. For [^{3}H]TdR incorporation and MTT assays, cells were seeded in quadruplicate in 96-well flat bottom plates at 1.2 x 10^4 cells/well or 6 x 10^3 cells/well for 24 h and 72 h treatments, respectively. For western blotting, MDA-MB-468 cells were seeded in T75 flasks at 1.0 x 10^6 cells/flask for 1 min, 10 min, and 30 min. For measuring T cell proliferation in the presence of breast cancer cells, MDA-MB-468 cells were seeded in T75 flasks at 1.0 x

10^6 cells/flask for 24 h, then cells were re-plated in 24-well flat bottom plates at 200,000 cells/well for 48 h and 72 h prior to the addition of Jurkat cells. To collect adherent cells from the tissue culture dish, 1 ml of TrypLE Express was added to each well and plates were incubated for 5 min at 37°C in a humidified 10% CO_2 incubator.

2.6.2 HMECs

For Ab staining, HMECs were seeded in 6-well flat bottom plates at 2.5 x 10^5 cells/well and cultured for 24 h. For MTT assays, HMECs were seeded in quadruplicate in 96-well flat bottom plates at 1.5 x 10^4 cells/well and cultured for 24 h.

2.6.3 Jurkat Cells

For Ab staining, 1 x 10^5 Jurkat cells were collected and stained. For measuring T cell proliferation in the presence of breast cancer cells, 5 x 10^4 Jurkat cells were co-cultured with MDA-MB-468 cells in a 24-well flat bottom plate for 48 h and 72 h.

2.7 MTT Assay

The viability of cells treated with apigenin or luteolin was determined using an MTT cell viability assay. MTT is a tetrazolium salt that gets reduced by succinate dehydrogenase within live cells to produce formazan, which can be dissolved by DMSO to produce a purple solution (293). The absorbance of each well is indicative of the succinate dehydrogenase activity of the cells in that well, which is relative to the cell number. Two hours before the end of the treatment period, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated at 37°C in a humidified 10% CO_2 incubator. 96-well plates were centrifuged for 5 min at 500 g and supernatants were discarded. DMSO (100 µl) was added to each well to solubilize formazan crystals produced by metabolically active cells. The plate was slowly shaken for 3 min at 550 rpm on a Microplate Genie (Montreal Biotech Inc., Montreal, QC). Absorbance was read at 570 nm using an Expert 96 microplate reader (Biochrom ASYS, Cambridge, UK). Percent viability of apigenin- or luteolin-treated cells was normalized to the medium control (100% viable cells), and calculated using the formula ([E/C] x 100), where E and C represent the absorbance readings of flavone (experimental) and medium (control) treated samples, respectively.

2.8 Flow Cytometry

All fluorescence data was acquired using a FACSCalibur flow cytometer using BD CellQuest^{TM} software (version 3.3; BD Biosciences, Mississauga, ON). For all fluorescence experiments, 1 x 10^4 counts per sample were analyzed using FCS Express software (version 3.0; De Novo Software, Thornhill, ON).

2.8.1 Annexin-V-FLUOS / PI Staining

After 24 h treatment with indicated concentrations of apigenin, medium was removed from each sample and transferred to a 5 ml round-bottom polystyrene tube (BD Biosciences). To lift cells from the tissue culture dish, 1 ml of TrypLE Express was added to each well and plates were incubated for 5 min at 37°C in a humidified 10% CO_2 incubator. Cells were transferred from wells into tubes containing their respective supernatants. Tubes were centrifuged for 5 min at 500 g, supernatants were discarded and cells were washed with 1 ml of PBS. Supernatants were removed and samples were resuspended in 50 µl of incubation buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$) plus 1 µg/ml PI and 2% Annexin-V-FLUOS labeling reagent (v/v). Tubes were incubated in the dark at room temperature (RT) for 10-15 min and post-incubation 0.45 ml of incubation buffer was added to each sample. Percent viability was calculated as the percentage of cells that stained negative for both Annexin-V-FLUOS and PI. Appropriate controls (unstained and single-stained) were performed in order to correct for the overlap in emission spectra of PI and Annexin-V-FLUOS stains, and compensation was performed during flow cytometric analysis.

2.8.2 Oregon Green Cell Proliferation Assay

Median fluorescence intensity (MFI) of each Oregon Green-stained sample was determined using flow cytometry. The number of cell divisions was calculated using the formula $n = \ln(MFI_{control}/MFI_{sample})/\ln 2$ where n is the number of cell divisions and $MFI_{control}$ is the MFI of the non-proliferative control (294).

2.8.2.1 Oregon Green Staining of Adherent Cell Lines

In order to synchronize cell cycles, cells were incubated in serum-free (sf) DMEM overnight. Cells were washed with warm PBS and incubated with sfDMEM containing 2 μM Cell Trace™ Oregon Green® 488 carboxylic acid diacetate for 1 h at 37°C in a humidified 10% CO_2 incubator. After incubation, samples were washed three times with cDMEM and incubated with cDMEM for 2-3 h. At this time, non-proliferative controls were collected and resuspended in 1% PFA ([w/v], in 1 x PBS) and all other samples were treated (DMSO [0.15%] and apigenin [30 μM]). After a 24 h or 72 h incubation with the indicated treatment, all samples were collected, fixed in 1% PFA, and analyzed by flow cytometry.

2.8.2.2 Oregon Green Staining of Suspension Cell Lines

Cells were collected and resuspended in warm 4 ml of PBS containing 2 μM Cell Trace™ Oregon Green® 488 carboxylic acid diacetate. Cells were incubated in the dark with Oregon Green containing PBS for 10 min at RT on a plate rocker. Four ml of FBS were added to cells to bind excess dye and tubes were centrifuged for 5 min at 500 g. Cells were resuspended in 10 ml of warm cRPMI and incubated for 30 min at 37°C in a humidified 5% CO_2 incubator. At this time, non-proliferative controls were collected and resuspended in 1% PFA. The remaining cells were seeded in T25 flasks and incubated overnight at 37°C in a humidified 5% CO_2. The following day cells were plated for T cell proliferation assays.

2.8.3 Cell Cycle Analysis

Cells were collected after 24 h incubations with indicated treatments (DMSO [0.15%] and apigenin [30 μM]), washed with ice cold PBS, resuspended in 500 μl ice cold PBS, and fixed by slowly adding 4.5 ml of ice-cold 70% EtOH while simultaneously vortexing the sample. All samples were then kept at -20°C for at least 24 h. Cells were then centrifuged, washed with 5 ml ice-cold PBS and resuspended in 0.5-1.0 ml cell cycle solution (0.2 mg/ml DNase-free RNase A, 0.02 mg/ml PI, and 0.1% Triton X-100 [v/v] in PBS). Samples were incubated in the dark at RT for 30 min. Samples were analyzed using flow cytometry and the percentage of cells in each stage of the cell cycle was

measured using ModFit LT software (Verity Software House, Topsham, ME). Gates were set to exclude debris and cell aggregates.

2.8.4 Antibody Staining

After 24 h treatment (DMSO [0.15%], IFN-γ [10ng/ml], apigenin [30 μM] and IFN-γ [10 ng/ml], and apigenin [30 μM]), supernatant containing nonadherent cells was removed from each culture and transferred to a 5 ml round-bottom polystyrene tube. One ml of TrypLE Express was added to each well, and plates were incubated for 5 min at 37°C in a humidified 10% CO_2 incubator. Cells were transferred from wells into tubes containing their respective supernatants. Tubes were centrifuged, and cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (0.2% NaN_3 [w/v], 1% BSA [w/v], in 1 x PBS). Samples were stained with 0.5 μg of either PE-conjugated Ab or isotype-matched PE-conjugated control Ab for 30 min in the dark at 4˚C. Following incubation cells were washed with FACS buffer twice. Each sample was resuspended in 0.5-0.8 ml 1% PFA and samples were analyzed using flow cytometry. During analysis with FCS Express software, a marker was placed over the area containing cells expressing a given molecule to determine the percentage of cells expressing that molecule. MFI was used to determine the level of expression of a given molecule on each cell. Unless otherwise indicated, all values for MFI and percent of cells expressing PD-L1 or IFN-γR were normalized by subtracting the value of the isotype control.

2.9 [^{3}H]TdR Incorporation Assay

Human breast carcinoma cells were pulsed with 0.2 μCi of [^{3}H]TdR for the last 6 h (MDA-MB-468, MDA-MB-231, MCF-7) or 18 h (SK-BR-3) of indicated treatment (DMSO [0.15%] and apigenin [30 μM]). To facilitate cell lysis, cells were frozen and thawed twice before harvesting onto fiberglass filter mats with a Titerek® Cell Harvester (Skatron Instruments, Sterling VA). Incorporation of [^{3}H]TdR into newly synthesized DNA was measured using a Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc., Mississauga, ON).

2.10 Protein Isolation

Treated cells (DMSO [0.15%], IFN-γ [10ng/ml], apigenin [30 μM] and IFN-γ [10 ng/ml], and apigenin [30 μM]) were collected, centrifuged at 500 g for 5 min at 4°C, and washed with 1 ml cold PBS. After wash, samples were resuspended in 35 μl of cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM Na_2HPO_4, 0.25% sodium deoxycholate [w/v], 0.1% NP-40 [v/v], 5mM EDTA, and 5 mM EGTA) containing protease and phosphatase inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 mM NaF, 1 mM PMSF, 1 mM DTT, 100 μM Na_3VO_4, 10 μM PAO, and 10 μg/ml aprotinin). Samples were incubated on ice for 15 min and centrifuged at 14,000 g for 10 min at 4°C. Supernatant containing cell proteins was collected and stored at -80°C for a maximum of 3 days.

2.11 Protein Quantification

Samples of total cell protein were quantified and equalized by colorometric assay. BSA was used to produce a protein standard curve (2.5 μg/ml – 40 μg/ml). Samples and standards were added to a 1 in 5 dilution of Bio-Rad Protein Assay Dye Reagent in ddH_2O and plated in quadruplicate in a 96-well flat bottom plate that was read at 570 nm with an Expert 96 Microplate reader. The protein concentration between samples was equalized and protein was denatured in SDS-PAGE sample loading buffer (200 mM Tris HCl [pH 6.8], 30% glycerol [v/v], 6% SDS [w/v], 15% β-ME [v/v], and 0.01% bromophenol blue [w/v]) that was heated to 95°C for 5 min and then stored at -80°C.

2.12 Western Blotting

Protein samples (10 μg) and prestained Bio-Rad protein ladder were separated on a 12% Tris-HCl acrylamide gel (12% acrylamide, 375 mM Tris-HCl [pH 8.8], 0.1% SDS [w/v], 0.1% APS [w/v], and 0.15% TEMED [v/v] with a 4% acrylamide stacking gel containing 125 mM Tris-HCl [pH 6.8], 0.1% SDS [w/v], 0.1% APS [w/v], and 0.3% TEMED [v/v]) at 200 V in SDS-PAGE running buffer (20 mM Tris-HCl [pH 8.3], 200 mM glycine, and 0.1% SDS [v/v]). After 1 h, protein was transferred to a nitrocellulose membrane using the iBlot® dry blotting system (Invitrogen). Membranes were blocked overnight at 4°C with 5% fat-free milk [w/v] in Tris-buffered saline (20 mM Tris-HCl

[pH 7.6], 200 mM NaCl) containing 0.05% Tween-20 [v/v] (TTBS). The following day, membranes were washed for 30 min with TTBS (changing wash every 5 min), then incubated with the indicated primary Ab for 1 h at RT or overnight at 4°C followed by a 30 min of wash with TTBS. Next, membranes were incubated with the indicated HRP-conjugated secondary Ab and then washed for 30 min with TTBS. After the final wash, membranes were incubated with enhanced chemiluminescence reagents for 1 min and used to expose X-ray film (Sci-Med Inc., Truro, NS), which was developed by a Kodak X-OMAT 1000A automated X-Ray developer. To account for any variation in protein loading, membranes were reprobed for actin, stripped using stripping buffer (62.5 mM Tris-HCl [pH 6.7], 2% SDS [w/v], and 100 mM β-ME), and probed for total protein levels of the protein under investigation. ImageJ software (version 1.45, National Institutes of Health, Bethesda, MD) was used to quantify the intensity of each protein band through densitometry.

2.13 T Cell Proliferation Assay

After 24 h treatment (DMSO [0.15%], IFN-γ [10ng/ml], apigenin [30 μM] and IFN-γ [10 ng/ml], and apigenin [30 μM]), MDA-MB-468 breast cancer cells were collected, washed with cRPMI, and 2 x 10^5 cells/well were replated into a 24-well flat bottom plate. Breast cancer cells were incubated at 37°C in a humidified 5% CO_2 incubator for 4 h to allow cells to adhere. Oregon Green-stained Jurkat T cells (5 x 10^4 cells/well) were then added to the adhered breast cancer cells and the co-culture was incubated at 37°C in a humidified 5% CO_2 incubator for 48 h or 72 h. After incubation, Jurkat T cells were transferred to 5 ml round-bottom polystyrene tubes, wells were washed once with PBS, and tubes were centrifuged at 500 g for 5 min at 4°C. Samples were resuspended in 1% PFA and their proliferation was analyzed by flow cytometry. The number of cell divisions was calculated using the formula $n = \ln(MFI_{control}/MFI_{sample})/\ln 2$ where n is the number of cell divisions and $MFI_{control}$ is the MFI of the non-proliferative control. Cell divisions were normalized to the medium control (294).

2.14 Statistical Analysis

Statistical analysis was performed using Student's t-test and one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons post-test, when appropriate, using GraphPad Prism analysis software (GraphPad Software Inc., La Jolla, CA). Data were considered significantly different when the *p* value was less that 0.05 (indicated by *). When the *p* value was greater than 0.05 data were determined not significant (denoted by ns).

CHAPTER 3

RESULTS

3.1 Low Dose Apigenin Does Not Have Toxic Effects on Breast Cancer Cells.

Apigenin has been previously shown to have anti-proliferative and toxic effects on a wide variety of cancer cell lines, including several breast cancer cell lines used in this study (125,126,131,132). A sub-cytotoxic concentration of apigenin was determined using MTT assays, in which the conversion of the MTT to colored formazan by mitochondrial succinate dehydrogenase is relative to the number of live cells. As shown in Figure 3.1A-D, low dose apigenin (10-30 μM) did not significantly reduce succinate dehydrogenase activity at 24 h in MDA-MB-468, MDA-MB-231, MCF-7 and SK-BR-3 human breast cancer cell lines. At 50 μM, apigenin began to cause a reduction in succinate dehydrogenase activity in MDA-MB-468 cells (Figure 3.1A). Conversely, 50 μM apigenin treatment of MCF-7 cells caused a significant increase in succinate dehydrogenase activity (Figure 3.1C; $p < 0.05$). Since other phytochemicals have previously been shown to reduce MTT in the absence of cells (295), apigenin was examined for this activity; however, when apigenin was incubated with MTT solution for 2 h in the absence of cells there was no detectable reduction of MTT to formazan (Table 3.1). MTT assays do not differentiate whether a decrease in cell number is due to cytostatic or cytotoxic effects. To confirm that 30 μM apigenin was not having cytotoxic effects on the breast cancer cells, Annexin-V/PI staining of apigenin-treated breast cancer cells was performed. Annexin-V binds to phosphatidyl serine, which is found on the outer membrane of cells undergoing apoptosis, and PI enters cells with compromised membrane integrity, as seen during late apoptosis or necrosis (296). Viable cells stain negative for both Annexin-V and PI. Annexin-V/PI staining showed that 30 μM apigenin caused only a minor reduction in MDA-MB-468 cell viability at 24 h (Figure 3.2B), which supported the findings of the MTT assay. To determine the effects of apigenin treatment at longer time points, MDA-MB-468 cells were treated with 10, 20, 30 and 50 μM apigenin for 48 and 72 h. MDA-MB-468 cells began to show a significant reduction in succinate dehydrogenase activity at 48 h when treated with 20-50 μM apigenin, and a further reduction was seen at 72 h (Figure 3.3; $p < 0.05$). The reduction in metabolic

activity indicates that longer exposure to apigenin has a cytostatic and/or cytotoxic effect on breast cancer cells and this effect becomes more prominent as time progresses.

3.2 Apigenin Reduces Breast Cancer Cell Proliferation by Causing G_2/M Cell Cycle Arrest.

Despite the lack of an effect of apigenin on breast cancer cell proliferation and/or viability at 24 h, 30 μM apigenin appeared to be reducing MDA-MB-468 breast cancer cell proliferation and/or causing cell death at later time points. To further investigate the effects of apigenin on breast cancer cell proliferation, [^{3}H]TdR incorporation assays were performed. Since [^{3}H]TdR is incorporated into the newly synthesized DNA of proliferating cells, an increase or decrease in [^{3}H]TdR incorporation indicates an increase or decrease in proliferation, respectively. Treatment of breast cancer cells with 30 μM apigenin caused a significant reduction in [^{3}H]TdR incorporation at 24 h and 72 h (Figure 3.4; $p < 0.05$). This data, along with the results from Annexin-V/PI staining experiments, indicate that at 24 h apigenin has cytostatic effects on MDA-MB-468 cells and prevents their proliferation. To confirm that this decrease in proliferation was caused by apigenin, breast cancer cells were stained with the fluorescent dye, Oregon Green, which binds to amine groups within the cell, and the quantity of the dye within the cells is halved by cell division, in turn halving the cells' fluorescence. After treatment with 30 μM apigenin for 24 h or 72 h, MDA-MB-468 cells were more fluorescent than those treated with the DMSO vehicle, indicating that the apigenin-treated cells had undergone fewer rounds of division than the cells treated with the DMSO vehicle (Figure 3.5). Previously, studies have shown that apigenin causes cells to arrest at the G_2/M stage of the cell cycle in various breast cancer cell lines (125,126). To confirm these findings with our cell line and doses of apigenin, cell cycle analysis with the DNA-intercalating dye PI, was performed on breast cancer cells treated with 30 μM apigenin for 24 h. The amount of fluorescence emitted by a cell indicates the quantity of DNA within that cell, and therefore the stage of the cell cycle. Cell cycle analysis determined that treatment of MDA-MB-468 cells with 30 μM apigenin significantly increased the percentage of cells in the G_2 stage of the cell cycle in comparison to cells treated with DMSO vehicle, indicating that the cells were arrested in the G_2/M stage (Figure 3.6; $p < 0.05$).

3.3 Apigenin Inhibits IFN-γ-induced PD-L1 Expression by Breast Cancer Cells.

IFN-γ induces PD-L1 surface expression in a variety of human cancer cell lines, including the MDA-MB-468 breast cancer cell line (172,237). To confirm that IFN-γ is able to upregulate PD-L1 expression and determine the dose of IFN-γ for maximal PD-L1 upregulation, MDA-MB-468 cells were incubated with 1, 10 or 100 ng/ml of IFN-γ for 24 h, and stained with Abs specific for PD-L1 or the appropriate isotype control. The amount of bound Ab was measured by flow cytometry, which determined that treatment with 10 ng/ml of IFN-γ caused optimal expression of PD-L1 by MDA-MB-468 breast cancer cells (Figure 3.7). Treatment with IFN-γ (10 ng/ml) or apigenin (10-30 μM) and IFN-γ (10 ng/ml) had no effect on MDA-MB-468 cell number at 24 h, as determined by MTT assay (Table 3.2 and 3.3). To determine whether apigenin downregulates IFN-γ-induced PD-L1 expression, MDA-MB-468 cells were treated with DMSO vehicle (0.15%), IFN-γ (10 ng/ml), apigenin (30 μM) and IFN-γ (10 ng/ml) or apigenin (30 μM) alone for 6, 12 or 24 h. PD-L1 expression was upregulated after 12 h of treatment with IFN-γ, but expression was highest after 24 h. At both 12 and 24 h, pretreatment with apigenin caused inhibition of IFN-γ-induced PD-L1 expression. Treatment of MDA-MB-468 cells with apigenin alone did not effect PD-L1 expression (Figure 3.8). Lower concentrations of apigenin (10 μM and 20 μM) did not significantly reduce IFN-γ-induced PD-L1 expression (data not shown). To ensure that the DMSO vehicle did not reduce IFN-γ-induced PD-L1 expression, MDA-MB-468 cells were treated with 0.15% DMSO vehicle 30 min prior to IFN-γ (10 ng/ml) treatment. Flow cytometric analysis determined that DMSO vehicle (0.15%) had no effect on IFN-γ-induced PD-L1 expression by MDA-MB-468 cells (Table 3.4)

Some breast cancer cells lines have constitutive PD-L1 expression while others are induced by IFN-γ to express PD-L1. MDA-MB-231 and SK-BR-3 cell lines constitutively express PD-L1 while MDA-MB-468 and MCF-7 cell lines only express PD-L1 when induced with IFN-γ (236,237,297). To compare the effects of apigenin on constitutive and IFN-γ-induced PD-L1 expression, MCF-7, SK-BR-3 and MDA-MB-231 cells were Ab stained for cell surface expression of PD-L1 (Figure 3.9). As expected, MDA-MB-468 and MCF-7 did not constitutively express PD-L1. Treatment with IFN-γ (10 ng/ml) induced PD-L1 expression significantly on MDA-MB-468 cells and

marginally on MCF-7 cells. Pre-treatment of these cells with apigenin (30 μM) completely inhibited IFN-γ-induced PD-L1 expression by both cell lines (Figure 3.8 and Figure 3.9A-C). MDA-MB-231 cells constitutively expressed PD-L1 at relatively high levels, and this constitutive expression was not significantly increased by IFN-γ treatment or decreased by apigenin treatment. Treatment of MDA-MB-231 cells with apigenin and IFN-γ led to an increase in PD-L1 expression per cell (Figure 3.9D-F). SK-BR-3 cells have low constitutive levels of PD-L1 expression that were significantly increased after treatment with IFN-γ, and reduced near to constitutive levels after treatment with apigenin and IFN-γ (Figure 3.9G-I). This data indicates that apigenin decreased IFN-γ-induced PD-L1 expression by breast cancer cells, but did not reduce constitutive expression of PD-L1 by breast cancer cells.

3.4 Combined Apigenin and IFN-γ Treatment Does Not Reduce IFN-γR Expression by Breast Cancer Cells.

The effect of IFN-γ and apigenin on IFN-γR surface expression was examined as a possible mechanism for the inhibition of IFN-γ-induced PD-L1 expression in the presence of apigenin. Flow cytometric analysis verified the constitutive expression of the IFN-γR on MDA-MB-468 cells and demonstrated that apigenin or IFN-γ treatment alone had minimal effect on IFN-γR expression (Figure 3.10B,D-F). When combined, apigenin and IFN-γ treatment did not significantly reduce IFN-γR expression in comparison to IFN-γ-treated MDA-MB-468 cells (Figure 3.10C,E,F). Therefore, reduction in IFN-γR on MDA-MB-468 cells is not a contributing factor to the decrease in PD-L1 expression seen on these cells after apigenin and IFN-γ treatment.

3.5 Apigenin Inhibits IFN-β-induced PD-L1 Expression by Breast Cancer Cells.

Schreiner et al. (2004) have shown that the pro-inflammatory cytokine IFN-β induces PD-L1 expression on monocytes and dendritic cells. To determine if IFN-β is capable of inducing PD-L1 expression by breast cancer cells, MDA-MB-468 cells were treated with DMSO vehicle (0.15%), IFN-β (20 ng/ml), apigenin (30 μM) and IFN-β (10 ng/ml) or apigenin (30 μM) alone for 24 h and stained for PD-L1 expression. Similar to IFN-γ, treatment of MDA-MB-468 cells with 20 ng/ml IFN-β caused a significant

increase in PD-L1 expression (Fig. 3.11 $p < 0.05$). MDA-MB-468 cells pre-treated with apigenin (30 μM) and subsequently treated with IFN-β (20 ng/ml) for 24 h showed no surface expression of PD-L1 by Ab staining (Figure 3.11). A dose response was performed to determine the concentration of IFN-β that induced optimal PD-L1 expression by MDA-MB-468 cells (data not shown). This data indicates that apigenin inhibited PD-L1 expression that is induced by IFN-β, as well as by IFN-γ.

3.6 The Pro-inflammatory Cytokines IL-1β, IL-6, IL-17A and IL-17F Do Not Induce PD-L1 Expression by Breast Cancer Cells.

The ability of the pro-inflammatory cytokines IFN-γ and IFN-β to induce PD-L1 expression suggested that other pro-inflammatory cytokines might also induce PD-L1 expression. After treatment with IL-1β (1, 10 and 100 ng/ml), IL-6 (20, 50, 100 ng/ml), IL-17A (10, 100 ng/ml) or IL-17F (10, 100 ng/ml) for 24 h, MDA-MB-468 cells were Ab stained for PD-L1 and their fluorescence was measured by flow cytometry. As shown in Figures 3.12, 3.13 and 3.14, neither the MFI nor the percentage of cells expressing PD-L1 increased significantly after treatment with IL-1β, IL-6, IL-17A or IL-17F. Although IL-6 did not upregulate PD-L1 expression by MDA-MB-468 cells, IL-6 did increase phospho-STAT3 in these cells, confirming that they express the IL-6 receptor and that the cytokine was active and able to induce signaling downstream of the IL-6 receptor (Figure 3.13C-D). These results demonstrate that PD-L1 expression cannot be upregulated by all pro-inflammatory cytokines.

3.7 The Apigenin Metabolite Luteolin Inhibits IFN-γ-induced PD-L1 Expression by Breast Cancer Cells.

The bioavailability of phytochemicals can be a hurdle in their usefulness as chemo-preventative and therapeutic agents. Since most phytochemicals will undergo phase I and phase II metabolism, it is important to know the effects that the metabolites of phytochemicals will have on the body and the disease being examined (298). To determine if metabolites of apigenin would have similar effects on PD-L1 expression compared to apigenin itself, the effect of luteolin, the primary metabolite of apigenin (112), on MDA-MB-468 cells was examined. MTT assays showed that luteolin had more

of an effect on the succinate dehydrogenase activity of MDA-MB-468 cells than apigenin. After a 24 h treatment 30 μM luteolin caused a significant decrease in MDA-MB-468 cell succinate dehydrogenase activity, which is likely due to a decrease in cell number. After treatment for 72 h, 10 μM luteolin significantly decreased MDA-MB-468 cell succinate dehydrogenase activity, which suggests a decrease in cell number (Figure 3.15A; $p < 0.05$). However, luteolin causes a reduction of MTT in the absence of live cells (295), so the effect seen in Figure 3.15A may under-represent the effect of the luteolin on MDA-MB-468 cell growth. To determine the effect of luteolin on PD-L1 expression, MDA-MB-468 cells were treated with DMSO vehicle (0.15%), IFN-γ (10 ng/ml), luteolin (30 μM) and IFN-γ (10 ng/ml) or luteolin (30 μM) alone and then Ab stained for PD-L1 expression. When the flow cytometer was gated on live cells, similar to apigenin, treatment with luteolin (30 μM) 30 min prior to IFN-γ treatment resulted in decreased IFN-γ-induced PD-L1 expression by MDA-MB-468 breast cancer cells (Figure 3.15B-D). Lower concentrations of luteolin (10 μM and 20 μM) did not significantly reduce IFN-γ-induced PD-L1 expression by MDA-MB-468 cells (data not shown).

3.8 Apigenin Did Not Reduce HMEC Cell Number, But Did Reduce IFN-γ-induced PD-L1 Expression by HMECs.

A previous report indicates that the cytostatic and cytotoxic effects of apigenin are limited to prostate carcinoma cell lines and there is no effect on normal cell lines (107). MTT assays were performed to determine if apigenin reduces the cell number of HMECs. Apigenin at concentrations as high as 50 μM did not reduce HMEC number; rather, concentrations of 30 and 50 μM apigenin caused a significant increase in succinate dehydrogenase activity, which is likely due to an increase in cell number (Figure 3.16A). Flow cytometric data showed that HMECs had very low levels of constitutive PD-L1 expression, but that these levels were significantly increased after treatment with IFN-γ (10 ng/ml). Similar to IFN-γ-induced PD-L1 expression by breast cancer cells, apigenin (30 μM) was able to reduce IFN-γ-induced PD-L1 expression on HMECs (Figure 3.16C). These data confirm the low toxicity of apigenin to normal cells, and demonstrates that apigenin downregulates IFN-γ-induced PD-L1 expression on both normal mammary epithelial cells and breast cancer cells.

3.9 Apigenin Decreased IFN-γ-mediated STAT1 Activation.

Engagement of the IFN-γR is commonly associated with activation of the transcription factor STAT1 (242). STAT1 has two phosphorylation sites, Tyr701 and Ser727 that are involved in its transcriptional activity (299). Previously, apigenin had been shown to inhibit IFN-γ-induced phosphorylation of STAT1 at both Tyr701 and Ser727 in N9 mouse microglial cells, although in primary microglial cells apigenin only inhibited phosphorylation of STAT1 at Tyr701 (124). To determine if apigenin inhibited IFN-γ-induced STAT1 activation in MDA-MB-468 cells, western blots were used to visualize the levels of phosphorylated STAT1 protein. This experiment confirmed that IFN-γ (10 ng/ml) induced phosphorylation of STAT1 at both Tyr701 and Ser727. IFN-γ-induced phosphorylation of STAT1 at Tyr701 occurred at an earlier time point (1 min) than IFN-γ-induced phosphorylation of STAT1 at Ser727 (30 min; Figure 3.17A and 3.18C, respectively). MDA-MB-468 cells did not show constitutive phosphorylation of STAT1 at Tyr701, but did show low levels of constitutive STAT1 phosphorylation at Ser727 that was increased by IFN-γ (10 ng/ml) treatment. This demonstrates that phosphorylation at these two locations is differentially regulated. Treatment of MDA-MB-468 cells with 30 μM apigenin prior to IFN-γ (10 ng/ml) treatment led to a significant reduction in STAT1 phosphorylation at Tyr701 at 1 min (Figure 3.17A). At 30 min, a slight decrease was seen in STAT1 phosphorylation at Ser727 after pretreatment with apigenin, but this decrease did not reach statistical significance (Figure 3.18C). This data suggests that inhibition of STAT1 activation may be involved in apigenin-mediated downregulation of IFN-γ-induced PD-L1.

3.10 Apigenin-mediated Reduction of IFN-γ-induced PD-L1 Expression by Breast Cancer Cells Leads to an Increase in Jurkat T cell Proliferation in the Presence of Breast Cancer Cells.

Activated T cells express PD-1 (the receptor for PD-L1) as a means of immune regulation, thereby preventing autoimmunity and preserving immune tolerance (228). IFN-γ-induced PD-L1 expressed on breast cancer cells binds to PD-1 on activated T cells and causes them to undergo apoptosis or enter into anergy. To determine whether the downregulation of IFN-γ-induced PD-L1 expression by apigenin affected the functional

activity of PD-L1, the proliferation of Oregon Green-labeled Jurkat T cells co-cultured with MDA-MB-468 breast cancer cells that had been previously treated for 24 h with DMSO vehicle (0.15%), IFN-γ (10 ng/ml), apigenin (30 μM) and IFN-γ (10 ng/ml), or apigenin (30 μM) alone was measured. In contrast with previous literature (300,301), our flow cytometric analysis determined that unstimulated Jurkat T cells constitutively expressed PD-1 (Figure 3.19). After being co-cultured with IFN-γ-treated MDA-MB-468 cells for 48 and 72 h, Jurkat cell proliferation was reduced, suggesting that IFN-γ-induced PD-L1 on the breast cancer cells interacted with PD-1 on the Jurkat cells and caused them to undergo apoptosis or enter into anergy. In contrast, this reduction in proliferation was eliminated when Jurkat cells were cultured with MDA-MB-468 cells treated with IFN-γ in the presence of apigenin (Figure 3.20). As seen in Figure 3.8, treatment of MDA-MB-468 cells with apigenin prior to IFN-γ treatment inhibited their expression of PD-L1. The data from Figure 3.8 in combination with the results of Figure 3.20 suggest that when MDA-MB-468 cells are unable to express PD-L1 they are also unable to inhibit Jurkat cell proliferation. This result may be due to reduced binding of PD-L1 on the breast cancer cells to PD-1 on the Jurkat cells.

To exclude the possibility that low levels of apigenin or IFN-γ may leech out of the MDA-MB-468 cells and have effects on Jurkat cell number, MTT assays were performed. Jurkat cells were treated with low levels of apigenin (3 μM) and IFN-γ (1 ng/ml). This experiment showed that treatment of MDA-MB-468 cells with low concentrations of apigenin and IFN-γ for 72 h did not affect the Jurkat cell number (Table 3.5).

Table 3.1. Apigenin Does Not Reduce MTT in the Absence of Cells.

	Vehicle	**A 10 µM**	**A 20 µM**	**A 30 µM**	**A 50 µM**
Average OD (570 nm)	0.21 ± 0.05	0.21 ± 0.02	0.19 ± 0.02	0.21 ± 0.02	0.23 ± 0.03

Apigenin (A) at indicated concentrations was incubated with MTT solution for 2 h. Data shown are the average optical density (OD) readings of triplicate wells ± standard deviation (SD).

Table 3.2. IFN-γ Does Not Reduce MDA-MB-468 Cell Number.

	Vehicle	**IFN-γ (10 ng/ml)**
% Succinate Dehydrogenase Activity	113.37 ± 20.49	98.88 ± 17.03

MDA-MB-468 cells were incubated with10 ng/ml IFN-γ for 24 h. The colorimetric MTT assay was used as a measure of cell number, and MTT solution was added to the cells for the last 2 h of incubation. Percent succinate dehydrogenase activity is relative to the medium control. Data shown are the average percent succinate dehydrogenase activity of 3 independent experiments ± standard error of the mean (SEM).

Table 3.3. Treatment with Apigenin and IFN-γ Does Not Reduce MDA-MB-468 Cell Number.

	Vehicle	A 10 μM + I	A 20 μM + I	A 30 μM + I
% Succinate Dehydrogenase Activity	95.85 ± 20.89	103.8 ± 10.27	103.4 ± 21.7	102.1 ± 39.06

MDA-MB-468 cells were incubated with apigenin at indicated concentrations and 10 ng/ml IFN-γ for 24 h. The colorimetric MTT assay was used as a measure of cell number, and MTT solution was added to the cells for the last 2 h of incubation. Percent succinate dehydrogenase activity is relative to the medium control. Data shown are the average percent succinate dehydrogenase activity of 3 independent experiments ± SEM.

Table 3.4. DMSO Vehicle Does Not Affect IFN-γ-induced PD-L1 Expression.

	Vehicle	IFN-γ	IFN-γ + Vehicle
PD-L1 Expression (MFI)	6.32	24.14	22.95
% Cells Expressing PD-L1	7.16	86.38	86.87

Data shown are MFI (n=1) and percentage of cells expressing PD-L1 (n=1).

Table 3.5. At Very Low Concentrations Apigenin and IFN-γ Do Not Reduce Jurkat Cell Number.

	Vehicle	A 3 µM	A 30 µM	I 1 ng/ml	I 10 ng/ml
% Succinate Dehydrogenase Activity	96.03 ± 5.94	99.68 ± 9.83	9.89 ± 0.34	112.47 ± 6.42	101.29 ± 4.22

Jurkat cells were incubated with the indicated concentrations of apigenin (A) or IFN-γ (I) for 72 h. The colorimetric MTT assay was used as a measure of cell number, and MTT solution was added to the cells for the last 2 h of incubation. Percent succinate dehydrogenase activity is relative to the medium control. Data shown are the average percent succinate dehydrogenase activity of triplicate wells ± SD.

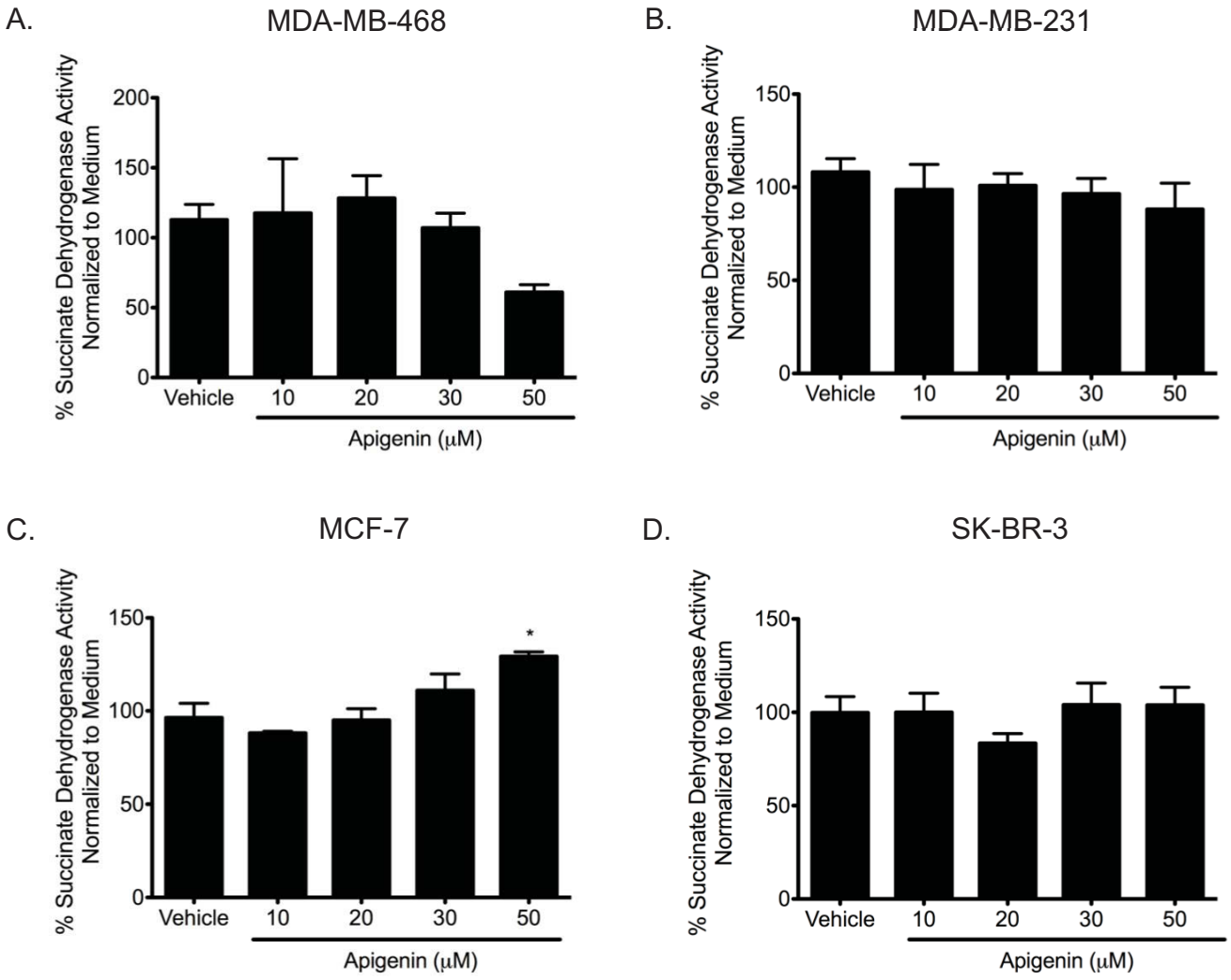


Figure 3.1. Low Dose Apigenin Does Not Reduce Breast Cancer Cell Number. **(A-D)** The human breast cancer cell lines **(A)** MDA-MB-468, **(B)** MDA-MB-231, **(C)** MCF-7, and **(D**) SK-BR-3 were incubated with the indicated concentrations of apigenin for 24 h. The colorimetric MTT assay was used as a measure of cell number, and MTT solution was added to the cells for the last 2 h of incubation. Percent succinate dehydrogenase activity is relative to the medium control. Data shown are the average percent succinate dehydrogenase activity of 3 independent experiments ± SEM; * $p < 0.05$ compared to DMSO vehicle control as determined by ANOVA with Tukey-Kramer post-test.

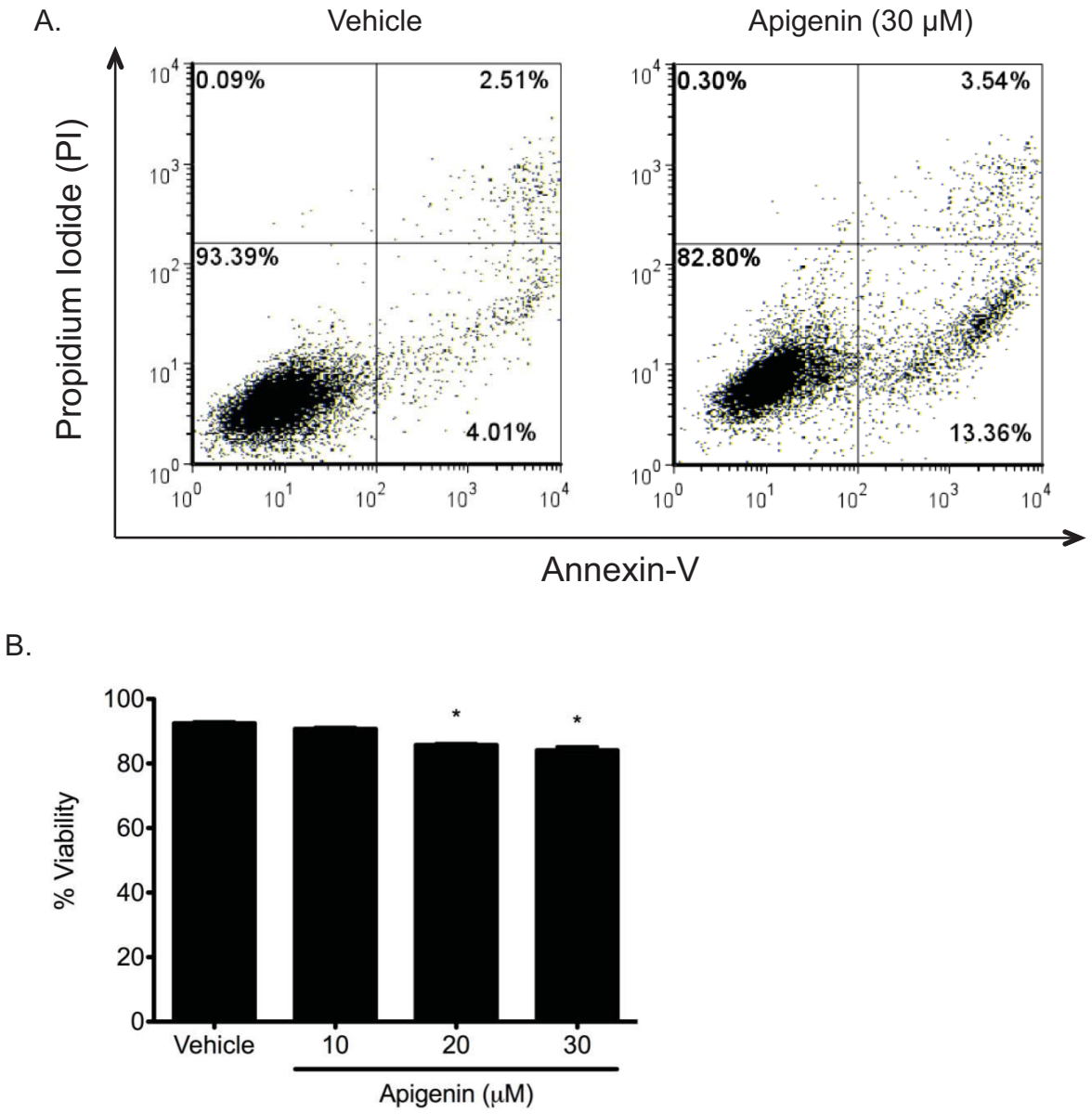


Figure 3.2. Low Dose Apigenin Causes Minimal Apoptosis/Necrosis in Breast Cancer Cell Cultures. MDA-MB-468 breast cancer cells were incubated with the indicated concentrations of apigenin for 24 h. Annexin-V/PI staining was performed, and the percent viability was based on the percentage of cells that stained negative for both Annexin-V and PI. Data shown are **(A)** flow cytometry histograms of vehicle and 30 μM apigenin samples from one representative experiment, and (**B)** the average percent viability of 3 independent experiments ± SEM; * $p < 0.05$ compared to DMSO vehicle control as determined by ANOVA with Tukey-Kramer post-test.

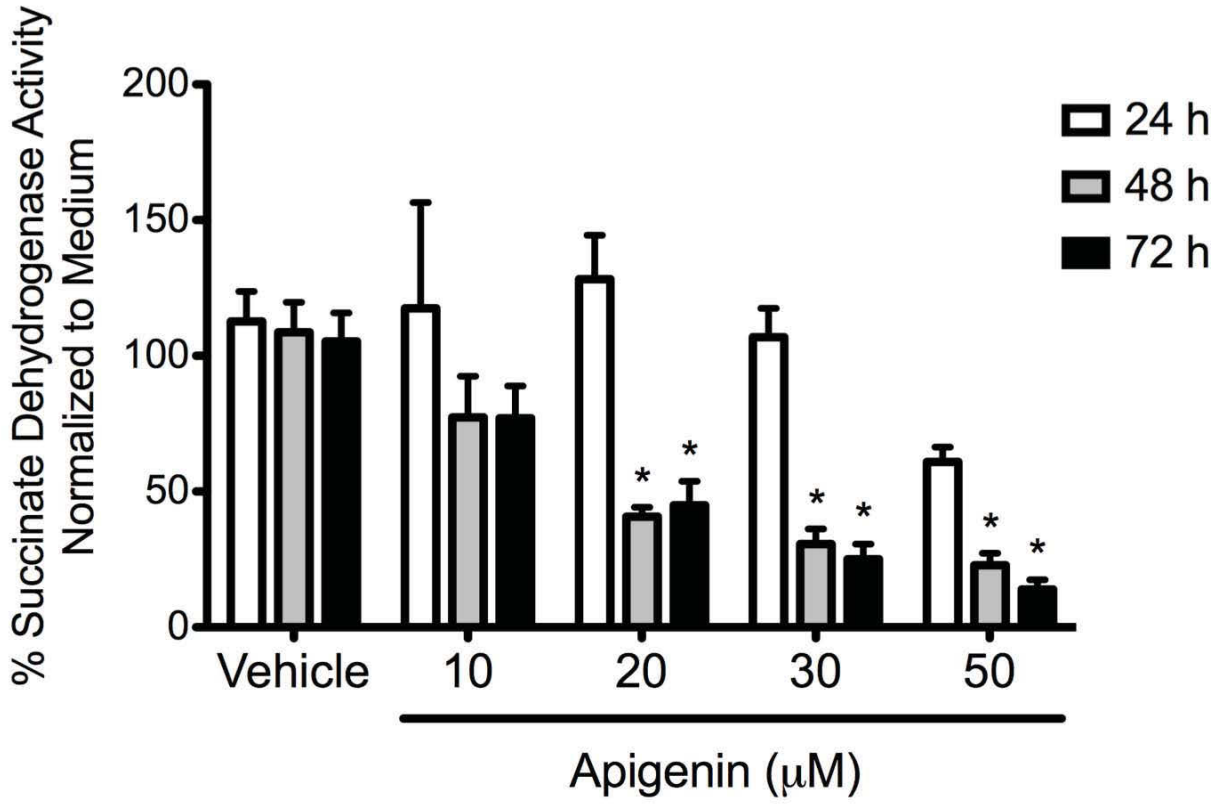


Figure 3.3. Low Dose Apigenin Reduces Breast Cancer Cell Number at Later Time-Points. MDA-MB-468 cells were incubated with the indicated concentrations of apigenin for 24 h, 48 h and 72 h. The colorimetric MTT assay was used as a measure of cell number, and MTT solution was added to the cells for the last 2 h of incubation. Percent succinate dehydrogenase activity is relative to the medium control. Data shown are the average percent succinate dehydrogenase activity of 3 independent experiments ± SEM; * $p < 0.05$ compared to the respective DMSO vehicle control as determined by ANOVA with Tukey-Kramer post-test.

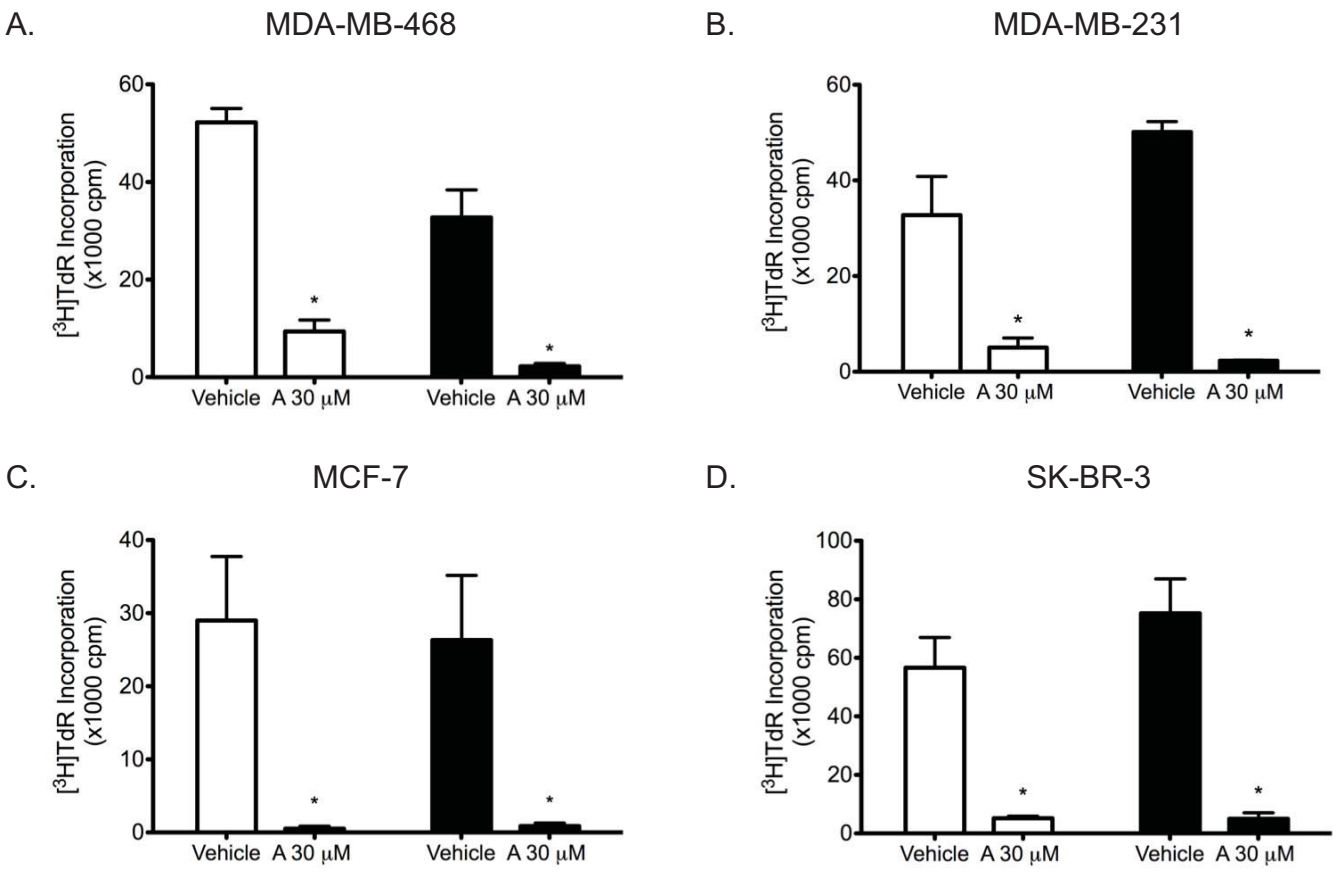


Figure 3.4. Apigenin Inhibits the Proliferation of Breast Cancer Cells. (A-D) Breast cancer cells were incubated with DMSO vehicle control or 30 μM apigenin (A) for 24 h (white bars) and 72 h (black bars), pulsed with [^{3}H]TdR for the last 6 h **(A-C)** or 18 h **(D)** of incubation and frozen and thawed prior to harvesting. Data shown are the mean cpm ± SEM of 3 independent experiments; * $p < 0.05$ compared to DMSO vehicle as determined by ANOVA with Tukey-Kramer post-test.

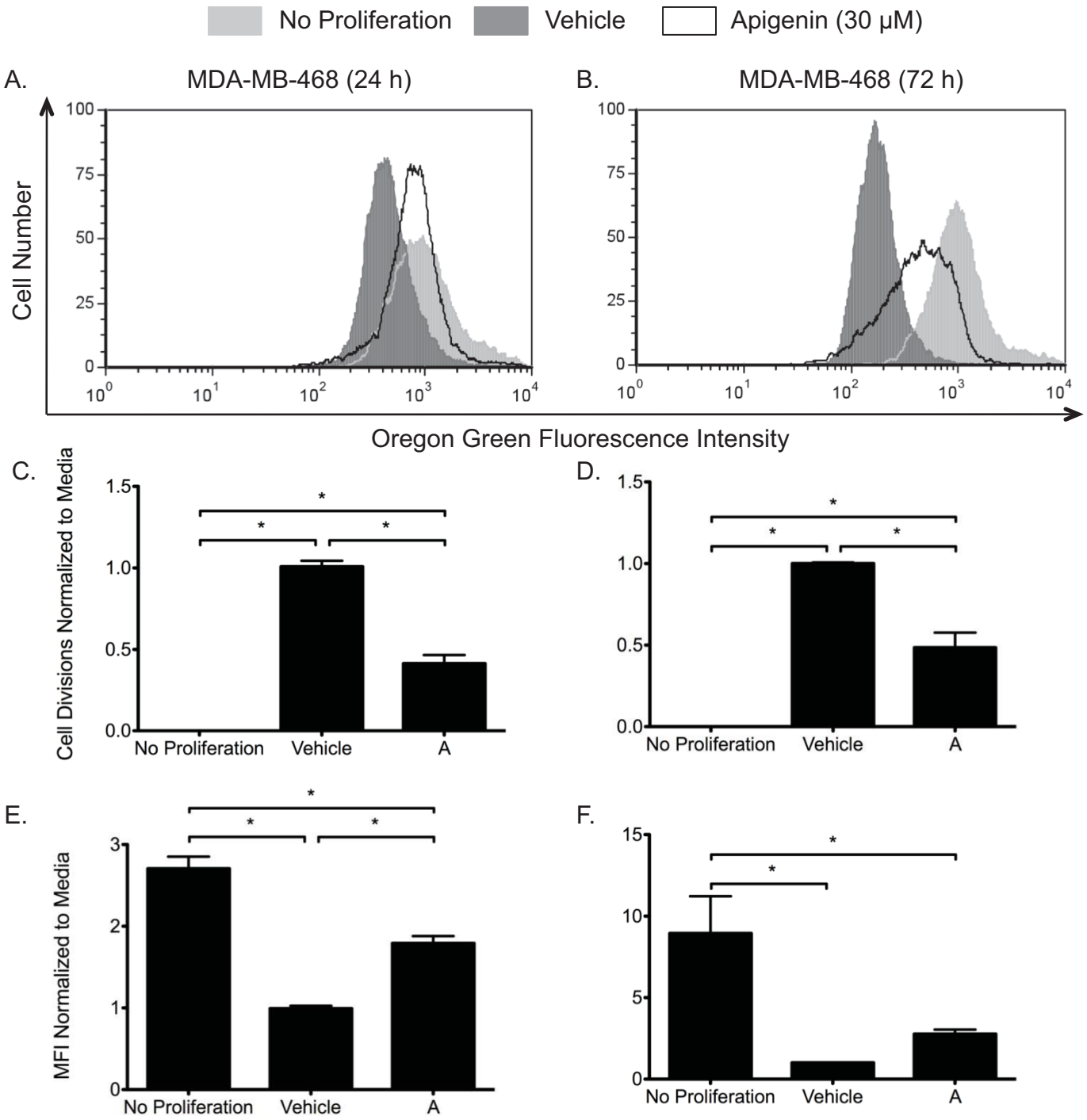


Figure 3.5. Apigenin Inhibits the Proliferation of Breast Cancer Cells. MDA-MB-468 breast cancer cells were stained with the fluorescent dye, Oregon Green. Cells were then harvested and fixed to act as a non-proliferative control or cultured in the absence or presence of 30 μM apigenin (A). After 24 h **(A,C,E)** and 72 h **(B,D,F)** cells were harvested, fixed and the level of fluorescence was determined by flow cytometry. Data shown are (**A & B)** from one representative experiment (n=3-4), **(C & D)** the average number of cell divisions normalized to medium control ± SEM from 3-4 independent experiment, (E & F) MFI normalized to medium control ± SEM from 3-4 independent experiment; * $p < 0.05$ as determined with ANOVA with Tukey-Kramer post-test.

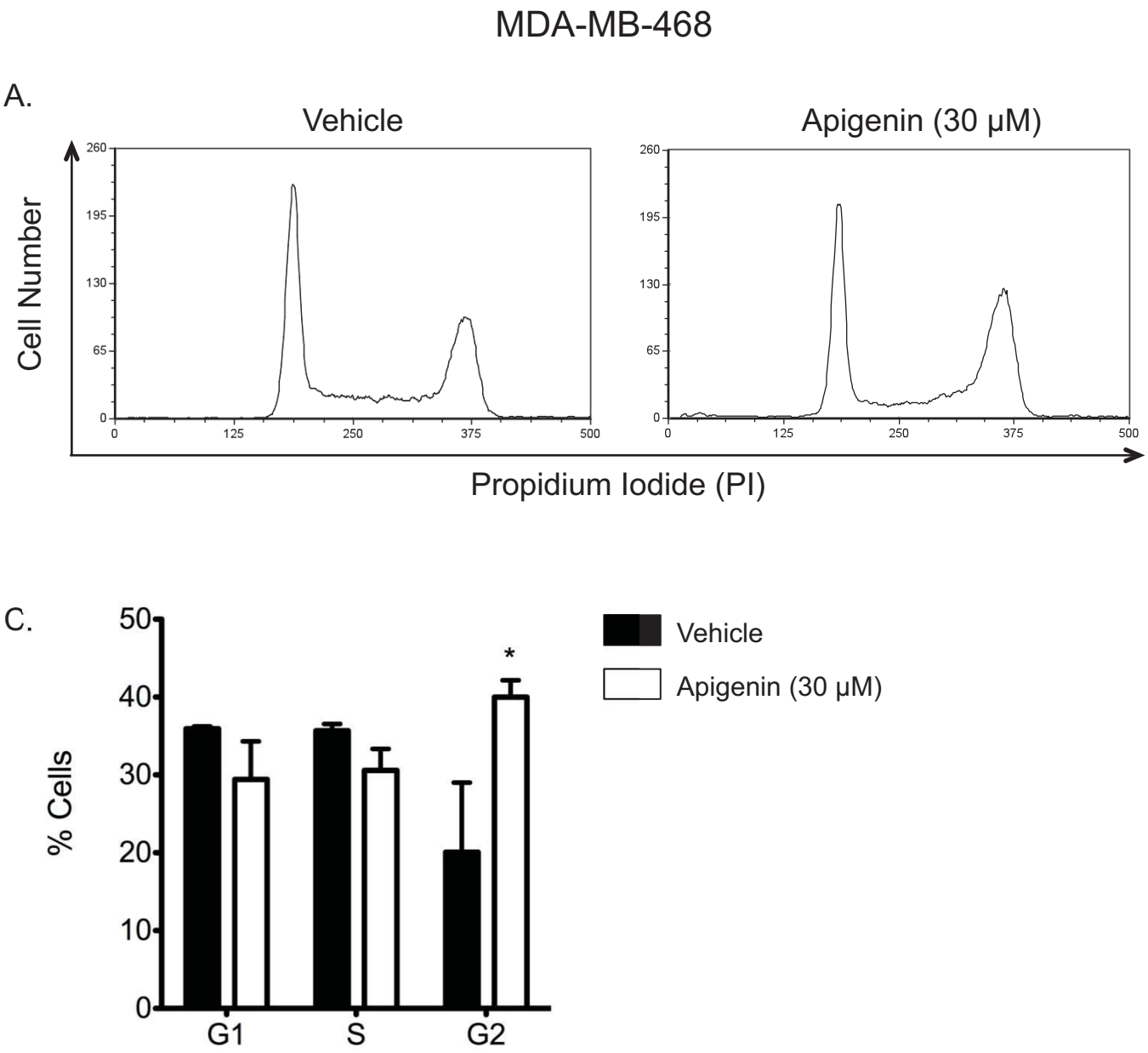


Figure 3.6. Apigenin Inhibits Breast Cancer Cell Entry Into G_2/M Phase of the Cell Cycle. MDA-MB-468 breast cancer cells were treated with 30 μM apigenin and harvested at 24 h. Following fixation in 70% ethanol for a minimum of 24 h, cells were stained with PI for 30 min prior to analysis by flow cytometry. The amount of PI fluorescence is proportional to the amount of DNA within a cell, indicating the phase of the cell cycle. Data shown are **(A)** flow cytometry histograms from one representative experiment and **(B)** the average percentage of cells in each phase of the cell cycle from 3 independent experiments ± SEM; * $p < 0.05$ compared to DMSO vehicle control as determined by ANOVA with Tukey-Kramer post-test.

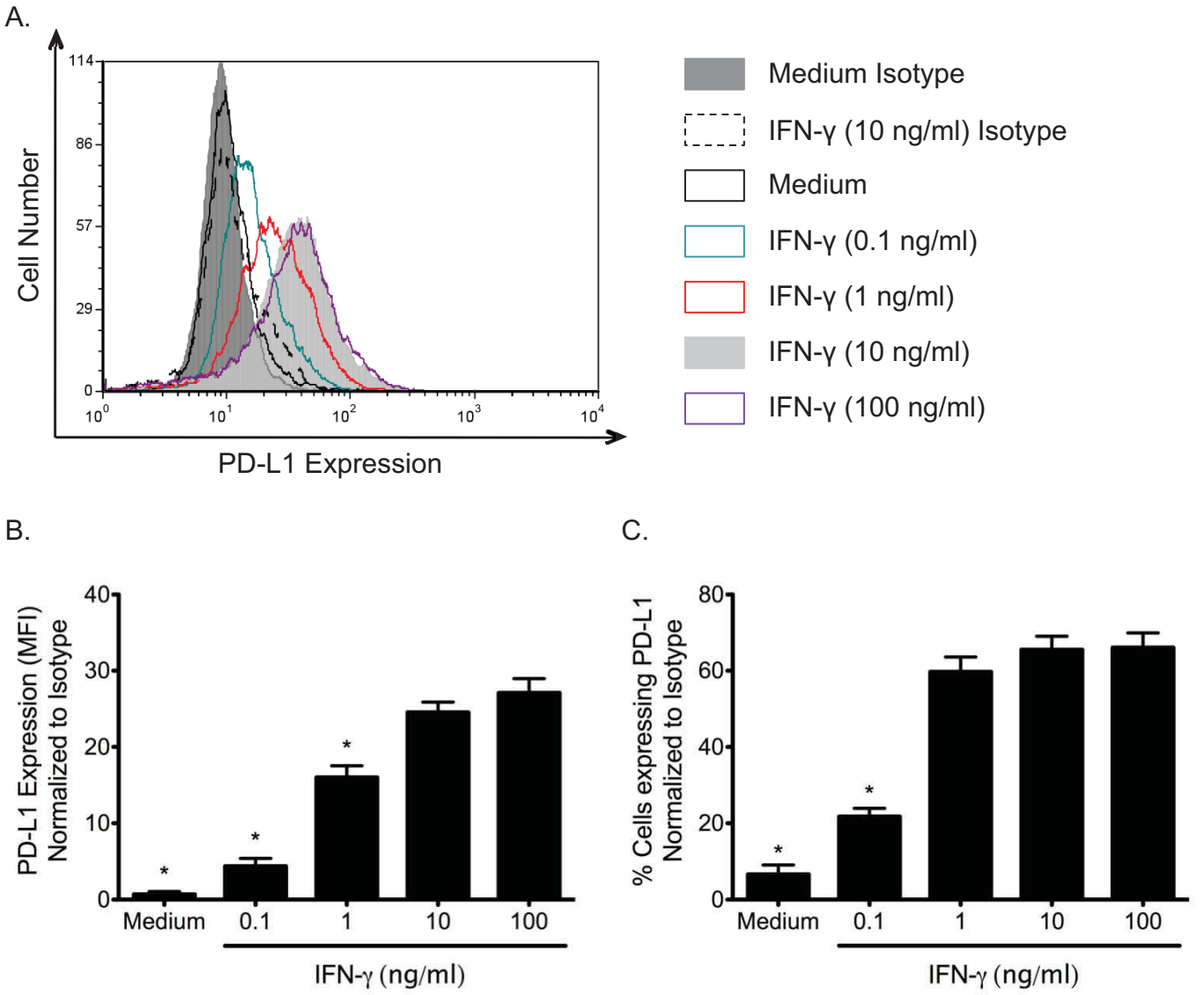


Figure 3.7. IFN-γ, At a Concentration of 10 ng/ml, Induces Optimal PD-L1 expression. (A-C) MDA-MB-468 human breast cancer cells were treated with the indicated concentrations of IFN-γ. After 24 h treatment, cells were harvested and stained with PE-anti-human PD-L1 or isotype control Abs. **(A)** Data shown are a flow cytometry histogram from one representative experiment, **(B)** the average MFI from 3 independent experiments ± SEM, and **(C)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM. All flow cytometry values have been normalized by subtracting the respective isotype control value; * $p < 0.05$ compared to 10 ng/ml IFN-γ treatment, as determined by ANOVA with Tukey-Kramer post-test.

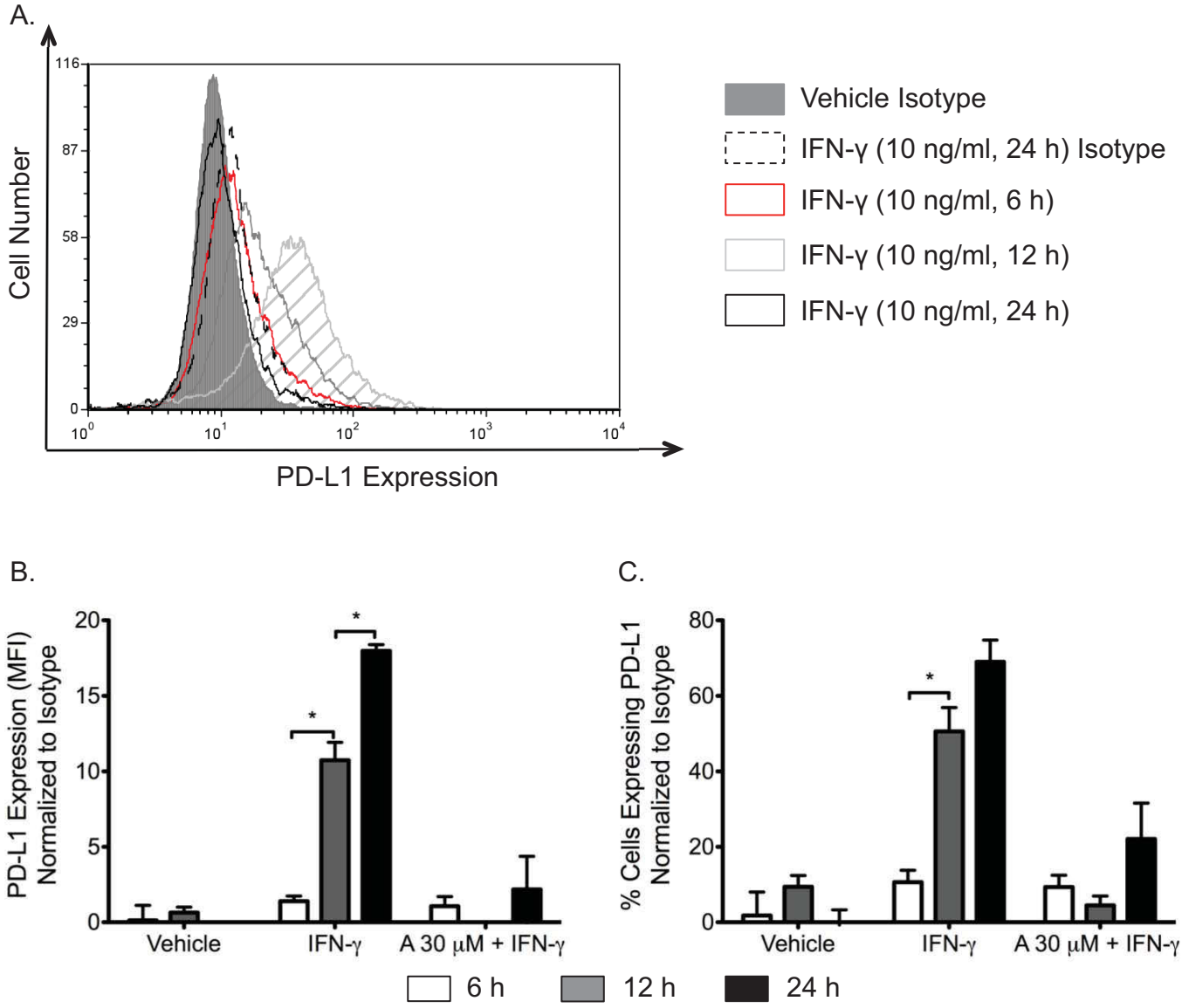


Figure 3.8. PD-L1 Expression is Induced by IFN-γ. MDA-MB-468 cells were incubated in the presence or absence of 30 μM apigenin (A) for 30 min, and then treated with 10 ng/ml IFN-γ for 6 (white), 12 (grey), or 24 h (black). After treatment cells were harvested and stained with PE-anti-human PD-L1 or isotype control Abs. PD-L1 expression was measured by flow cytometry. Data shown are **(A)** a flow cytometry histogram from one representative experiment, **(B)** the average MFI from 3 independent experiments ± SEM, and **(C)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM. Apigenin alone did not effect PD-L1 expression by MDA-MB-468 cells. All flow cytometry values have been normalized by subtracting the respective isotype control value; * $p < 0.05$ as determined by ANOVA with Tukey-Kramer post-test.

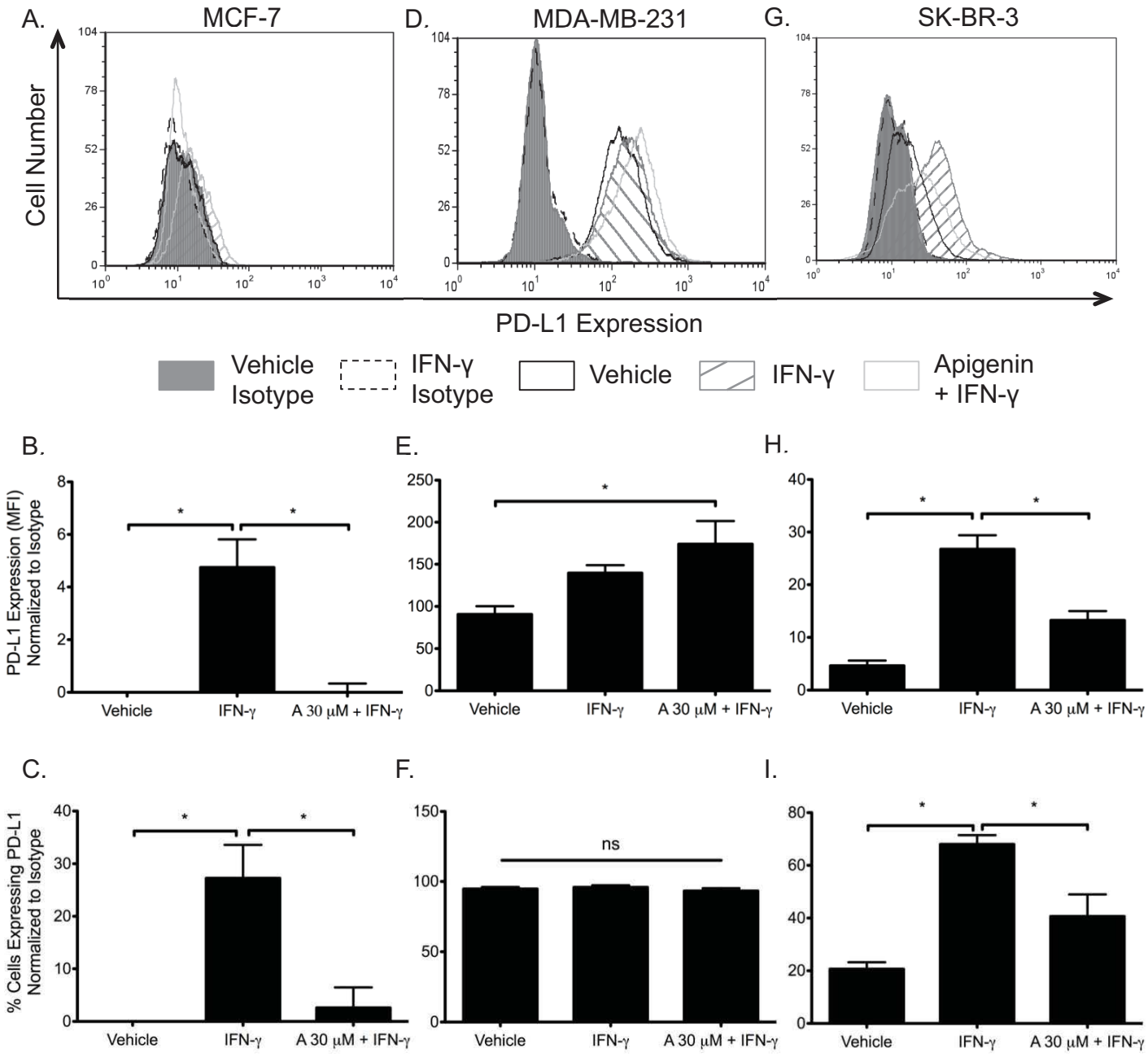


Figure 3.9. Apigenin Downregulates IFN-γ-induced PD-L1 Expression, But Not Constitutive PD-L1 Expression by Breast Cancer Cells. Breast cancer cells were incubated in the presence or absence of 30 μM apigenin (A) for 30 min, and then treated with 10 ng/ml human IFN-γ. After a 24 h treatment, cells were harvested and stained with PE-anti-human PD-L1 or isotype control Abs. PD-L1 expression was measured by flow cytometry. Data shown are **(A, D & G)** flow cytometry histograms from one representative experiment, **(B, E & H)** average MFI from 3 independent experiments ± SEM, and **(C, F & I)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM for (**A-C)** MCF-7, **(D-F)** MDA-MB-231 and **(G-I)** SK-BR-3 breast cancer cells. All flow cytometry values have been normalized by subtracting the respective isotype control value; * $p < 0.05$ as determined by ANOVA with Tukey-Kramer post-test.

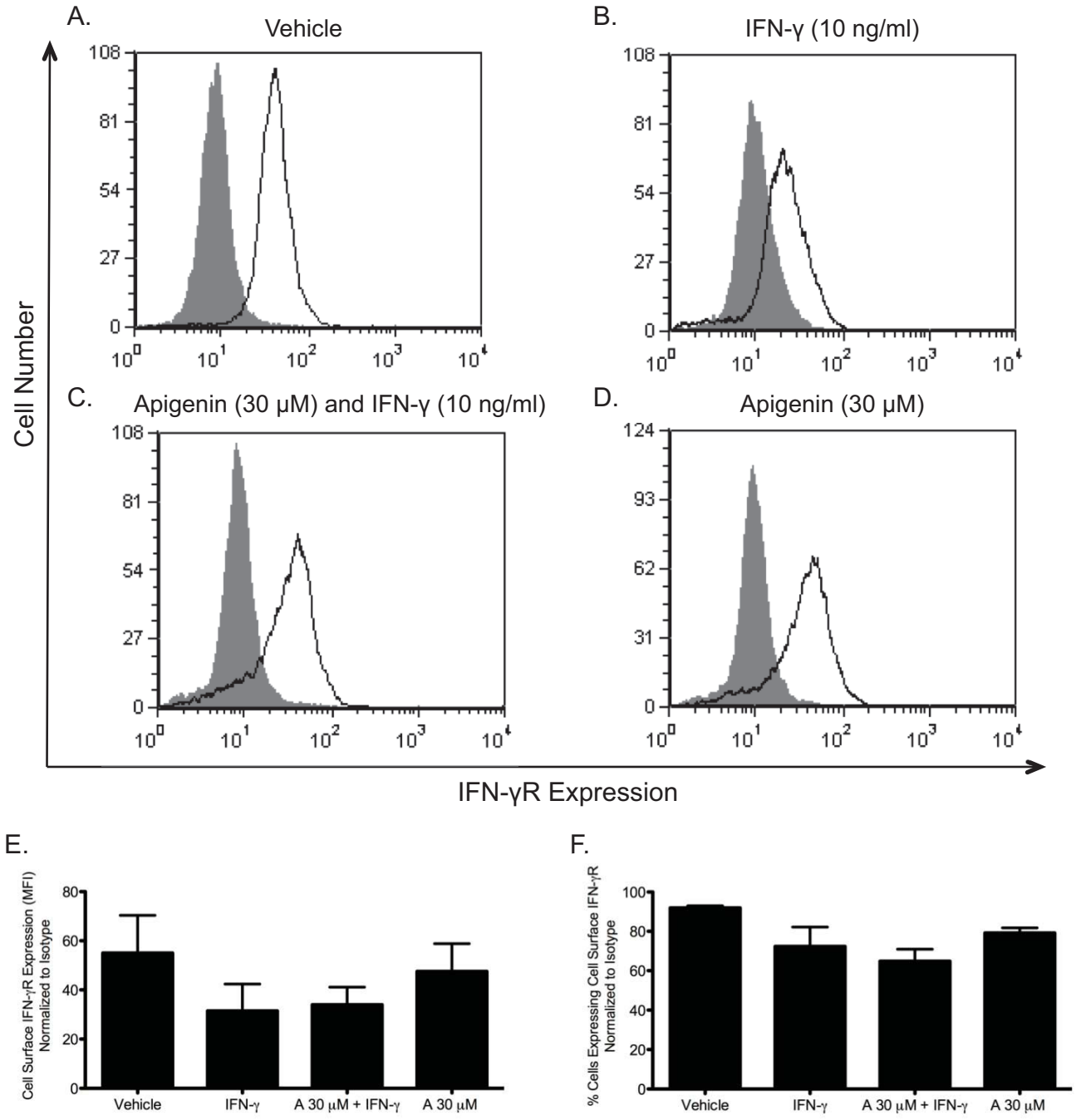


Figure 3.10. Apigenin Does Not Substantially Affect IFN-γ Receptor (R) Expression by Breast Cancer Cells. MDA-MB-468 cells were incubated in the presence or absence of 30 μM apigenin (A) for 30 min, and then treated with 10 ng/ml IFN-γ. After a 24 h treatment, cells were harvested and stained with PE-anti-human IFN-γR or isotype control Abs. IFN-γR expression was measured by flow cytometry. Data shown are **(A-D)** flow cytometry histograms of each treatment from one representative experiment, **(E)** average MFI from 3 independent experiments ± SEM, and **(F)** the average percentage of cells expressing IFN-γR from 3 independent experiments ± SEM. All flow cytometry values have been normalized by subtracting the respective isotype control value.

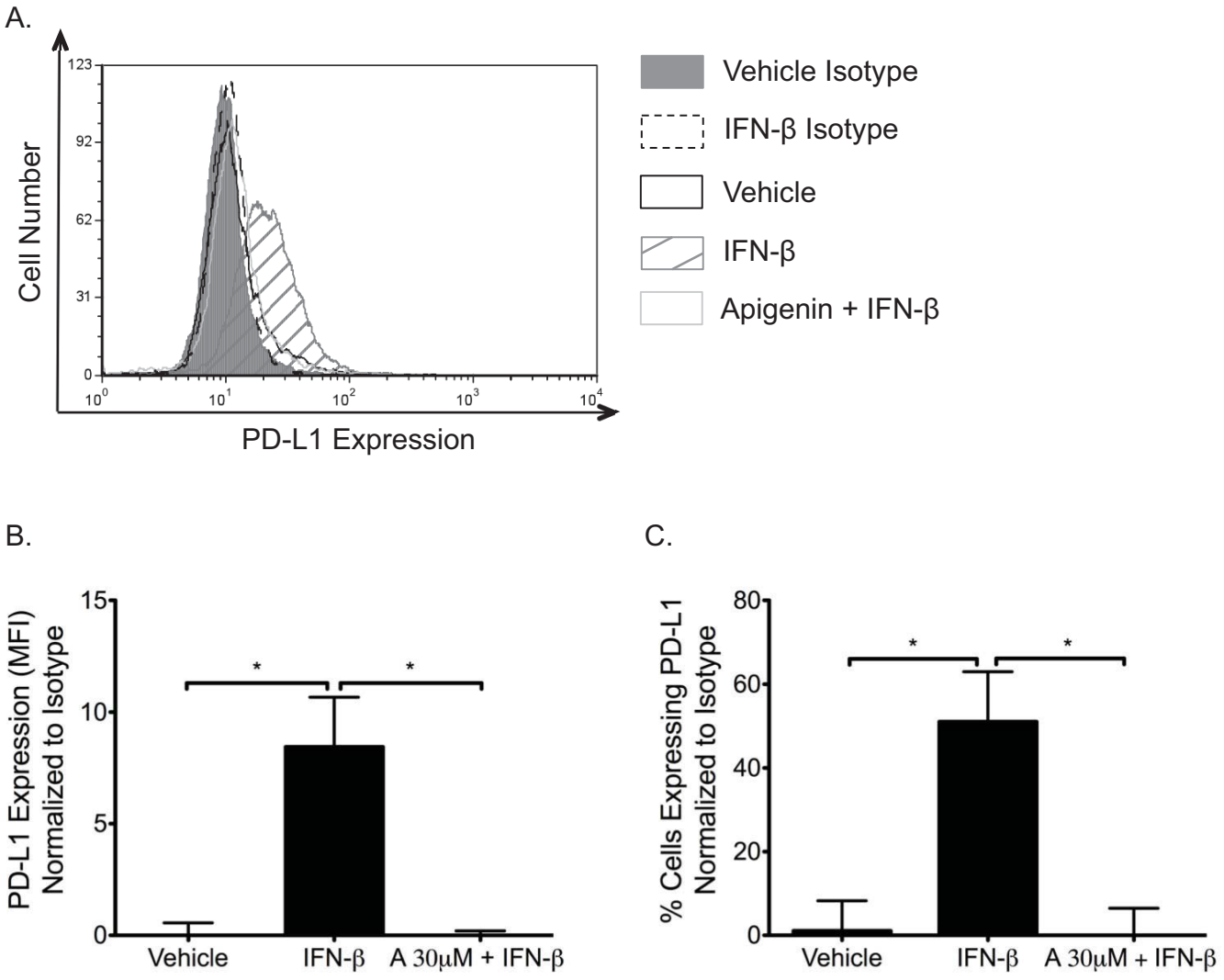


Figure 3.11. Apigenin Downregulates IFN-β-induced PD-L1 Expression by Breast Cancer Cells. MDA-MB-468 cells were incubated in the presence or absence of 30 μM apigenin (A) for 30 min and then treated with 20 ng/ml IFN-β. After a 24 h treatment, cells were harvested and stained with PE-anti-human PD-L1 or isotype control Abs. PD-L1 expression was measured by flow cytometry. Data shown are **(A)** a flow cytometry histogram from one representative experiment, **(B)** average MFI from 3 independent experiments ± SEM and **(C)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM. Apigenin alone did not effect PD-L1 expression by MDA-MB-468 cells. All flow cytometry values have been normalized by subtracting the respective isotype control value; * $p < 0.05$ as determined by ANOVA with Tukey-Kramer post-test.

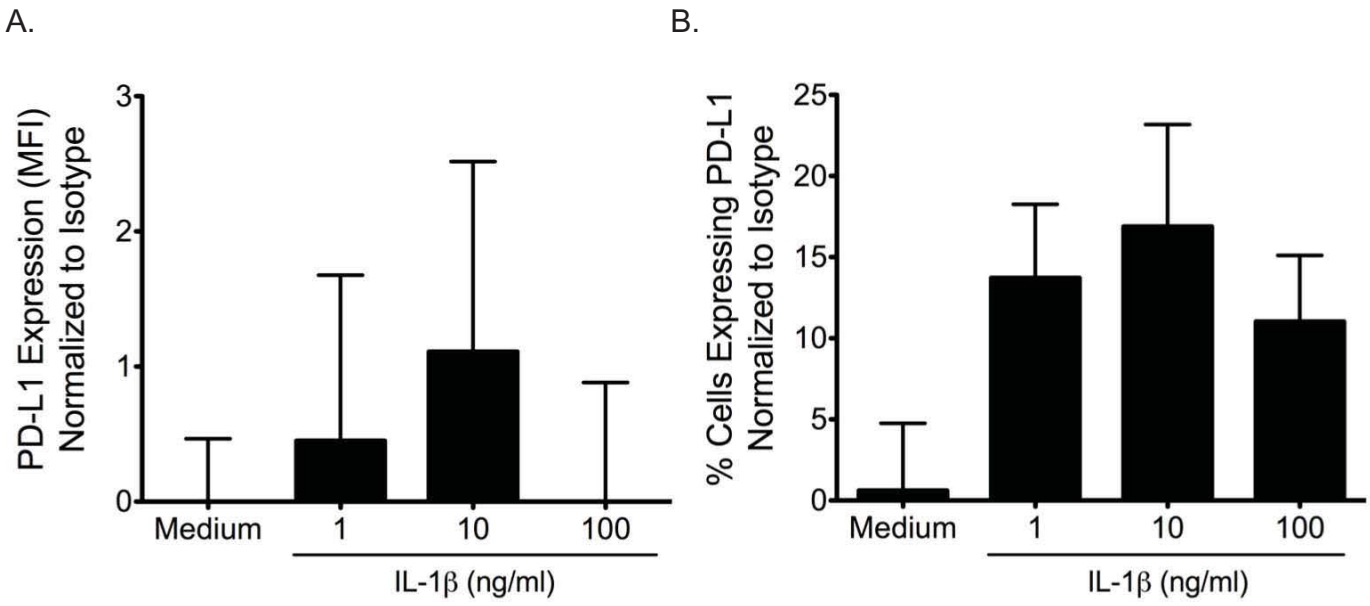


Figure 3.12. IL-1β Does Not Upregulate PD-L1 Expression by Breast Cancer Cells. MDA-MB-468 cells were incubated in the presence or absence of 1, 10 or 100 ng/ml of IL-1β. After 24 h, cells were harvested and stained with PE-anti-human PD-L1 or isotype control Abs. PD-L1 expression was measured by flow cytometry. Data shown are **(A)** the average MFI from 3 independent experiments ± SEM and **(B)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM. All flow cytometry values have been normalized by subtracting the respective isotype control value.

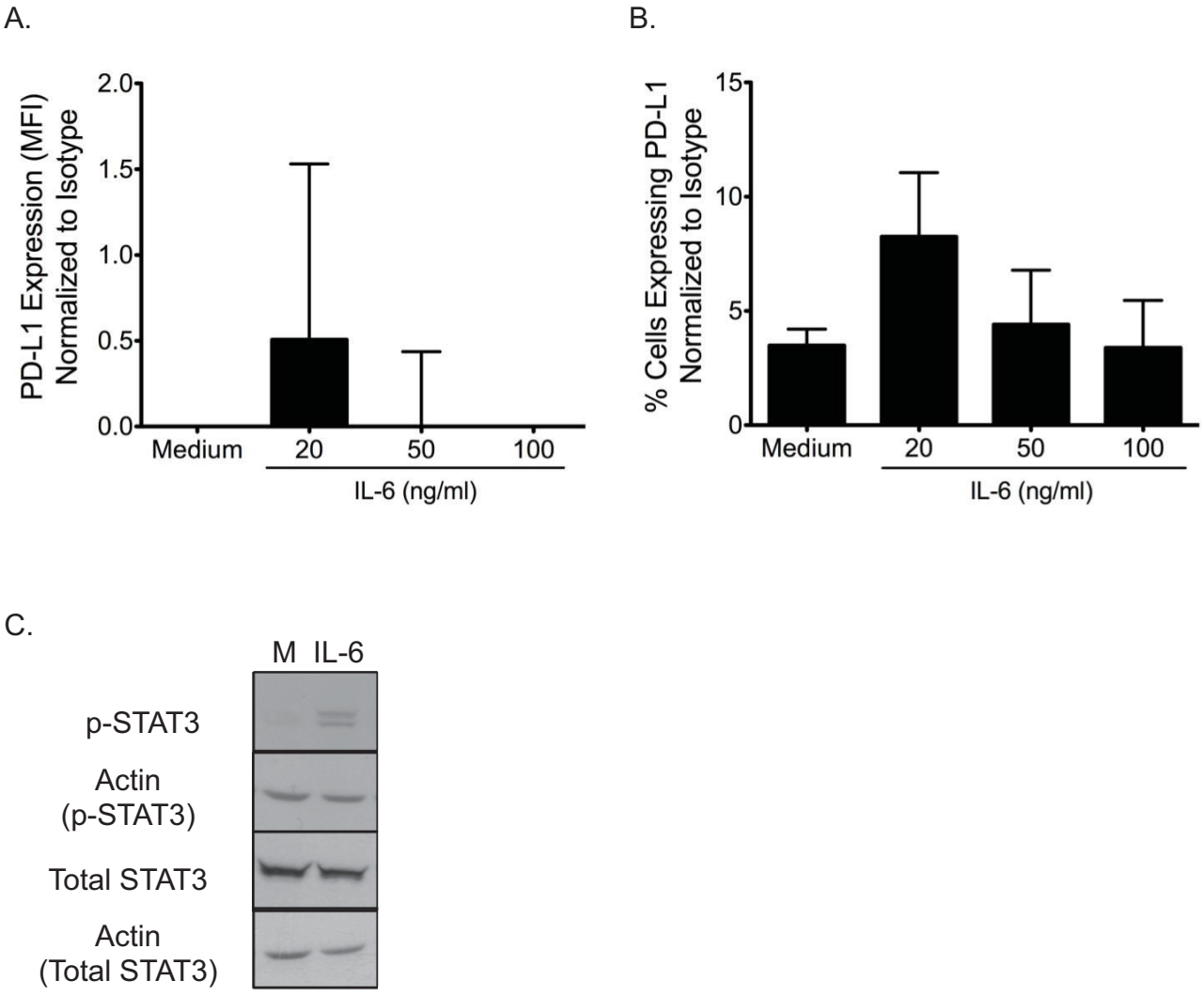


Figure 3.13. IL-6 Does Not Upregulate PD-L1 Expression by Breast Cancer Cells. (A & B) MDA-MB-468 cells were incubated in the presence or absence of 20, 50 or 100 ng/ml of IL-6. After a 24 h treatment, cells were harvested and stained with PE-anti-human PD-L1 or isotype control Abs. PD-L1 expression was measured by flow cytometry. Data shown are **(A)** the average MFI from 3 independent experiments ± SEM and **(B)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM. **(C & D)** MDA-MB-468 cells were incubated with medium (M) or 100 ng/ml of IL-6. After 1 h, cells were harvested, protein was isolated, and protein was separated by western blotting. Membranes were probed with the indicated Abs and the appropriate secondary Ab. Data shown are **(C)** one representative western blot (n=3) and **(D)** the average density of each band from 3 independent experiments ± SEM, as determined by densitometric analysis. All flow cytometry values have been normalized by subtracting the respective isotype control value; * $p < 0.05$ compared to medium as determined by ANOVA with Tukey-Kramer post-test.

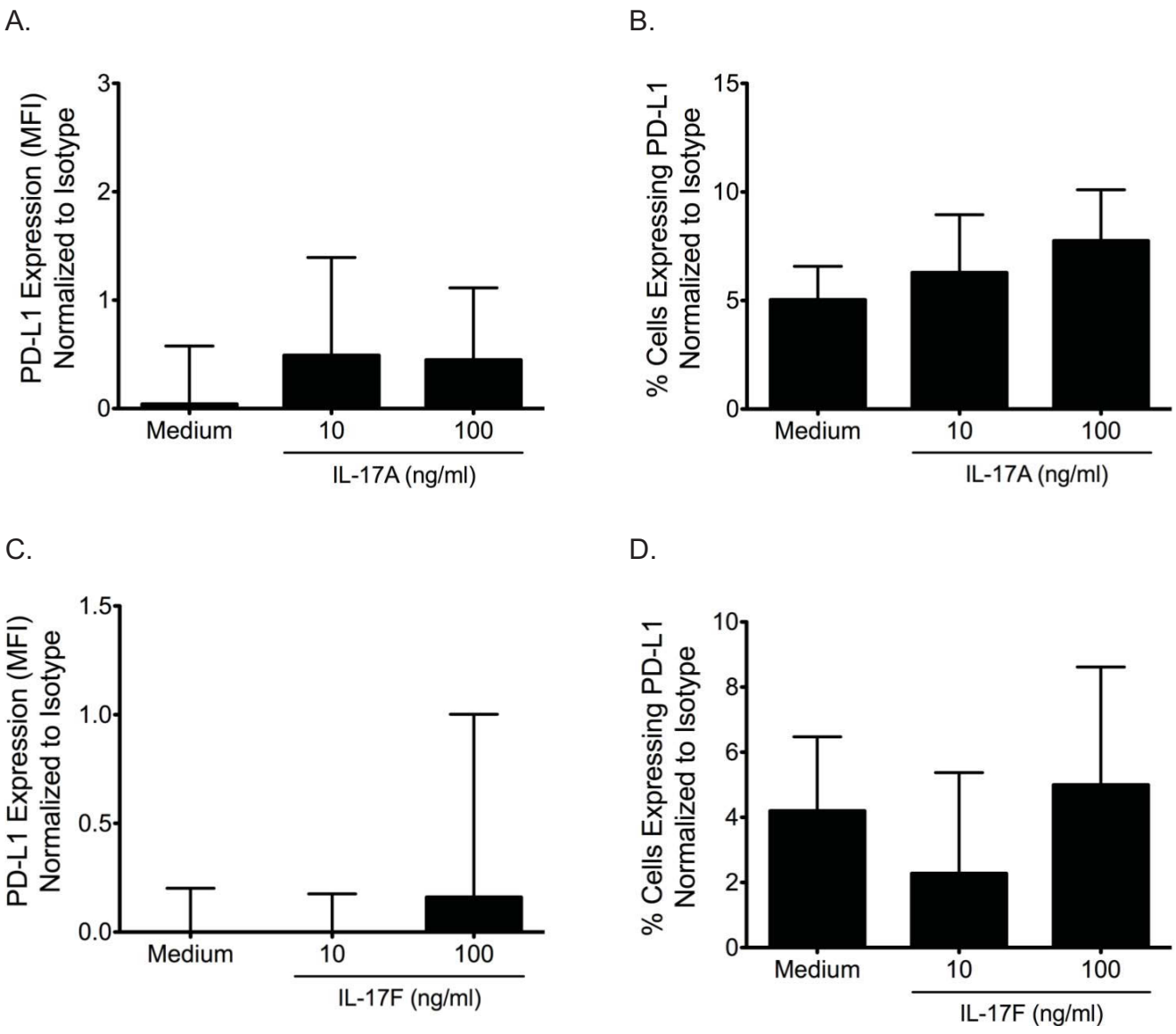


Figure 3.14. IL-17A and IL-17F Do Not Induce PD-L1 Expression by Breast Cancer Cells. MDA-MB-468 cells were incubated in the presence or absence of the indicated concentrations of **(A-B)** IL-17A or **(C-D)** IL-17F. After 24 h treatment, cells were harvested and stained with PE-anti-human PD-L1 or isotype control Abs. PD-L1 expression was measured by flow cytometry. Data shown are **(A & C)** the average MFI from 3 independent experiments ± SEM and **(B & D)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM. All flow cytometry values have been normalized by subtracting the respective isotype control value.

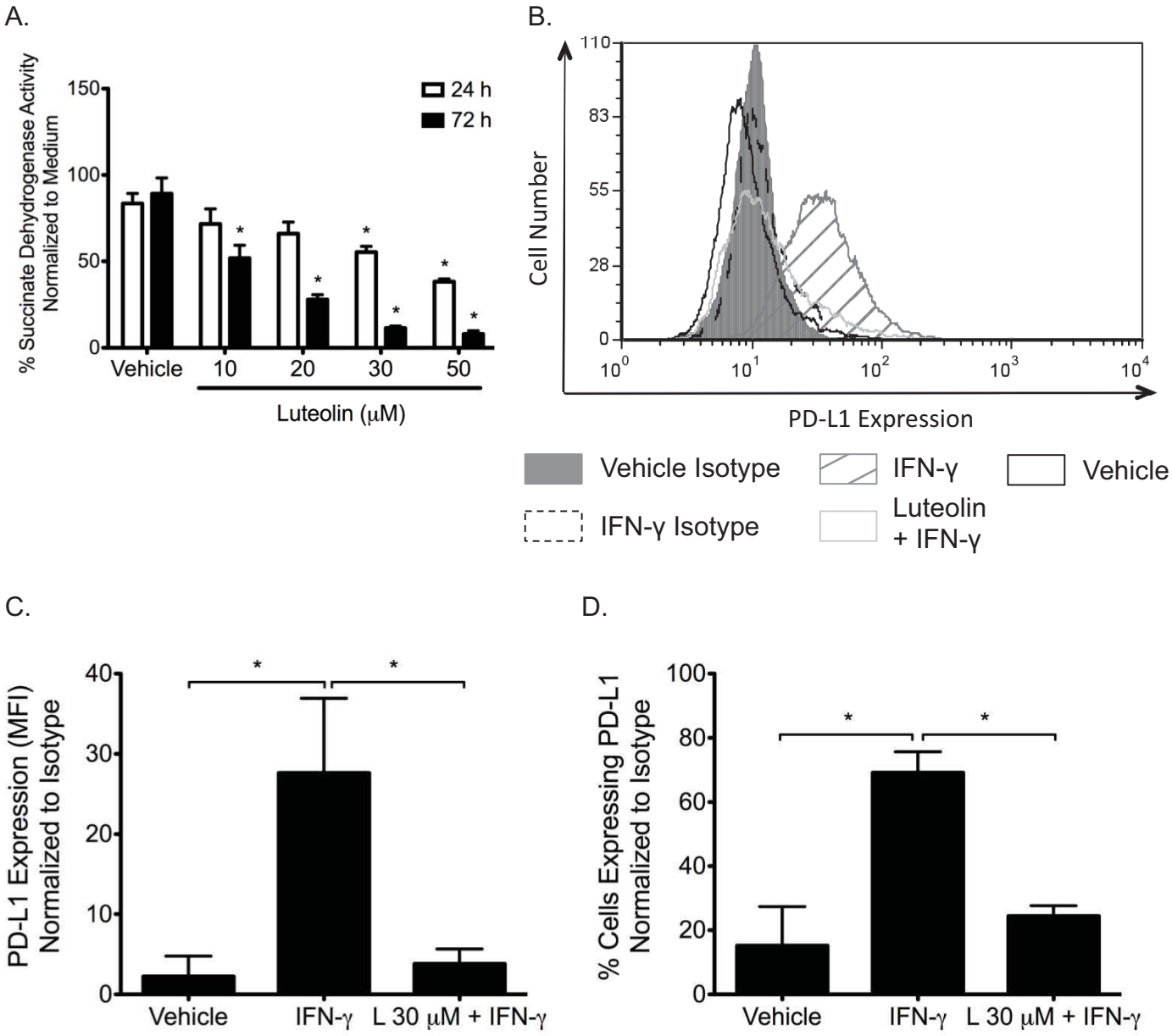


Figure 3.15. Luteolin Downregulates IFN-γ-induced PD-L1 Expression by Breast Cancer Cells. (A) MDA-MB-468 breast cancer cells were incubated with the indicated concentrations of luteolin for 24 or 72 h The colorimetric MTT assay was used as a measure of cell number. Percent succinate dehydrogenase activity is relative to medium control; * $p < 0.05$ compared to DMSO vehicle control by ANOVA with Tukey-Kramer post-test. **(B-D)** MDA-MB-468 breast cancer cells were incubated in the presence or absence of 30 μM luteolin (L) for 30 min, and then treated with 10 ng/ml IFN-γ. After 24 h treatment, cells were harvested and stained with PE-anti-human PD-L1 or isotype control Abs. PD-L1 expression was measured by flow cytometry. Data shown are **(B)** the flow cytometry histogram from one representative experiment, **(C)** the average MFI from 3 independent experiments ± SEM and **(D)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM. Luteolin alone did not effect PD-L1 expression by MDA-MB-468 cells. All flow cytometry values have been normalized by subtracting the respective isotype control value; * $p < 0.05$ determined by ANOVA with Tukey-Kramer post-test.

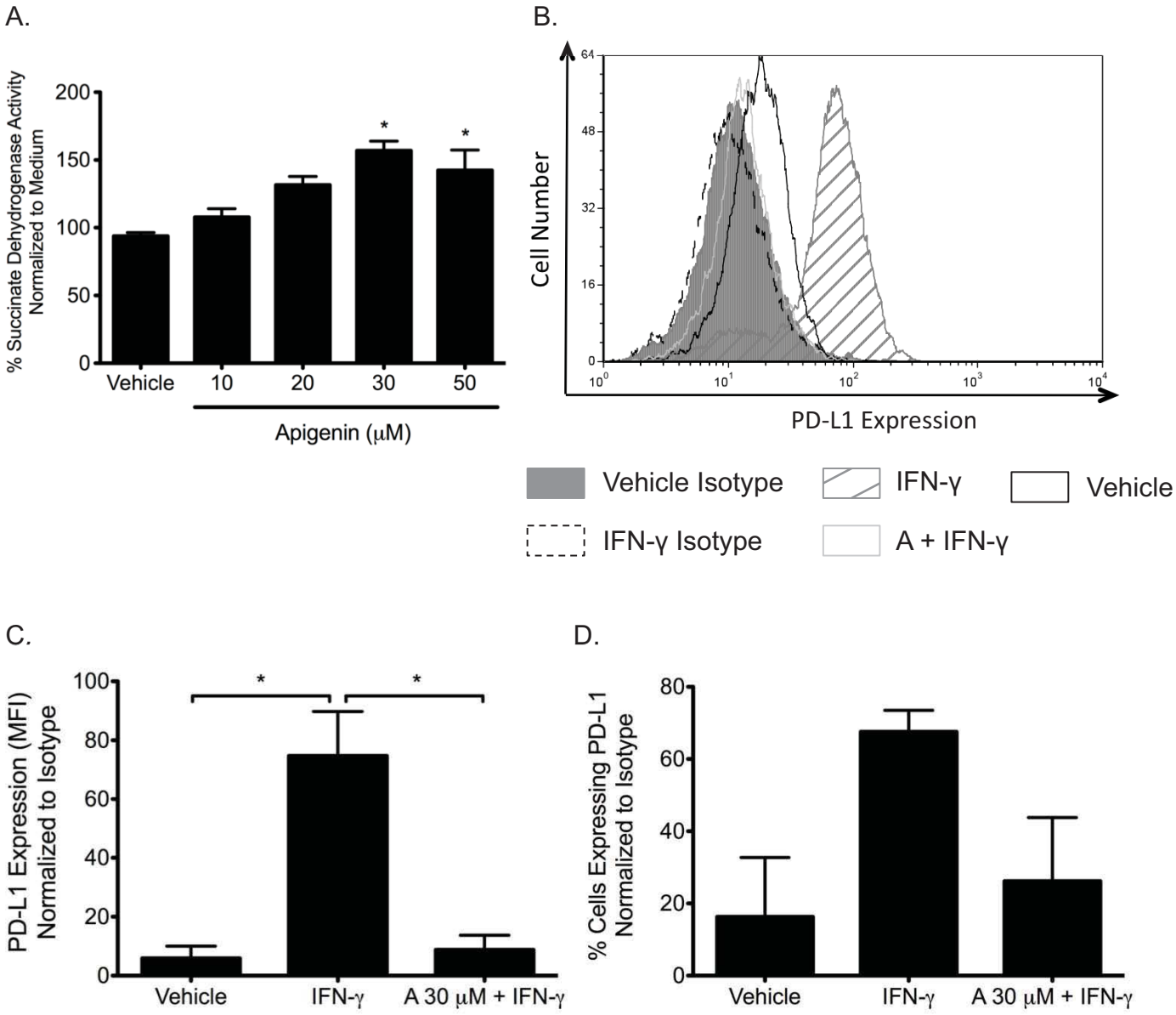


Figure 3.16. Apigenin Does Not Kill Normal Mammary Epithelial Cells (HMECs) but Inhibits IFN-γ-induced PD-L1 Expression. (A) The colorimetric MTT assay was used as a measure of cell number of HMECs that were incubated with the indicated concentrations of apigenin for 24 h. Percent viability is relative to the medium control; * $p < 0.05$ compared to DMSO vehicle control by ANOVA with Tukey-Kramer post-test. **(B – D)** HMECs were incubated in the presence or absence of 30 μM apigenin (A) for 30 min, and then treated with 10 ng/ml human IFN-γ. After 24 h, cells were harvested and stained with PE-anti-human PD-L1 or isotype Abs. PD-L1 expression was measured by flow cytometry. Data shown are **(B)** a flow cytometry histogram from one representative experiment, **(C)** the mean fluorescence intensity (MFI) averaged from 3 independent experiments ± SEM and **(D)** the average percentage of cells expressing PD-L1 from 3 independent experiments ± SEM. Apigenin alone did not effect PD-L1 expression by HMECs. All flow cytometry values have been normalized by subtracting the respective isotype control value; * $p < 0.05$ determined by ANOVA with Tukey-Kramer post-test.

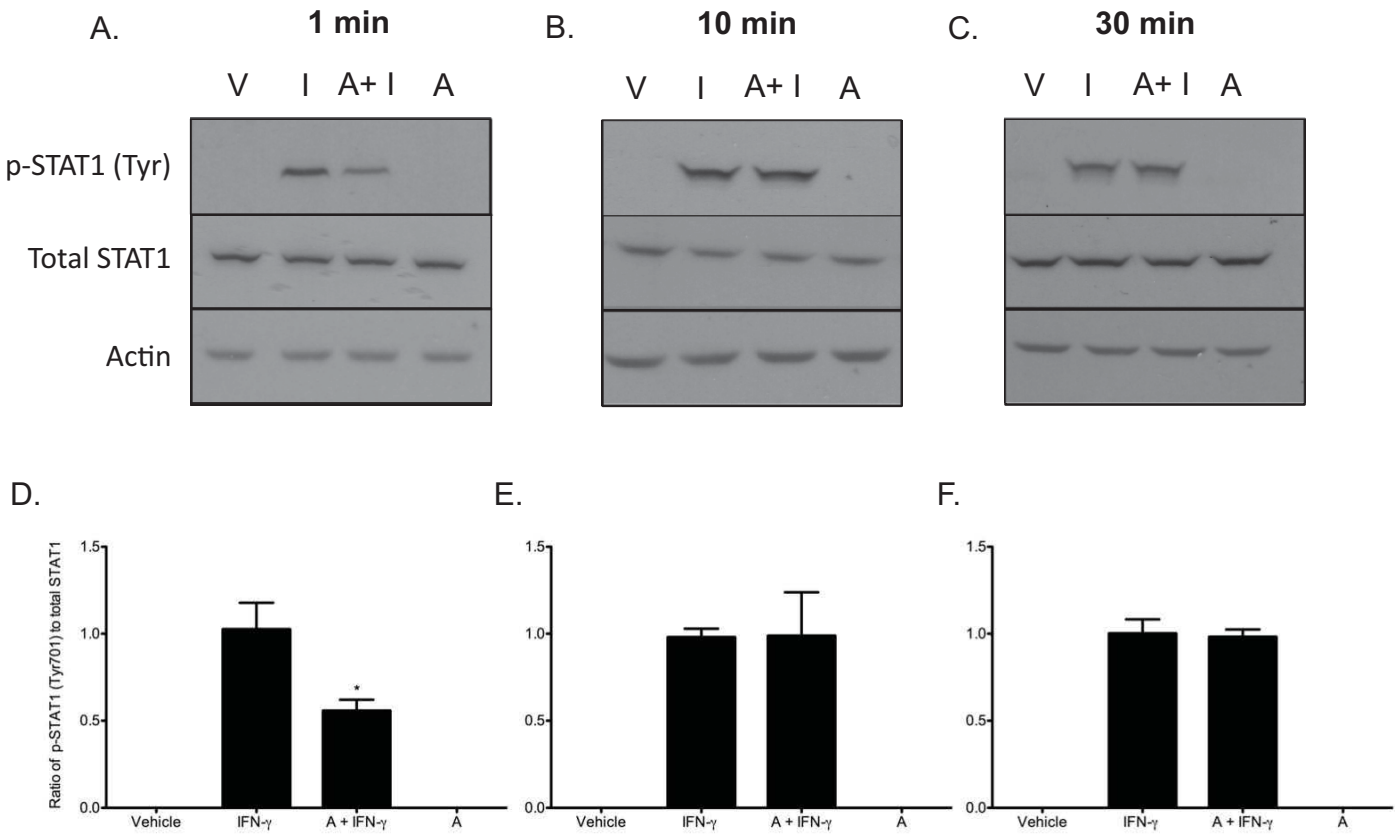


Figure 3.17. Apigenin Inhibits Early IFN-γ-induced STAT1 Phosphorylation at Tyr701 in Breast Cancer Cells. MDA-MB-468 cells were incubated for 30 min in the presence of the DMSO vehicle (V) or 30 μM apigenin (A) followed by treatment with 10 ng/ml IFN-γ (I) for **(A&D)** 1 min, **(B&E)** 10 min or **(C&F)** 30 min. Cells were then harvested, protein was isolated and protein was separated by western blotting. Membranes were probed with the indicated Abs and the appropriate secondary Ab. Data shown are **(A-C)** one representative western blot from each time point (n=3) and **(D-F)** the average density of phosphorylated STAT1 (Tyr701) normalized to total STAT1 from 3 independent experiments ± SEM as determined by densitometric analysis; * $p < 0.05$ compared to IFN-γ treatment as determined by ANOVA with Tukey-Kramer post-test.

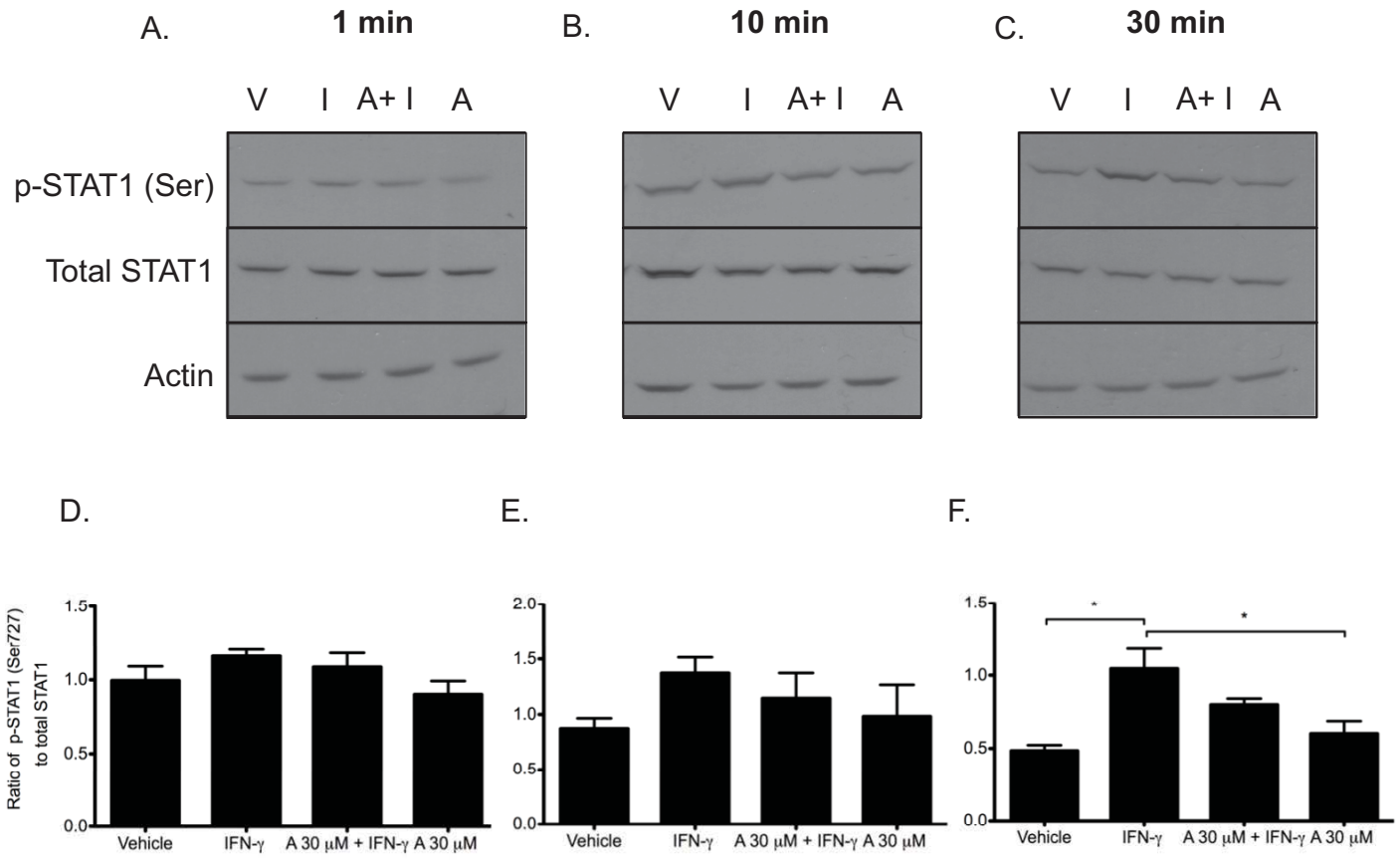


Figure 3.18. Apigenin Does Not Inhibit Early IFN-γ-induced STAT1 Phosphorylation at Ser727 in Breast Cancer Cells . MDA-MB-468 cells were incubated for 30 min in the presence of DMSO vehicle (V) or 30 μM apigenin (A) followed by treatment with 10 ng/ml IFN-γ (I) for **(A&D)** 1 min, **(B&E)** 10 min or **(C&F)** 30 min. Cells were then harvested, protein was isolated and protein was separated by western blotting. Membranes were probed with the indicated antibodies and the appropriate secondary antibody. Data shown are **(A-C)** one representative western blot from each time point (n=3) and **(D-F)** the average density of phosphorylated STAT1 (Ser727) normalized to total STAT1 from 3 independent experiments ± SEM as determined by densitometric analysis; * $p < 0.05$ as determined by ANOVA with Tukey-Kramer post-test.

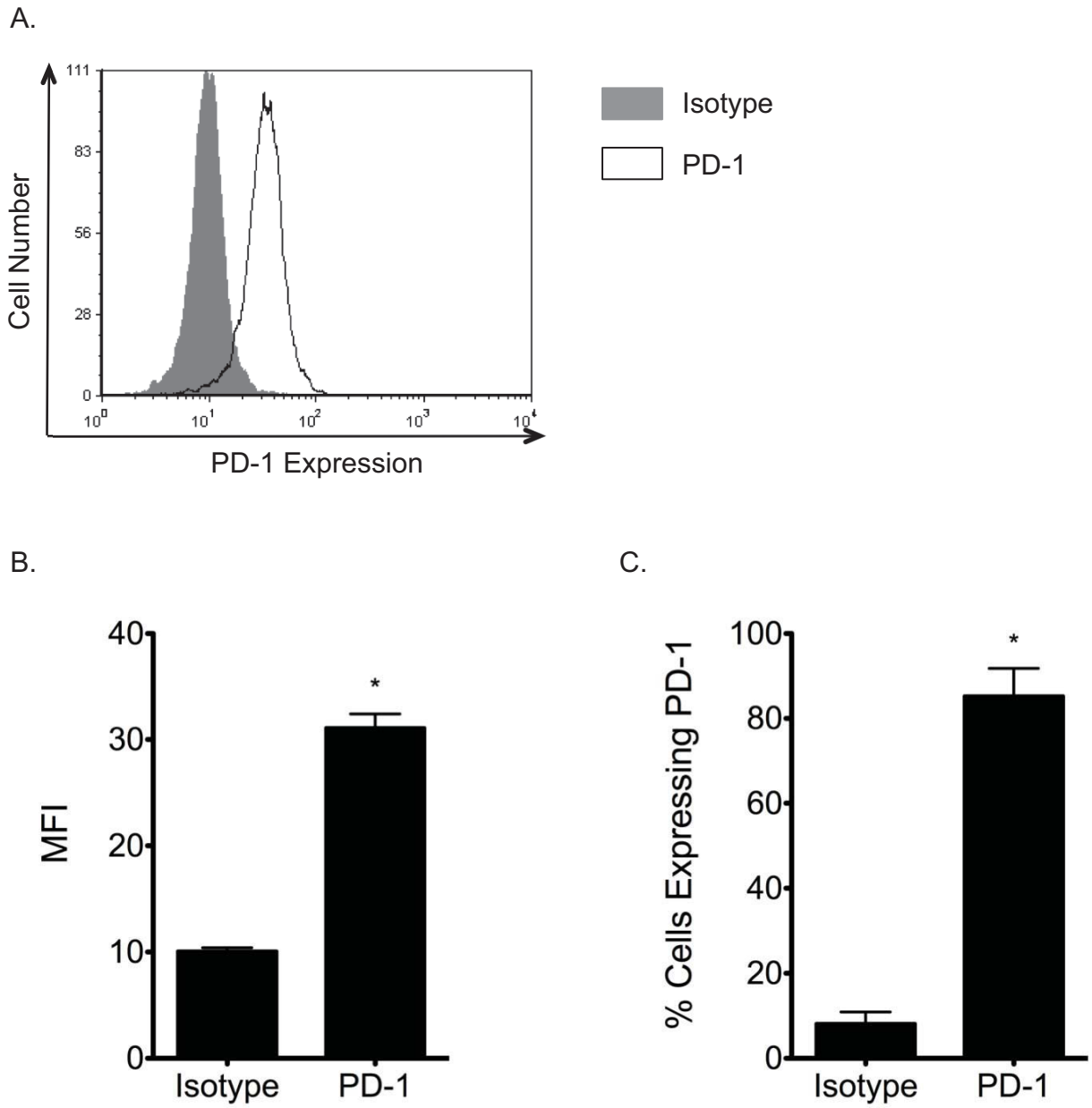


Figure 3.19. Jurkat T cells Constitutively Express PD-1, the PD-L1 Receptor. Jurkat cells were stained with PE-anti-human PD-1 or isotype control Abs. PD-1 expression was measured by flow cytometry. Data shown are **(A)** a flow cytometry histogram from one representative experiment, **(B)** average MFI from 3 independent experiments ± SEM, and **(C)** the average percentage of cells expressing PD-1 from 3 independent experiments ± SEM; * $p < 0.05$ compared to isotype control as determined by a paired t-test.

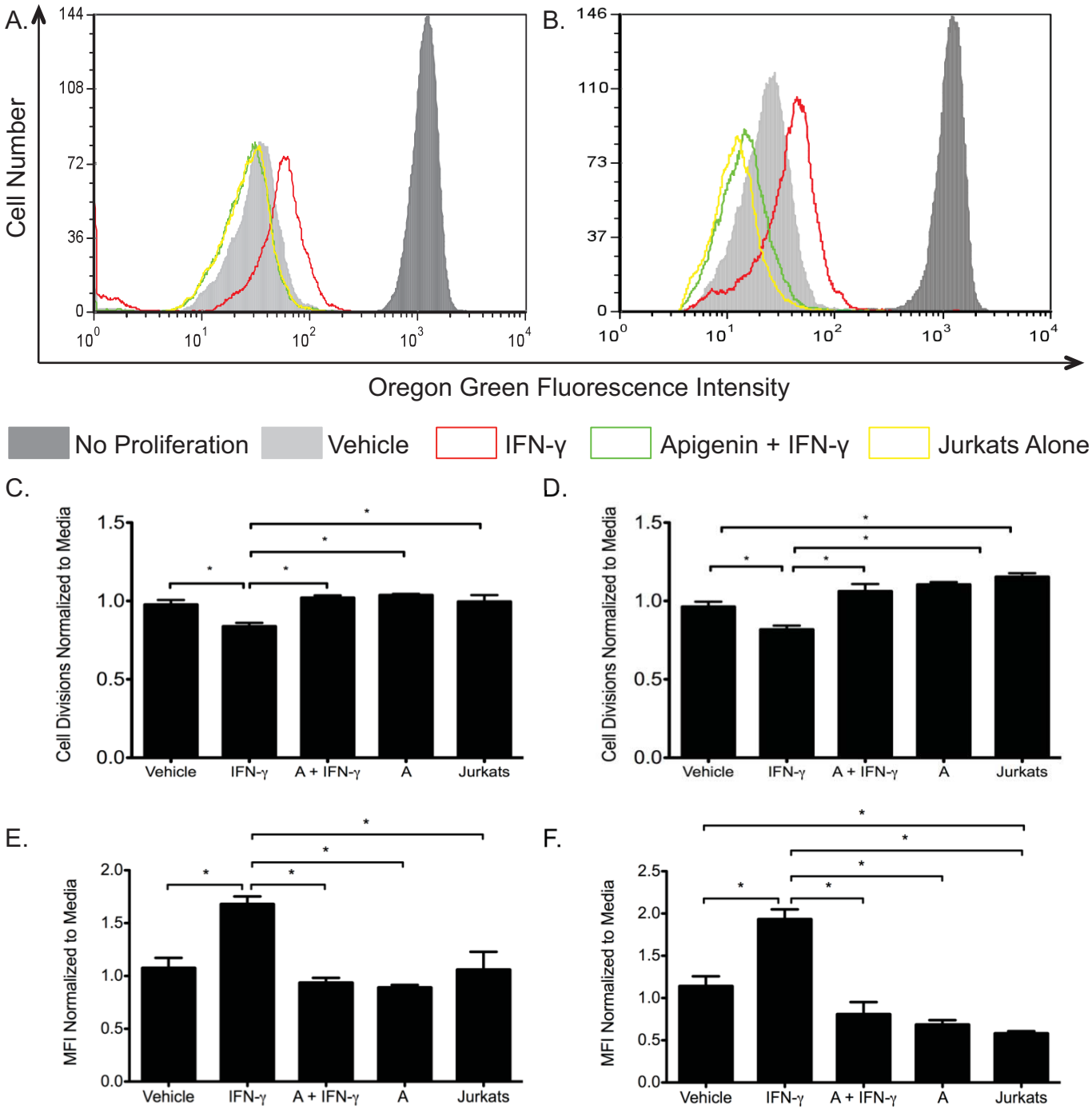


Figure 3.20. Co-treatment with Apigenin Reduces the Inhibition of Jurkat T cell Proliferation by IFN-γ-treated Breast Cancer Cells. Jurkat cells were stained with the fluorescent dye, Oregon Green. Jurkat cells were then co-cultured with MDA-MB-468 breast cancer cells that had been treated as indicated and washed prior to co-culture. After 48 h **(A,C,E)** and 72 h **(B,D,F),** Jurkat cells were collected, fixed with 1% PFA and the level of fluorescence was determined by flow cytometry. (**A&B)** Data shown are from one representative experiment, **(C&D)** the average number of cell divisions normalized to the number of cell divisions seen in the medium control ± SEM from 3 independent experiments, and **(E&F)** The average MFI of of each sample normalized to the average MFI of the medium control; * $p < 0.05$ as determined by ANOVA with Tukey-Kramer post-test.

CHAPTER 4

DISCUSSION

Phytochemicals such as apigenin have been extensively studied for their chemopreventative properties. This investigation furthers our understanding of apigenin by analyzing a possible role for apigenin in regulating an anti-tumor immune response. The T cell inhibitory PD-L1/PD-1 pathway is currently under investigation as a target for novel immunotherapies (267). This is the first study to look at the effects of a phytochemical on the PD-L1/PD-1 pathway and demonstrate the immunomodulating potential of apigenin treatment on breast cancer cells.

4.1 Cytostatic and Cytotoxic Properties of Apigenin

Apigenin has both anti-proliferative and pro-apoptotic effects on a variety of breast cancer cell lines (106). This study therefore used a subcytotoxic concentration of apigenin in order to study its effects on cell surface proteins.

MTT assays showed that treatment with 30 μM apigenin for 24 h did not reduce the cell number in cultures of any tested cell lines, including breast cancer cell lines and human mammary epithelial cells. The ER-positive breast cancer cell line MCF-7 and mammary epithelial cells showed an increase in cell number after treatment with 50 μM and 30 μM apigenin, respectively (Figure 3.1C & Figure 3.16A). This is consistent with the ability of apigenin to act as an ER agonist (139). Previous literature has indicated that both MCF-7 cells and mammary epithelial tissue express the ER (139,302). Le Bail and colleagues demonstrated that apigenin at low concentrations (< 50 μM) stimulated proliferation of MCF-7 cells (139). At higher concentrations, apigenin overcame this stimulatory effect and began to exert its anti-proliferative properties independent of ER status (139). These data imply that treatment with lower doses (< 50 μM) of apigenin could have adverse effects on patients with ER-positive tumors. MTT data suggests that 30 μM apigenin did not reduce HMEC number (Figure 3.16). This result is consistent with previous studies that show apigenin (10-40 μM) to be less toxic to normal prostate cells than carcinoma cells (107). Also, *in vivo* studies show that rats fed an apigenin rich diet (50 mg/kg daily for 10 days) display no signs of toxicity, which suggests that

apigenin is non-toxic to a variety of normal cells (108). At a concentration of 50 μM, apigenin began to reduce the cell number of the triple negative breast cancer cell line MDA-MB-468 (Figure 3.3), which was consistent with a study performed on cultured murine microglia cells in which apigenin began to demonstrate cytotoxic effects at a concentration of 50 μM after 24 h (124). In general, MDA-MB-468 cells are the most sensitive, of the cells tested, to phytochemical-mediated cytotoxicity (303,304).

While in a rat *in vivo* model the half-life of apigenin is relatively long (91.8 h) in comparison to other phytochemicals, the effects of its metabolites are still critically important (110). This investigation looked at the effects of the phase I metabolite of apigenin, luteolin, and found that luteolin caused a significant reduction in the cell number in cultures of MDA-MB-468 cells after treatment with 30 μM luteolin for 24 h (Figure 3.15A). This same decrease in cell number was not seen after treatment with apigenin, indicating that luteolin is more potent in terms of cytotoxicity than apigenin. The cytotoxic effects of luteolin and apigenin vary between cell types and cell lines (305–307). This is possibly reliant upon the dependence of a certain cell type or line on the molecular targets of apigenin and luteolin. At 48 h, luteolin and apigenin had similar cytotoxic effects on human colorectal cancer cells, hepatocytes, and hepatoma cells (305,306). Apigenin is less cytotoxic toward rat hepatocytes than luteolin, but luteolin has less of a cytotoxic effect than apigenin on hepatoma cells (307). The active nature of luteolin suggests that apigenin-mediated effects can outlast the presence of apigenin itself.

Staining of MDA-MB-468 cells with Annexin-V/PI showed that treatment with 30 μM apigenin for 24 h caused minimal (~10%) induction of apoptosis compared to cells treated with the DMSO vehicle (Figure 3.2.). Although these data may seem contradictory to previous reports that apigenin is strongly apoptotic to cancer cells, many of these results are based on the use of higher concentrations of apigenin and evaluations that were performed at later time points. At 24 h, apigenin-mediated apoptosis was seen in the breast cancer cell line SK-BR-3, leukemia cells, and lung cancer cells, but the concentrations of apigenin used were 100, 50, and 80-160 μM, respectively (125,133,308). This study showed that 30 μM apigenin caused a decrease in MDA-MB-468 cell number after a 48 h treatment, as determined by MTT assay (Figure 3.3).

Although Annexin-V/PI staining was not performed at 48 h, the effects of apigenin on other cancer cell types suggest that this decrease in cell number may be partly due to cytotoxicity. Apoptosis was seen in SK-BR-3 breast cancer cells after treatment with 50 μM apigenin for 48 h and MDA-MB-453 breast cancer cells underwent apoptosis after treatment with 40 μM apigenin for 36 h (21,125).

[^{3}H]TdR incorporation and Oregon Green cell proliferation assays both showed a reduction in breast cancer cell proliferation after 24 and 72 h of apigenin treatment (Figure 3.4 & Figure 3.5). This reduction in cell proliferation corresponds to previous reports of apigenin-mediated reduction in proliferation of breast, cervical, pancreatic, leukemia, and colon cancer cells (125,127–130). At 24 h, the data obtained from the Oregon Green cell proliferation assay suggest that the breast cancer cells underwent less than one round of division after treatment with 30 μM apigenin (Figure 3.5C). MDA-MB-468 cells were serum starved prior to Oregon Green staining, which synchronizes their cell cycle, so this result is likely not due to proliferation of a portion of the cells. However, the Oregon Green cell proliferation assay is based on fluorescence intensity. A cell arrested during the mitotic stage of cell cycle may be larger than cells in other stages, which may reduce fluorescence intensity within the cell leading to the appearance of half of a cell division (309). The lack of decrease in cell number seen by MTT assay after treatment of breast cancer cells with 30 μM apigenin for 24 h does not correspond with the findings of these other proliferation assays. One potential explanation is that apigenin is actually stimulating succinate dehydrogenase activity within the breast cancer cells, which is masking the decrease in proliferation and cell number in the MTT assay. Another possible explanation is that apigenin itself is causing reduction of the MTT; however, the effects of apigenin (10-50 μM) on MTT solution after a 2 h incubation in the absence of cells was analyzed and no changes were seen, so this is an unlikely cause of this discrepancy (Table 3.1).

Cell cycle analysis performed at 24 h suggests that treatment with 30 μM apigenin causes MDA-MB-468 cells to arrest at G_2/M stage (Figure 3.6). This corresponds to previous literature that demonstrated G_2/M cell cycle arrest of breast, colon, liver, pancreatic, and leukemia cancer cells after treatment with apigenin. Based on these previous studies, the potential mechanism by which apigenin may cause G_2/M arrest

includes p21 activation, stabilization of p53, suppression of CDK regulators, and inhibition of MAPK pathway activation (125,126). In general the anti-proliferative and pro-apoptotic properties of apigenin in this study of breast cancer cells correspond to findings previously published in other cell lines.

4.2 Effects of Apigenin on PD-L1 Expression

Breast cancer cells can express PD-L1 constitutively or be induced by cytokines to express PD-L1. The most common and effective upregulator of PD-L1 is IFN-γ (234). This study confirmed previous findings that MDA-MB-468 and MCF-7 cells have no constitutive PD-L1 expression, but can be induced by IFN-γ to express cell surface PD-L1 protein, while MDA-MB-231 and SK-BR-3 cells constitutively express PD-L1 (Figure 3.7 & Figure 3.10) (236,237). IFN-γ induces marginal PD-L1 expression on ER-positive MCF-7 cells than on triple negative MDA-MB-468 cells, which agrees with clinical studies that analyzed PD-L1 expression by human breast tumors. Ghebeh and colleagues demonstrated a correlation between increased PD-L1 expression and lack of ER expression (297). Flow cytometric data showed that constitutive PD-L1 expression by SK-BR-3 can be increased after treatment with IFN-γ (Figure 3.10G-I). While IFN-γ is often associated with anti-tumor effects such as reduction in proliferation, suppression of angiogenesis, and increase in apoptosis of cancer cells, IFN-γ is still found within the tumor microenvironment (310–312). The IFN-γ within the tumor microenvironment comes from activated T cells (313). Despite anti-tumor properties of IFN-γ, cancer cells use IFN-γ to reduce the anti-tumor immune response through upregulation of PD-L1 expression (172). The PD-1/PD-L1 pathway is a better immunotherapy target than IFN-γ itself because of the anti-tumor properties of this pro-inflammatory cytokine (310–312).

Treatment for 24 h with 30 μM apigenin completely inhibited IFN-γ-induced PD-L1 expression by MDA-MB-468, MCF-7, and SK-BR-3 cells, but this treatment had no effect on constitutive PD-L1 expression on MDA-MB-231 and SK-BR-3 cells (Figure 3.8, Figure 3.10). Apigenin may affect IFN-γ-induced PD-L1 expression but not constitutive PD-L1 expression because these two states of PD-L1 expression are regulated differently. In multiple myeloma cells, IFN-γ-induced upregulation of PD-L1 is dependent on STAT1 activation, which is consistent with many IFN-γ-mediated signaling

pathways (240). However, constitutive expression of PD-L1 by macrophages is STAT1-independent since PD-L1 expression in STAT1-deficient macrophages is normal (314). Since apigenin has previously been shown to inhibit IFN-γ-induced STAT1 activation (124), this could be the reason for apigenin affecting IFN-γ-induced PD-L1 expression and not constitutive expression. Downregulation of breast cancer cell PD-L1 expression by apigenin is predicted to prevent the induction of anergy in tumor-specific T cells and make the cancer cells more susceptible to elimination by the anti-tumor immune response (Figure 4.1).

Flow cytometric data indicated that HMECs may have very low levels of constitutive PD-L1 expression, which is significantly increased after treatment with IFN-γ (Figure 3.16B-D). Dong and colleagues found that breast tissue does not constitutively express PD-L1 (172). The level of constitutive PD-L1 expression seen in our investigation was very low and varied between experiments; therefore, the discrepancy between our study that of Dong *et al.* could be due to differences in PD-L1 expression between individuals. PD-L1 is expressed on a variety of non-hematopoietic cells. A study on normal expression of PD-L1 on mouse tissues found constitutive PD-L1 expression by heart, pancreatic, small intestinal, and placental tissues (232). In non-malignant human cells, PD-L1 was moderately expressed by tracheal, bronchial, and alveolar epithelial cells, and PD-L1 was upregulated on gastric epithelial cells by persistent infection (315,316). Similar to breast cancer cells, treatment of HMECs with 30 μM apigenin inhibited IFN-γ-induced PD-L1 expression (Figure 3.16B-D). The upregulation of IFN-γ-induced PD-L1 on HMECs suggests that mammary epithelial cells may use this immunoinhibitory pathway to control inflammation within normal mammary tissue. The implications of these findings are that apigenin may exacerbate an inflammatory response by decreasing immunoinhibitory effects of the PD-L1/PD-1 pathway.

Flow cytometric analysis of the effects of the apigenin metabolite luteolin on IFN-γ-induced PD-L1 expression showed that 30 μM luteolin also inhibited IFN-γ-induced PD-L1 expression (Figure 3.15B-D). The ability of luteolin to inhibit IFN-γ-induced PD-L1 expression by breast cancer cells increases the potential for apigenin to have clinical benefits because even after apigenin undergoes phase I metabolism, its major metabolite

may continue to exert the immunostimulatory effect caused by inhibition of PD-L1 expression.

Overall, apigenin shows potential to regulate the anti-tumor immune response through inhibition of IFN-γ-induced PD-L1 expression by breast cancer cells. Several other cancer cell types such as multiple myeloma, oral squamous carcinoma, and uveal melanoma upregulate PD-L1 as a method of immune evasion (240,317,318). Apigenin should be tested to determine whether it is capable of inhibiting IFN-γ-induced PD-L1 expression in other cancer cell types.

4.3 Effect of Pro-inflammatory Cytokines on PD-L1 Expression

A panel of pro-inflammatory cytokines were tested for their ability to induce PD-L1 expression by MDA-MB-468 cells (Figure 3.11-Figure 3.14). Aside from IFN-γ, the only other cytokine tested that induced significant PD-L1 expression was IFN-β (Figure 3.11). This was not surprising as published reports show that IFN-β induces PD-L1 expression, although to a lesser extent than IFN-γ, on monocytes, dendritic cells, and endothelial cells (230,234). Interestingly, IFN-β is often used during the treatment of the autoimmune disease multiple sclerosis, and recently it was shown that patients who received IFN-β treatment showed an increase in PD-L1 mRNA transcripts and a reduction in disease progression (219). These results suggest that IFN-β-induced PD-L1 expression is contributing to the beneficial effects of IFN-β on the progression of multiple sclerosis by potentially inducing T cell anergy and apoptosis. Apigenin was also able to inhibit IFN-β-induced PD-L1 expression (Figure 3.11). This suggests that apigenin may have negative effects on patients with multiple sclerosis due to its inhibitory effect on IFN-β-induced PD-L1 expression. Other cytokines that have been shown to be able to upregulate PD-L1 expression include the anti-inflammatory cytokines IL-10 and IL-27 (235,319). IL-10 induces PD-L1 expression by human monocytes, although the mechanism has yet to be determined (235). IL-27 upregulates PD-L1 expression by naïve $CD4^+$ T cells in a STAT1-dependent manner and inhibited differentiation of naïve $CD4^+$ T cells into T_H17 cells (319). Inhibition of IL-10- and/or IL-27- mediated PD-L1 expression by apigenin may enhance the immune response in diseases associated with these cytokines. IL-10 and IL-27 were not used in this

investigation, but the ability of these cytokines to induce PD-L1 expression on breast cancer cells and other malignant cells should be examined in the future, as both cytokines are present in the tumor microenvironment (320,321).

4.4 Mechanism of Apigenin-Mediated Inhibition of Inducible PD-L1 Expression

IFN-γ signals through the IFN-γR (242). Treatment of MDA-MB-468 cells with IFN-γ and apigenin did not cause a significant decrease in IFN-γR1 surface expression when compared to MDA-MB-468 cells treated with only IFN-γ (Figure 3.10). Treatment of MDA-MB-468 cells with IFN-γ alone did appear to reduce IFN-γR1 surface expression, but this decrease was not significant (Figure 3.10). This result is consistent with literature that shows that IFN-γ promotes endocytosis of the IFN-γR1 subunit and transports the receptor subunit to the nucleus, but the IFN-γR2 subunit remains primarily at the cell surface (322). These results demonstrated that apigenin-mediated reduction in IFN-γ-induced PD-L1 expression by MDA-MB-468 cells was not due to a reduction in IFN-γR1 surface expression by these cells. While IFN-γ signaling is commonly associated with the JAK/STAT1 pathway, alternative pathways have been implicated in IFN-γ-induced upregulation of PD-L1 (242). Western blotting showed a significant decrease in p-STAT1 (Tyr701) after pre-treatment with apigenin and treatment with IFN-γ for 1 min (Figure 3.17A&D). At 30 min apigenin appeared to reduce p-STAT1 (Ser727), but this decrease was not significant (Figure 3.18C&F). These results are consistent with findings in fibroblast cells that showed that after IFN-γ stimulation, STAT1 phosphorylation at the Tyr701 residue occurred at an earlier time point than Ser727 phosphorylation (299). Zhu and colleagues also found that Tyr701 and Ser727 phosphorylation events occurred independently of each other, but that both were dependent on JAK2 activity (299). STAT1 activation is therefore likely involved in IFN-γ-induced upregulation of PD-L1 in breast cancer cells, which corresponds to findings that IFN-γ-mediated upregulation of PD-L1 in multiple myeloma cells is also dependent on STAT1 phosphorylation (240).

These results also match data showing that apigenin inhibits IFN-γ-induced STAT1 phosphorylation at both Tyr701 and Ser727 locations (124). Other phytochemicals that inhibit STAT1 activation include EGCG, myricetin, and delphinidin

(323,324). This result makes these phytochemicals other possible candidates for inhibiting IFN-γ-induced PD-L1 expression. The other molecules shown to be involved in the IFN-γR pathway in multiple myeloma cells were MyD88, TRAF6, MEK, and ERK (240). The involvement of these molecules has not yet been studied in breast cancer cell lines. Apigenin also inhibited IFN-β-induced PD-L1 expression, suggesting that it is either inhibiting a molecule that is common to both the IFN-γ and IFN-β signaling pathways or two different signaling molecules. Since JAK1 is involved in phosphorylation of STAT1 during signaling through both the IFN-γR, and the IFN-α/β receptor (IFNAR), JAK1 is very likely the target for apigenin-mediated inhibition of both IFN-β- and IFN-γ-induced PD-L1 expression (242,325). IFN-α also signals through the IFNAR receptor and has been shown to induce PD-L1 expression in endothelial cells (230). The results of my investigation suggest that apigenin inhibits PD-L1 expression induced by IFN-α, IFN-β, and IFN-γ through the inhibition of STAT1 activation.

4.5 Functional Consequence of Apigenin-Mediated Inhibition of IFN-γ-induced PD-L1 Expression

Inhibition of IFN-γ-induced PD-L1 expression by MDA-MB-468 breast cancer cells was associated with reduced breast cancer cell-mediated suppression of Jurkat T cell proliferation (Figure 3.20). PD-L1 expression has been shown on a variety of tumors and high PD-L1 expression by tumor tissue indicates poor prognosis in a variety of cancer types, including renal, esophageal, and ovarian cancer (177,261,262). A similar experiment to the functional assay performed in this investigation was done with IFN-γ-stimulated uveal melanoma cells that were co-cultured with activated Jurkat cells for 48 h (318), but in this case the readout was IL-2 production instead of Jurkat cell proliferation. Yang and colleagues showed that IL-2 production is significantly decreased, by half, after co-culture of Jurkat cells with IFN-γ-treated melanoma cells (318). The results of our investigation were not as dramatic as seen by Yang and colleagues, possibly because assessing IL-2 production is a more sensitive indicator of PD-L1 modulation. Measuring of IL-2 levels would not have worked in our system because the Jurkat cells were not activated during this assay. Apigenin treatment also caused an increase in Jurkat cell proliferation above that seen in Jurkat cells co-cultured with untreated breast cancer cells

(Figure 3.20), which suggests that PD-L1/PD-1 interactions are not the only method by which these breast cancer cells are reducing T cell proliferation, and that apigenin also has other effects. Other possible means of cancer immune evasion in this system include Fas ligand-mediated killing of Jurkat cells and generation of inhibitory cytokines such as IL-10 and TGF-β by the breast cancer cells (326–328). A study on AML shows the importance of both the PD-1/PD-L1 and TIM3/galectin-9 pathways in the induction of T cell exhaustion (266). In our system, the TIM3/galectin-9 pathway probably does not play a major role in reduction of Jurkat cell proliferation as TIM3 levels on Jurkat cells are relatively low in the absence of PMA stimulation (329). These results of investigation support future study in an *in vivo* rodent model of breast cancer and suggest that the ability of apigenin to inhibit IFN-γ-induced PD-L1 may be a significant mechanism involved in the role of apigenin as a chemopreventative and immunotherapeutic agent.

4.6 Limitations

All scientific investigations have limitations, and the following are limitations of this study that need to be considered. The MTT data for the effects of luteolin may be underestimated because of the ability of luteolin to reduce MTT to formazan dye in the absence of cells (295). Another major limitation of this study is the use of cell lines instead of primary cells. This limitation is particularly apparent when using breast cancer cell lines and the Jurkat T leukemia cell line instead of clinical isolates and peripheral blood T cells. The use of Jurkat cells in the functional assay is not ideal. Although these cells are often used to study T cell signaling, Jurkat cells have many different characteristics from primary T cells. One key difference that could affect our functional assay is that Jurkat cell proliferation is IL-2-independent and does not require antigen stimulation, and the inhibitory effects of PD-1 signaling involve a reduction in IL-2 production, which in primary T cells should contribute to a decrease in cell proliferation (330) This could be weakening the effect that IFN-γ-induced PD-L1 expression has on Jurkat T cell proliferation in the functional assay compared to primary T cells. The functional data also has to be interpreted with caution because until PD-L1 or PD-1 is blocked in this model, the increase in Jurkat cell proliferation cannot be attributed to PD-L1 downregulation alone. This may be done through the introduction of a blocking

antibody or siRNA to PD-1 or PD-L1. Also, using breast cancer cells that constitutively express PD-L1, such as MDA-MB-231 cells, would confirm that apigenin-mediated downregulation of PD-L1 was causing the increase in T cell proliferation, and that this increase was not do to other apigenin-mediated effects. Flow cytometric analysis of MDA-MB-468 cells after they were incubated with Oregon Green Jurkat cells showed that they were slightly fluorescent, which suggested that some Jurkat cells remained attached to the breast cancer cells (data not shown). In the future efforts should be made to remove the Jurkats cells, so that they are included in the analysis. However, notably these Jurkat cells underwent the same amount of proliferation as Jurkat cells that were incubated with medium treated MDA-MB-468 cells and were able to be removed. In spite of the limitations of this report, significant progress has been made in this field and will inform future research in this area.

4.7 Future Directions

The next objective of this study will be to develop a better understanding of the mechanism by which apigenin inhibits IFN-γ- and IFN-β-induced PD-L1 expression in breast cancer cells. Based on studies of IFN-γ-induced PD-L1 expression in multiple myeloma cells and dermal fibroblasts, molecules that are of interest for further investigation include ERK, PI3K/Akt, MyD88 and TRAF6 (240,241). Previous studies show that IFN-β can activate STAT1, but western blotting will be needed to confirm that this is happening in breast cancer cells during IFN-β-induced upregulation of PD-L1. IFN-γ signaling regulates a variety of anti-tumor pathways including suppression of angiogenesis and reduction in cancer cell proliferation and survival (310–312). Our results suggest that apigenin may inhibit these pathways as well through inhibition of STAT1 activation, which may have detrimental effects to the IFN-γ-mediated anti-tumor response. Therefore, the effects of apigenin on the anti-tumor properties of IFN-γ should be examined. Further development of the *in vitro* functional assay is also needed in order to eliminate some of the limitations of this experiment. The use of primary T cells in this system may better demonstrate the effect of decreased PD-L1 expression on T cell proliferation. Primary murine T cells would be isolated from mice with mammary tumors that inducibly express PD-L1 such as the murine mammary carcinoma cell line 4T1

(Coombs *et al*, unpublished data). These T cells would then be stained with Oregon Green, incubated with 4T1 cells that were previously treated (DMSO [0.15%], IFN-γ [10ng/ml], apigenin [30 μM] and IFN-γ [10 ng/ml], and apigenin [30 μM]), and T cell proliferation would be analyzed by flow cytometry. Also, blocking PD-L1/PD-1 interactions in this system will confirm the importance of these interactions. Since several other cancer cell lines also express IFN-γ-induced PD-L1 (172), the effects of apigenin on the expression of PD-L1 in these cell lines will be examined. Also of interest is the effect of EGCG, myricetin, and delphinidin on IFN-γ-induced PD-L1 expression as these phytochemicals also inhibit STAT1 activation (323,324). Ghebeh and colleagues demonstrated that doxorubicin downregulates constitutive PD-L1 expression by breast cancer cells (236). Future research on the effects of apigenin treatment in combination with doxorubicin on PD-L1 expression and breast cancer progression should therefore be performed. Lastly, results similar to those seen in this study have been obtained *in vitro* using mouse mammary carcinoma cell lines (Coombs *et al*, unpublished data). These mouse mammary carcinoma cell lines will be useful to study the effects of apigenin on immune regulation and cancer progression *in vivo*.

4.8 Conclusions

The results obtained from this investigation suggest that the dietary phytochemical apigenin reduces IFN-induced PD-L1 expression by breast cancer cells, potentially making these cells more susceptible to anti-tumor immune responses. Apigenin at 30 μM did not reduce the cell number of tested breast cancer cell lines, but did slow the proliferation of these cells. As has been previously reported for other non-malignant cell lines, apigenin was non-toxic to normal epithelial cells. Pre-treatment of malignant and non-malignant breast cells with apigenin or luteolin completely inhibited IFN-γ- and IFN-β-induced PD-L1 expression. Apigenin-mediated inhibition of IFN-γ-induced PD-L1 expression by breast cancer cells decreased the ability of breast cancer cells to suppress Jurkat T cell proliferation, which may cause the breast cancer cells to be more susceptible to an anti-tumor immune response.

This investigation found that in addition to chemopreventative properties, apigenin may act as an immunomodulatory agent. The ability of apigenin to reduce

inducible PD-L1 expression by breast cancer cells makes these cells more susceptible to eradication by tumor-specific T cells. Clinical trials are currently underway to study the effects of PD-L1/PD-1 inhibitors on cancer progression (267). These data in combination with the non-toxic effects of apigenin on normal epithelial cells suggests that apigenin may have synergistic effects if it were to be used in conjunction with current chemotherapies and be able to reduce the negative side effects of chemotherapeutic agents by lowering the doses needed to achieve a beneficial effect.

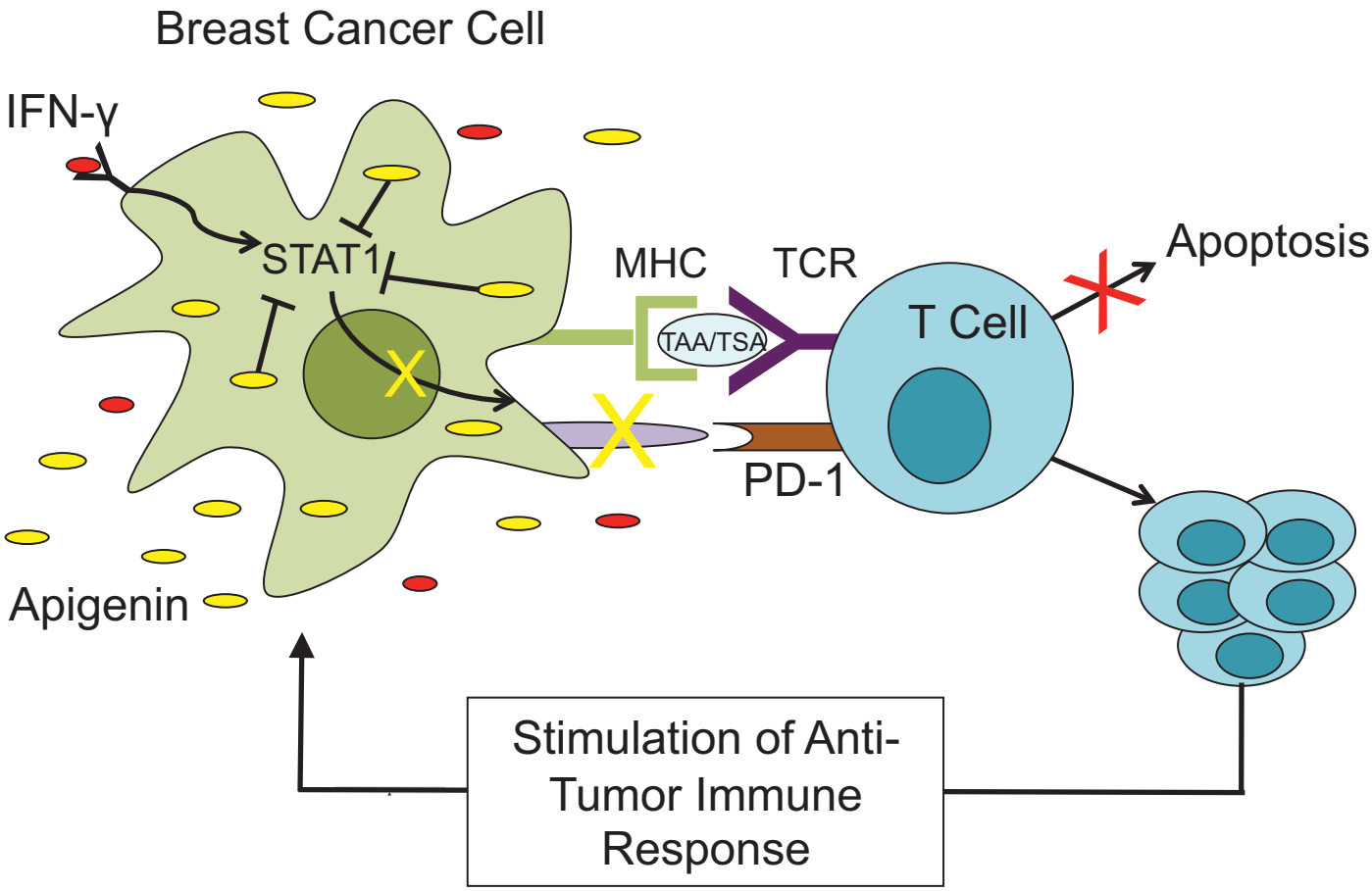


Figure 4.1. Proposed Model of Apigenin-mediated Inhibition of IFN-γ-induced PD-L1 Expression. Apigenin inhibits IFN-γ-induced STAT1 phosphorylation at both Tyr701 and Ser727 residues. This prevents PD-L1 expression by breast cancer cells. Tumor-specific T cells can then elicit an anti-tumor immune response against TAAs and TSAs by the cancer cells, and cause eradication of cancer cells.

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