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Cucurbitacin B: A Potential Natural Agent for Targeting Tumor Immune Cell Population

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CUCURBITACIN B: A POTENTIAL NATURAL AGENT FOR TARGETING TUMOR IMMUNE CELL POPULATION

A Thesis

by

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ABSTRACT

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Immune checkpoint blockade (PD1, PDL-1 and CTLA-4) immunotherapies have emerged as the breakthrough in cancer treatment. However, some malignancies show marginal response. One factor that influences the efficacy of immunotherapy is the development of immunosuppressive tumor microenvironment (TME), caused by infiltration of myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAM) into the tumor, facilitating metastatic tumor growth and immunotherapy resistance. We have identified Cucurbitacin B (Cuc. B), a potent small molecule that targets TAM and MDSCs and inhibits Stat3, CSF-1R, and PI3Kγ signaling axis at lower doses compared to the pharmacological inhibitor of PI3Kγ (IPI-549). IL-4 polarization of TAM caused a loss of the phagocytic capacity which we observed to be restored by Cuc. B treatment. Cuc. B treatment also inhibited the expression of PDL-1 in TAMs. Taken together, our results suggest that Cuc. B has the potential to reprogram TAM and MDSCs *via* targeting Stat3, CSF-1R, and PI3Kγ. Therefore, Cuc B could be a novel therapeutic modality in improving tumor immunity and checkpoint blockade PD-1/CTLA-4 immunotherapy against non-responsive malignancies.

DEDICATION

To God be the glory, great things He has done. Dedication to the Anning family.

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CHAPTER I

INTRODUCTION

1.1 Statement of the Problem

The incidence of cancer is on the ascendency with an estimation of 1.9 million new cases and an estimated cancer-related death of 609,360 in the United States for the year 2022. On the world scale, nearly 10 million cancer-related mortalities and about 20 million cases were recorded in the year 2020 (Siegel et al. 2022). Although there has been an insurgence of new therapeutic modalities for the management of cancer, resistance to cancer therapy remains a bane of successful cancer therapeutics (Ahmad 2013; Gatenby and Brown 2020; Quagliano, Gopalakrishnapillai, and Barwe 2020). The state of Texas alone has estimated new cases of 129,770 with an estimated death of 41, 810. The 5-year survival rate for all cancers is 67%, and solid tumors such as pancreatic, liver, and intrahepatic bile duct cancer remain low at 11% and 20 % respectively (Siegel et al. 2022).

Despite the breakthroughs presented by the emergence of immune checkpoint blockade (i.e. PDL-1 (programmed death-ligand 1), PD-1R (programmed death-1 receptor), CTLA-4 (cytotoxic T lymphocyte antigen 4)) immunotherapies, various solid tumors such as pancreatic and liver cancers show only a marginal response (Ribas and Wolchok 2018; Sangro et al. 2021; Yang 2015; Yap et al. 2021). A key component that has been identified to contribute to the attenuation of the efficacy of immunotherapy is the development of an immunosuppressive tumor microenvironment (TME) (Petitprez et al. 2020). Upon infiltration of tumor immune cell

populations such as the myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAM) into the tumor sites, various mechanisms are initiated which culminate in metastatic tumor growth and also contribute to immunotherapy resistance(Gabrilovich 2017; Noy and Pollard 2014). Therefore, small molecules which have the capacity to target various immune cell populations in the tumor microenvironment such as MDSCs, and TAM could be used in enhancing checkpoint blockade immunotherapy response and by extension, enhance tumor immunity against non-responsive tumors.

1.2 Statement of the Purpose

In this study, we have selected a very potent small molecule Cucurbitacin B (Cuc. B) which targets STAT3, CSF-1R, and PI3Kγ at lower doses compared to existing specific small molecule inhibitors (Xu et al. 2020; Zhou et al. 2017). Various studies have suggested its potent chemopreventive and chemotherapeutic efficacy alone or in combination with chemotherapeutic drugs against various types of cancers (Garg, Kaul, and Wadhwa 2018; Luo et al. 2019; Mao et al. 2019). However, no study has explored the effect of Cuc. B tumor immunity in cancer. Our novel observations provided us with strong evidence that Cuc. B reprograms TAMs and MDSCs *via* targeting STAT3, CSF-1R, and PI3Kγ and these pathways have been shown to induce immunosuppression in various cancers (Han et al. 2021; Valero et al. 2021; Zou et al. 2020). By employing the use of *in-vitro* culture systems, we propose the M1 phenotype promotion of Cuc. B with a concomitant abrogation of M2 phenotype. On the premise of this compelling evidence, we hypothesize that, Cuc. B treatment could be a novel therapeutic modality in improving tumor immune surveillance and improving checkpoint blockade PD-1/CTLA-4 immunotherapy against non-responsive malignancies.

CHAPTER II

REVIEW OF LITERATURE

2.1 Cancer and Cancer Epidemiology

Cancer is a malignancy characterized by uncontrolled growth of cells due to either the inactivation of tumor suppressors or the activation of protooncogenes, where the cells lose their functional features and have the tendency of invading other sites to cause deleterious health effects (Garg et al. 2018). As rightly discussed in their review, "the hallmarks of cancer", Hanahan & Weinberg (2000) described the six cardinal qualities of cancers namely; limitless replicative potential, evading apoptosis, sustained angiogenesis, insensitivity to anti-growth signals, self-sufficiency in growth signals and tissue invasion and metastasis. Currently, cancer remains the world's leading cause of mortality with nearly 10 million cancer-related deaths and about 20 million new cases have been recorded worldwide in 2020 (Sung et al. 2021). There are an estimated 1.9 million new cases and 609,360 cancer-related deaths in the United States alone for the year 2022 (Siegel et al. 2022). Despite the improving 5-year survival rate for all combined cancers which currently stands at 67%, solid tumors such as pancreatic, liver, and intrahepatic bile duct cancer remain low at 11% and 20 % respectively.

Cancer risk factors are multifaceted, ranging from environmental factors such as air pollution, and exposure to ultraviolet and ionizing radiation, to lifestyles such as tobacco

smoking, alcohol intake, infection like the H. pylori infection, hepatitis (B and C) virus, and other infections and carcinogenic exposures (Sung et al. 2021)

2.2 Tumor Microenvironment (TME)

The TME represents various cell types and structures located within the tumor niche that evolve with the tumor cells and promote the development and growth of the tumor (Junttila and de Sauvage 2013). Within the TME are vascular networks which are produced in response to signals from the tumor, fibroblasts for the maintenance of tissue structural framework, and immune cells such as neutrophils, dendritic cells, T-cells, and monocytes as well as signaling molecules produced by both the tumor and normal cells (Junttila and de Sauvage 2013). Primarily the TME and the tumor cells have bidirectional communication to promote both normal cellular and tissue homeostasis as well as the growth and development of the tumor cells (Quail and Joyce 2013).

Studies have shown that the TME composition changes constantly and this dynamism stems from the changes in the environmental conditions as well as varying oncogenic signals which ensue as the tumor progresses. These changes result in the evolution of the microenvironment toward a pro-tumorigenic state while disrupting the normal cellular interaction within healthy normal adult tissues (Quail and Joyce 2013). During carcinogenesis, immune components such as macrophages and fibroblasts inhibit the growth of immune components of the tumor cells. As the tumor develops, these immune components are then polarized into phenotypes *via* the synthesis of cytokines, chemokines, growth factors, and proteases that have pro-tumorigenic roles which include but are not limited to tumor growth,

invasion of cancer cells, and angiogenesis (Denton, Roberts, and Fearon 2018; Hanahan and Weinberg 2011).

Hypoxia is one of the major hallmarks of the TME and facilitates the growth and metastasis of tumors. Within the developing tumor, there is acute vascular collapse secondary to high interstitial pressure and immature blood vessel formation which culminates in TME hypoxia (Horsman et al. 2012). The vascular endothelial growth factor (VEGF) is one of the proangiogenic cytokines produced by tumor cells to promote angiogenesis under low nutrient and hypoxic conditions which is very typical of the TME (Claesson-Welsh and Welsh 2013). Aside from promoting tumorigenesis, the TME also plays a pivotal role in therapy resistance (Shree et al. 2011; Vitale et al. 2019). The subsequent paragraphs highlight some components of the TME.

2.2.1 Tumor Associated Macrophages (TAMs)

Macrophages are key components of the innate immune system that facilitate immune responses *via* antigen presentation and phagocytosis (Chen et al. 2015). Macrophages found in the tumor milieu may either be tumoricidal (M1) or pro-tumorigenic (M2), but in tumors there is an increase in the polarization of M1 towards the M2 phenotype in the microenvironment as tumors transition to advanced stages (Mantovani and Sica 2010).

TAMs form a key component of the ecology of tumor cells that exhibit pro-tumoral properties. *Via* their immunosuppressive and angiogenic enhancing properties, TAMs can ensure the evolution of tumor cells into malignant tumors by promoting tumor cell growth, motility, invasion, and metastasis (Noy and Pollard 2014). Additionally, TAMs release various growth factors, pro-tumorigenic proteases, and cytokines within the TME which suppresses T-cell

effector functions and promotes the progression of tumors into malignancy. By creating an inflammatory environment, macrophages can promote tumor growth and subsequently promote tumor invasion, migration and angiogenesis during malignant transformation (Qian and Pollard 2010; Szefel, Danielak, and Kruszewski 2019).

Phenotypically, M1 macrophages due to their immunosuppressive effect express various receptors, cytokines, and effectors that mediate their immunosuppressive action. Some of these which are rightly classified as M1 markers include *iNOS/NOS2*, *Il-12, TNF-α, IL-6, IL-12, CD80,* and *CD86*. Pro-tumorigenic M2 macrophages are also characterized by various markers such as *Arg1*, *IL-10, Ym1, FIZZ1,Mrc 1, PPARγ, TGFβ* (Mantovani et al. 2002; Sica and Mantovani 2012).

2.2.2 Myeloid Derived Suppressor Cells (MDSCs)

MDSCs, as defined by Gabrilovich et al., (2017), refer to myeloid cell populations including macrophages and dendritic cells which contribute to generating immunosuppressive TME. (Gabrilovich et al. 2007). Myeloid cells originate from pluripotent hematopoietic stem cells after a series of differentiation, and they have the sole function of promoting both innate and adaptive immunity. However, during cancer, myeloid cells are polarized to MDSCs which contrary to regular myeloid cells, exert an immunosuppressive effect within the TME by inhibiting other immune cells such as the effector T cells and the natural killer cells properties (Gabrilovich 2017; Gabrilovich, Ostrand-Rosenberg, and Bronte 2012; Szefel et al. 2019). Growth factors and chemokines such as the granulocyte–macrophage colony stimulating factor (GM-CSF), CSF-1, and CCL-2 secreted by the tumor cells promote the myeloid cells recruitment (Pollard 2009; Pylayeva-Gupta et al. 2012). MDSCs induce $CD8^+$ cytotoxic T

lymphocyte (CTL) dysfunction which is correlated with poor cancer prognosis(Lechner et al. 2013).

Various growth factors (chemokines and cytokines) such as endothelial growth factor (VEGF), colony stimulating factor (CSF1), macrophage-colony stimulating factor (M-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), stem cell factor (SCF), cyclo-oxygenase2 (COX-2), prostaglandin E2 (PGE2), interleukin-6 (IL-6) and IL-1β secreted by the tumor cells promote the differentiation of monocytes into MDSCs (Dufait et al. 2015; Lechner, Liebertz, and Epstein 2010). Through the induction of reactive oxygen species, these myeloid lineages have been shown to induce nitration of TCR/CD8, resulting in the failure of $CD8^+$ T cells to bind the peptide MHC resulting in tumor escape (Nagaraj et al. 2007). Another suggested mechanism by which MDSCs sufficiently suppresse anti-tumor immunity is *via* arginase-1 and iNOS mediated depletion of L-arginine and production of reactive oxygen and nitrogen species, key components for T-cell receptor maturation needed for T-cell proliferation and interaction with tumor antigens (Szefel et al. 2019; Talmadge 2007). In coherence with this fact, the inhibition of MDSCs was seen to be characterized by the inhibition of inducible nitric oxide synthase (iNOS) and arginase-1 by Dufait *et al*., (2015).

Various phenotypic markers that characterize MDSCs include CD11b, CD11c, CD 206, IL-1β and TNF-α (Umemura et al. 2008). By utilizing RT-PCR Lechner *et al*., (2010) successfully characterized the various immune factors expressed by MDSC which included arginase1, *iNOS, COX-2, TNF-α, VEGF, TGF-β, IL-4, IL-6* and *IL-1β*, using RT-PCR (Lechner et al. 2010). The hallmark of popular chemotherapeutic agents such as gemcitabine, 5-fluorouracil and docetaxel is the depletion of MDSCs (Kodumudi et al. 2010; Vincent et al. 2010).

2.2.3 T Cells

During inflammatory events and cancers, naïve T cells are activated and differentiated into effector T-cells to elicit effector roles to clear implicated antigens after which some of the T-cells persists and are differentiated into memory cell which downregulate effector T-cell function as a regulatory mechanism. The immunosuppressive nature of the TME curtails the effector roles of $CD8^+$ T cells *via* a variety of mechanisms, with a common one being immune checkpoint blockade *via* programmed cell death-1 (PD-1) (Wherry et al. 2007). During tumor progression, T_{reg} cells infiltrate the tumor and promotes tumor immune escape by inhibiting cytotoxic T cell and B cell proliferation. Inhibition of antigen presentation by dendritic cells is also a key immunosuppressive mechanism of Treg cells (Quail and Joyce 2013). T_{reg} cells suppress the function of Th1/TH2/Th17 lymphocytes thereby causing overall immunosuppression (Szefel et al. 2019).

2.2.4 Natural Killer (NK) cells

As a key component of the innate immune system, natural killer cells are one of the principal effector cells against tumor cells. Key cytokines such as interferon γ (IFNγ) released by NK cells promote helper T cell polarization (Chiossone et al. 2018). Also, recruitment of other immune cells such as dendritic cells into solid tumors is enhanced by NK cells *via* the release of some chemokines such as CCL5, XCL1, and 2. NK cells are however key culprits of the immunosuppressive effect of MDSCs and T_{reg} cells during cancer where they inhibit the cytotoxicity of NK cells on tumor cells (Quail and Joyce 2013).

2.2.4 Cancer-associated Fibroblasts (CAFs)

Fibroblasts are major connective tissues responsible for the synthesis and degradation of extracellular matrix components. Contrary to the regulatory roles in inflammation and epithelial differentiation as well as promotion of wound healing by normal fibroblasts, CAFs produce chemokines and growth factors that promote endothelial cell recruitment (Kalluri and Zeisberg 2006). CAFs have been implicated in the initiation, progression, and metastasis of cancer through but not limited to driving HGF and TGFβ expression, stromal cell-derived factor 1 (SDF-1) secretion, and promotion of the release of metastatic genes respectively (Grum-Schwensen et al. 2005; Kuperwasser et al. 2004; Orimo et al. 2005). Most tumors are characterized by an aberrantly over-expression CAFs with distinctive properties from fibroblasts. CAFs express various markers such as α- smooth muscle actin (α-SMA), vimentin, fibroblast activation protein (FAP), fibroblast specific protein (FSP), and other fibroblast-associated proteins (Micke and Ostman 2004; Paulsson et al. 2009).

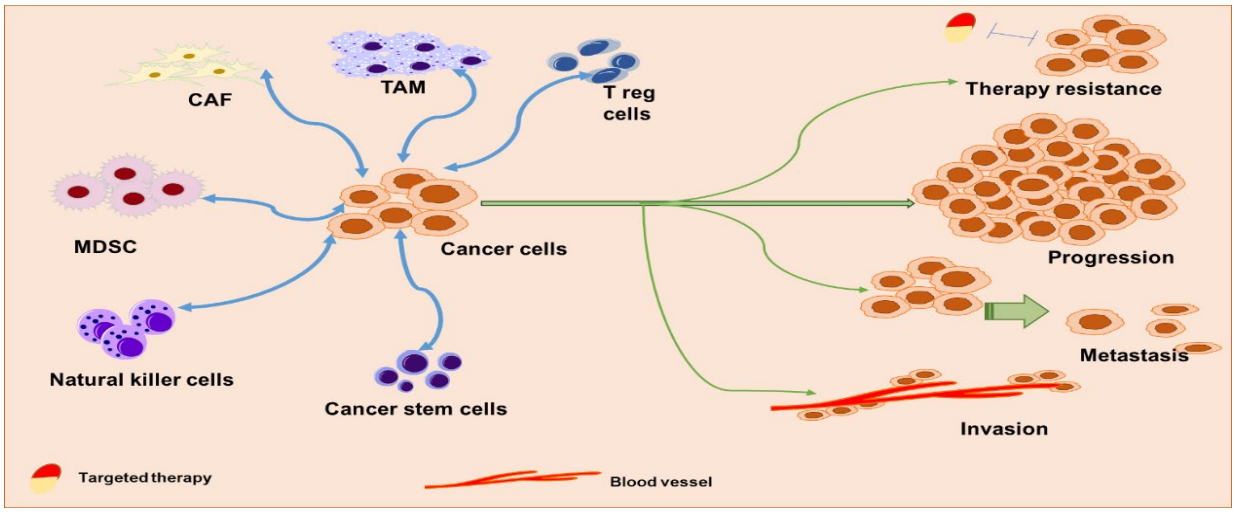

Figure 1.1: Schematic representation of the interplay between components of the tumor microenvironment and tumor cells:
During tumorigenesis, cytokines produced by the tumor cells induces the transformation of various immune cells in the TME which in turn promotes the progression, invasion, metastasis and therapy resistance of the tumor cells. TAM: Tumor Microenvironment, MDSC: Myeloid derive suppressor cells, CAF: Cancer Associated Fibroblast, T reg cells: Regulatory T cells.

2.3 Current Cancer Therapies

Various treatment strategies are adopted based on the type and the stage of cancer advancement. To enhance the effectiveness and response to therapy, some clinicians prescribe treatment regimens that may be a combination of these treatment options. At the core of every cancer treatment option is the aim to completely remove tumorous tissues without causing harm to the normal neighboring tissues. However, a compromise to settle for the reduction of tumors and associated complications to a subclinical state is made when absolute remission is not a viable option (Wang, Lei, and Han 2018). Increasing knowledge in cancer dynamics coupled with various technological advancements have revolutionized cancer treatment strategies over time. We discuss some of the treatment strategies as follows.

2.3.1 Surgery

Surgical procedures involve the removal of cancerous tissues which may end either in the total or partial removal of the cancerous tissues depending on the advancement level of the cancer and the affected areas during surgery. Surgical procedures are often used for biopsies during screening and are the mainstay for localized, early-stage tumors (Wyld, Audisio, and Poston 2015). Gradually, surgical procedures are becoming commonplace in the treatment of non-hematological cancers notable of them is cervical cancer (Peng et al. 2016; Pu et al. 2013).

One of the emerging technologies in surgical oncology is robotic surgery. Particularly in neurosurgery, robot-assisted technologies are utilized in the excision of deep benign astrocytoma. Computer tomography images in the form of interactive 3D displays have aided surgeons in performing complex procedures with greater ease and precision. Robotic surgeries have become the mainstay for biopsies and treatment of breast, abdominal, gynecological, and urological cancers (Hashizume and Tsugawa 2004).

A major disadvantage of surgery as a treatment option for cancer is the incidence of recurrence of cancer shortly after a surgical procedure. For instance, within five years after surgery, 1 in 4 colorectal cancer patients are likely to have recurrence with signs of colon cancer metastasis (van der Bij et al. 2009; Coffey et al. 2003).

2.3.2 Radiotherapy

Cancer radiotherapy is a common treatment modality in cancer management with over 50% of cancer patients known to have had radiation therapy. Patients either receive radiotherapy as a standalone therapy or as a combination therapy with surgery or chemotherapy(Delaney et al. 2005). Radiotherapy in the clinical setting involves the use of an external beam for deep tissue deposition of energy *via* high-energy photons while avoiding the exposure of radiation to the skin, with a typical treatment schedule spanning over 8 weeks where treatment is given in daily fractions. Brachytherapy is a form of internal radiotherapy where the source of radiation is implanted in the tumor (Allen, Her, and Jaffray 2017). Chemoradiation, the concurrent administration of chemo and radiation therapy has been used clinically for the management of various cancers. In chemoradiation, DNA modifying agents (cisplatin and temozolomide) and antimetabolite (gemcitabine and 5-fluorouracil) along with radiosensitizers are administered for improving clinical outcomes against cancer (Wahl and Lawrence 2017).

A major clinical challenge associated with radiation therapy is the induction of hypoxic TME. O_2 is a known radiosensitizer that enhances cancer cell death during radiotherapy by producing free radicals within the tumor cells. Tissue hypoxia in cancer cells causes a reduction in the generation of free radicals needed for the induction of DNA damage (Rey et al. 2017). Clinical application of radiotherapy is also limited to primary tumor with very little use when

tumors have metastasized since therapy for metastasized tumors requires a systemic approach (Ganesh and Massagué 2021). Also, the increased risk of toxicity is a major limitation associated with radiotherapy as the incidence of major side effects such as leukopenia, radiation dermatitis, anorexia, alopecia, oral mucositis and just to mention but a few increases with increasing radiation intensity and duration, thereby placing limitations on the therapeutic window (Bradley et al. 2015).

2.3.3 Chemotherapy

Chemotherapy involves the administration of various cytotoxic chemical agents which are relatively more toxic to pathogens to eradicate tumors or alleviate tumor burden, and this often involves the intravenous administration of cytotoxic drugs (Anon 1937; Nygren 2001). These cytotoxic agents are classified based on their mechanism of action and briefly discussed below are some general classifications of commonly used clinical chemotherapeutic agents. Alkylating agents and alkylators-related agents such as cyclophosphamide and cisplatin respectively elicit their cytotoxicity *via* DNA binding, where they disrupt DNA activity. Antimetabolites (gemcitabine and 5-fluorouracil) are also a class of chemotherapeutic agents that share similar structural characteristics with various biomolecules. These agents can then be incorporated into various cellular synthetic pathways and eventually either halt the synthesis of essential DNA and RNA components of the tumor cells or result in the synthesis of a non-functional end product(Sauter and Gillingham 2020). Topoisomerase inhibitors inhibit the enzyme topoisomerase which is required for the induction of transient single or double-stranded breaks in DNA during DNA replication, thereby inhibiting the overall replicative process within the tumor. Microtubule interacting agents are a class of cytotoxic agents that disrupts normal microtubule formation. Thus, these classes of cytotoxic agents result in an abnormal

microtubular formation which results in the inhibition of eukaryotic cell proliferation thereby promoting cell death (Steinmetz and Prota 2018).

Administration of the chemotherapeutic agents is often used clinically in combination with different agents that attack the cancer cells at different stages of the tumor development to elicit a synergistic effect (Hellyer and Wakelee 2020). The use of chemotherapy can either be adjuvant therapy or neoadjuvant therapy. Adjuvant therapy is administered after initial therapy such as surgery or radiotherapy to eradicate the remaining tumor cells. In neoadjuvant therapy, chemotherapeutic agents are administered preoperatively to reduce tumor size to render them operable (Leow et al. 2014; Miller et al. 2014). Chemotherapy has been the mainstay for the management of most malignancies but developing chemoresistance is a major challenge encountered by onco-clinicians. The molecular mechanism of drug resistance includes increased drug metabolism, increased ABC transporter (P-glycoprotein) that cause efflux of drugs from cancer cells, and increased DNA repair capacity (Bukowski, Kciuk, and Kontek 2020). Various organ toxicity (cardiotoxicity, nephrotoxicity, neurotoxicity and hepatotoxicity) are major challenges associated with the use of chemotherapeutic agents (Gramatyka 2014; Iki and Urabe 2000; Miller et al. 2010; Oun, Moussa, and Wheate 2018). Therefore, the discovery of new non-toxic drugs and the development of new strategies which can reduce the organ toxicity of chemotherapy are in urgent need.

2.3.4 Hormonal Therapy

The use of hormonal therapy (HT) has gained popularity in both gynecological and non-gynecological cancers. HT is based on the premise that some cancers are characterized by an underlying hormonal dysregulation and by correcting this dysregulation, homeostasis can be restored to halt the onset, progression as well as metastasis of cancer. Often in gynecological

cancers, there is a cessation in the synthesis of ovarian hormones in menopausal women whereas younger patients tend to have premature ovarian insufficiency necessitating the need to replace these lacking hormones (Deli, Orosz, and Jakab 2020). In managing breast and other gynecological cancer aromatase inhibitor (tamoxifen) estrogen and/or progestogen preparations are administered either alone or in combination to improve clinical outcomes (Barchiesi et al. 2020; Drăgănescu and Carmocan 2017). In non-gynecological cancers such as prostate, thyroid, and bladder cancer, various hormonal blockers and hormonal regulators are used in their management (Jastrzebska, Gietka-Czernel, and Zgliczyński 2001; Oottamasathien and Crawford 2003).

2.3.5 Targeted Therapies

In cancer therapeutics, targeted therapy involves targeting specific molecular components using drugs to inhibit the growth and proliferation of cancer cells (Lee, Tan, and Oon 2018). As cancer cells develop, they evolve various mechanisms to elude immune surveillance, resulting in their unregulated and uncontrolled growth (Hanahan and Weinberg 2011). In targeted therapy, various molecular alterations that distinguish cancerous cells from normal cells are identified and serve as points of attack using specific agents that attack these alterations (Røsland and Engelsen 2015). These molecular targets include ligands, receptors, genes, growth factors, and various pathways involved in cell cycle, angiogenesis, cell growth, survival, metastasis, and apoptosis (Saijo 2010). The identification of molecular heterogenicity between normal healthy cells and cancerous cells employs the use of various molecular tools for the accurate selection of genes, proteins, and other molecules as targets for therapy(Lee et al. 2018).

The various categories of agents used in targeted therapy include monoclonal antibodies (e.g. Nivolumab), small molecules (e.g. sorafenib), vaccines, and gene therapy(Lee et al. 2018).

Developing resistance is also a major drawback associated with targeted therapy. Since targeted therapy is directed towards a specific molecular target, it becomes inefficacious when there is heterogeneity in the target within the tumor. Moreover, cancer cells can also evolve to be independent of the molecular target resulting in their continuous growth and aggressiveness despite therapy (Lee et al. 2018).

Immunotherapy is currently one of the targeted therapies which has shown very promising results in the management of liver and pancreatic cancer. The human immune system has a cytotoxic potential mediated by tumor-specific T cells that target tumor cells (Kalbasi and Ribas 2020). However, tumor cells evolve to bypass this innate immune response by enhancing the upregulation of the ligands to the inhibitory receptors located on the surface of the cytotoxic T-cells, thereby inhibiting their activation. CTLA-4 and PD1 are key regulatory checkpoints whose ligands are aberrantly overexpressed in TME to suppress cytotoxic T-cell activity (Pardoll 2012). Some evolving cancer therapies aim at targeting the evasive mechanism of tumors by enhancing tumor immune surveillance. Some of these agents have already received Food and Drugs Administration (FDA) approval and are either used individually or in combination with other anti-cancer agents.

Ipilimumab is one of such therapeutic molecules that promote anti-tumor immunity by targeting cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) while activating T cells (Sharma et al. 2011). Nivolumab and lambrolizumab, an antibody to the programmed death 1 (PD1) receptor and ligand respectively are also anti-tumor immune-enhancing agents that promote anti-tumor immunity(Hamid et al. 2013; Wolchok et al. 2013). Cetuximab and bevacizumab, which are inhibitors of EGFR and VEGF angiogenesis respectively are also FDA-approved drugs that are used in the clinical management of cancer (Wilkes 2018).

2.4 Cucurbitacin B.

Cucurbitacin B (Cuc. B) is a natural small molecule in the Cucurbitaceae family which are chemically characterized by the tetracyclic nucleus, 19-(10→9β)-abeo-5α-lanostane-5-ene and a molecular weight of 558.7. The botanical source includes the roots, fruit, and seeds of cucurbitaceous plants such as *Citrullus, Cucumins, Lagenaria and Bryonia* species. Cucurbitacins are crystalline, hydrophobic nature, and readily soluble in organic solvents (Enslin and Rehm 1958; Garg et al. 2018; Kaushik, Aeri, and Mir 2015). Cucurbitacins have been used as anti-inflammatory, analgesic, anti-infective and anti-cancer agents across countries in the Asian subcontinent (Arora and Kaushik 2016; Mallick et al. 2017).

In numerous pre-clinical investigations, Cuc. B has demonstrated encouraging anti-cancer effectiveness against colon, breast, liver, and lung malignancies. Cuc. B has also showed that it has a similar impact as several effective anti-cancer medications (Jayaprakasam, Seeram, and Nair 2003). The same investigation by Jayaprakasam *et al*., (2003) also produced evidence of the anti-inflammatory and the lipid peroxidation inhibitory effect of Cuc. B. There is also evidence that suggests a pleiotropic effect of Cuc. B, inducing apoptosis and cell cycle arrest (Tannin-Spitz et al. 2007).

Cuc. B has been shown to inhibit the activation of both Janus kinase2 (JAK2) and STAT3 signaling pathway and this contributes to the induction of apoptosis and growth inhibition of various cancerous cells (Escandell et al. 2008; Sun et al. 2005; Xie et al. 2016). Zhou *et al*., (2017) reported that Cuc. B in pancreatic cancer where experimental findings showed that Cuc. B effectively inhibits pancreatic cancer cell proliferation by arresting the cells in the G2/M phase of the cell cycle. This growth inhibition was due to the inhibitory effect of Cuc. B on EGFR expression as well as other downstream signaling components such as PI3K/Akt/mTOR and

STAT3 (Zhou et al. 2017). Through its inhibitory effect on PI3K/Akt/mTOR pathway, Cuc. B inhibits epithelial-mesenchymal transition (EMT), a major factor that promotes tumor metastasis, induced by TGF-β which is a regulator of cell growth and differentiation (Yuan et al. 2022). A similar inhibitory effect of Cuc. B in tumor metastasis was observed in hepatoma cells *via* the inhibition of the PI3K/AKT signaling pathway (Zhou X. *et al*., 2012). Findings by Mao *et al*., (2019) also suggested an antiproliferative and apoptotic effect of Cuc. B in colorectal cancer *via* regulation of B-cell translocation gene 3 (BTG3) methylation level (Mao et al. 2019). However, evidence addressing the role of Cuc. B in cancer immunotherapy is very lacking.

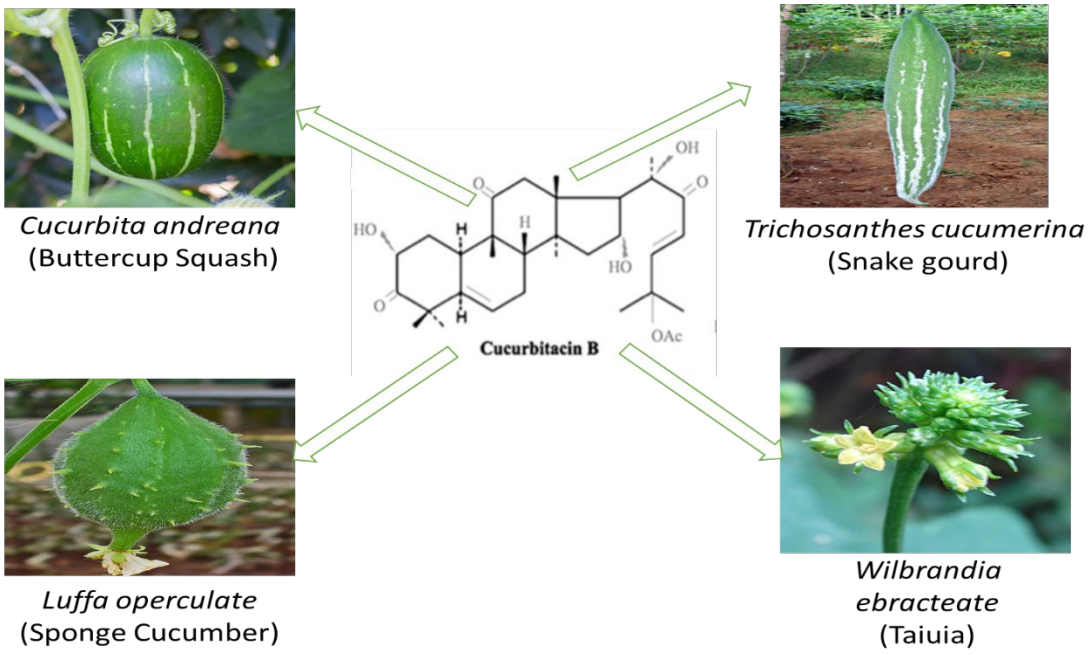

Figure 1.2: Structure and images of the botanical sources of Cucurbitacin B

2.5 Key Molecular Targets of Cuc. B

2.5.1 Phosphoinositide-3-kinaseγ (PI3Kγ) Pathway

Phosphoinositide-3-kinaseγ (PI3Kγ) is a subclass of the PI3K lipid kinase family which act together with the Akt pathway to promote cellular growth and survival. The PI3K/Akt pathway is typically active in a cellular environment where nutrient and oxygen supply is deficient, which exemplifies the TME, making it a key pathway to target in cancer therapy(Porta, Paglino, and Mosca 2014). PI3K along with other signaling components such as the mammalian target of rapamycin (mTOR) and AKT enhances growth, angiogenesis, metastasis as well as therapy resistance in cancer (Porta et al. 2014).

Targeted pharmacological and genetic inhibition of PI3Kγ increased the infiltration of cytotoxic T-cell population and reduce the TAMs and MDSC population within the TME and enhances the immune checkpoint blockade immunotherapy against cancer (De Henau et al. 2016; Evans et al. 2016; Kaneda et al. 2016; Kraehenbuehl et al. 2022).

2.5.2 CSF-1R Signaling Pathway

CSF-1R is a transmembrane tyrosine kinase receptor that stimulates the formation of macrophages and/or granulocytes from hematopoietic precursors (Smith et al. 1995). This receptor which is encoded by the c-fms proto-oncogene stimulates the differentiation and proliferation and of monocytes/macrophages whiles sustaining the protumorigenic effect of TAMs (Fujiwara et al. 2021; Stanley et al. 1983). CSF1-R is activated by the colony-stimulating factor 1 receptor (CSF-1R) which is a polypeptide growth factor (Rettenmier, Roussel, and Sherr 1988). The overexpression of the CSF-1 ligand and its receptor has been observed in various cancers. CSF-1 induces down-regulation of granulocyte-specific chemokine expression in CAFs.

This crosstalk mediated by HDAC2 causes a decreased granulocyte recruitment to tumors thereby enhancing the tumorigenicity (Kumar et al. 2017).

CSF-1 promotes polarization of macrophages into the protumorigenic M2-TAMs. A study by Fujiwara and colleagues showed that BMDM chemotaxis is enhanced upon CSF1-mediated phosphorylation of ERK ½ in BMDMs which also promotes polarization towards M2 phenotype (Fujiwara et al. 2021). Targeting CSF1/CSF1R signaling pathway, increased stimulation of $CD8^+$ T cells with a concomitant reduction in the polarization, chemotaxis, and survival of TAMs (Fujiwara et al. 2021).

2.5.3 STAT 3 Pathway

STAT 3 is a subtype of the family of signal transducers and activators of transcription (STATs), which are latent cytoplasmic transcription factors that regulate transcription either directly or indirectly *via* the transduction of extracellular signals (Darnell 1997). The biological functions of STATs include regulations of cell cycle progression, proliferation, differentiation, and apoptosis (Battle and Frank 2002; Bromberg and Darnell 2000; Silva 2004). Various growth factors (chemokine and cytokines) such as interleukin 6, interferons and EGFphosphorylate STATs. STAT3 phosphorylation can occur at either the tyrosine site, as an early event where the transcription factors are activated or at serine site (Ser-727) for the enhancement of transcriptional activity (Turkson and Jove 2000). The phosphorylated STAT 3 then undergoes homodimerization or heterodimerization and subsequently translocates into the nucleus to promote transcription of various genes linked to proliferation, invasion and apoptosis inhibition of cancer cells (Sun et al. 2005).

The transcriptional activity of STAT is highly regulated in normal cells however in tumor cells, STAT is dysregulated and the constitutive activation of STAT particularly STAT3 plays a

key role in the increased proliferation of tumor cells (Lehmann et al. 2003; Turkson and Jove 2000). The constitutive activation of STAT3 as observed by Bromberg et al. (1999) is capable of mediating the transformation of cells and formation of tumors in nude mice. Dysregulated STAT3 activation has also been associated with the pathogenesis of hematological and solid tumors, presenting STAT3 as a good target in cancer therapy (Csomós et al. 2021; Yu and Jove 2004). In this thesis, we for the first time evaluated the effect of Cuc. B on PI3Kγ/CSF-1R/Stat3 signaling pathways in tumor cells and how targeting these signaling components impact on the phenotypes of MDSc and TAMs.

CHAPTER III

MATERIALS AND METHODS

3.1 Cell lines, reagents, and treatments

Murine macrophages cell line Raw 264.7 was obtained from American Type Culture Collection (ATCC, TIB-71, Manassas, VA) and cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated FBS (Life Technologies, Grand Island, NY), 1x Antibiotic-Antimycotic and incubated at 37°C in a humidified environment of 5% CO_2. Cuc. B was purchased from Cayman chemical (Ann Arbor, MI) and dissolved in Dimethyl Sulfoxide (DMSO, Sigma, St. Louis MO). Interleukin-4 (IL-4) (Sigma, St. Louis, MO) was dissolved in 1X PBS and used at a concentration of 20 ng/mL. GAPDH (21185), AKT (9272s), iNOS (13120s), PI3K (p110) gamma (4252s), CD206/MRC1 (24595s), p-STAT3 (94994s), p-CSF-1R-MCSFR (3155s), MCSF(28917s) PDL-1 (13684s) were purchased from cell signaling. STAT3 (ad119352) was purchased from Abcam. pAKT (sc 514032), Arg 1 (sc47715) were purchased from Santa Cruz Biotechnology. A complete list of reagents used in this study is shown in supplementary (Table 2).

3.2 MTT Assay

A colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazoliumbromide (MTT) was used to determine the cytotoxicity of Cuc. B towards Raw 264.7 murine macrophage

cells. Briefly, cells were seeded at a density of 5×10^3 /well in 96-well plates and cultured until cells were 75-80% confluent. Confluent cells were then treated with the respective compounds as indicated and followed with the addition of MTT at a concentration of 5mg/mL at a volume of 20 µL per well for at least 2 hours at 37°C. Media was then removed and replaced with DMSO (150µL per well) and incubated at room temperature for 15 minutes on a shaker. Optical density (OD) was recorded at 570nm using a spectrophotometer.

3.2 Endotoxin Assay

Endotoxin concentration was determined with ToxinSensor Endotoxin detection system (GenScript, Piscataway, NJ) to account for any potential endotoxin contamination following manufacturer's instructions and endotoxin levels were found to be below 0.01eu/mL (0.004 eu/mL) endotoxin unit for Cuc. B.

3.4 *In-vitro* isolation of BMDMs from murine bone marrow

We used discarded C57BL/6J mice from our approved UTRGV-IACUC protocol to isolate BMDMs. Briefly, BMDMs were isolated using a protocol developed by the Liza Makowski Lab (UTHSC, Memphis, TN). Briefly, bone marrow cells from the femur and tibia bone of C57BL/6 J mice were isolated and grown in two 150mm plates containing 20mL BMDM complete media (RPMI supplemented with 10% FBS, 1% pen./strep, 10 mM HEPES, 2mM GlutaMAX, 20ng/mL recombinant mouse M-CSF) after RBC lysis using RBC lysate buffer. Ten mL BMDMs complete media was added to each plate on day 4 and the cells were allowed to grow for 2 more days after which the cells were passage into plates for further treatments and analysis. Raw 264.7 cells were also used in generating TAMs as described by Liu *et al*., (2019).

3.5 *In-vitro* differentiation of MDSCs from murine bone marrow

MDSCs were generated according to the protocol by Weber *et al*., (2020) and (Marigo et al. 2010). Briefly, bone marrow cells from the femur and tibia bone of C57BL/6 J mice were isolated and 2.5×10^6 cells were culture in 100mm plates with 10mL MDSC complete media (RPMI supplemented with 10% FBS, 1% pen./strep, 10 mM HEPES, 1mM sodium pyruvate, 1mM non-essential amino acids, 40ng/mL recombinant mouse IL-6 and 40ng/mL recombinant mouse GM-CSF) and treated with the needed compounds after day 4 for as required.

3.6 Western immunoblot analysis

Cells were seeded in 60mm or 100 mm plates and treated with the required treatments and treatment duration after cells attached and grew 75-80% confluent. After treatment duration, cells were collected in RIPA lysis Buffer System (Santacruz Biotech., Dallas, TX), sonicated, centrifuged and a bicinchoninic acid (BCA) assay was used to quantify 40µg protein from the supernatant. The protein was then separated using 10% SDS-PAGE with a subsequent transfer of the separated proteins unto a PVDF membrane. Proteins were then blocked in 10% skim milk in TBST (0.1 % Tween 20-Tris buffered saline) at room temperature for one hour after which the primary antibodies were incubated with the membranes overnight. Membranes were washed in TBST buffer and incubated in HRP-conjugated secondary antibodies for an hour at room temperature. Membranes were incubated in chemiluminescence reagents for 30 seconds and images were taken using ChemiDoc^{TM}MP (Bio-rad, Hercules, CA).

3.7 Quantitative real time PCR (qRT-PCR)

Total RNA was extracted from cells after treatment as described earlier using TRIzol (Life Technologies, Carlsbad CA). The purity and concentration of isolated RNA was determined using Nanodrop One^{C} (Thermo Scientific, Madison, WI). 2000ng RNA was used to

synthesize cDNA RNA using High-Capacity cDNA Reverse Transcription Kit (Thermofisher, Graiciuno, LT). RT-PCR was ran using CFX 96 Real Time System™ (Bio-Rad Inc., Singapore) to determine the expression level of various genes normalized to the fold change of GAPDH.

3.8 Immunofluorescence Assay

Cells were cultured in Lab-Tek IICC2 4-well chamber slide system (Thermo Fisher Scientific, Rochester, NY). After duration of treatment, cells were fixed with 4% Paraformaldehyde for 15 minutes at 4°C and permeabilized with 0.2% triton X-100 for 5 minutes. Cells were then blocked using 10% donkey serum at room temperature for 1 hour and then incubated in the respective primary antibody overnight at 4°C. Cells were then washed in TBST (1x tris-buffered saline, 0.1% tween 20) and incubated in the secondary antibody (Alexa Fluor 488-conjugated antibody, Jackson ImmunoResearch) for one hour at room temperature, washed and mounted in antifade mounting medium with DAPI (Vectashield, Burlingame, CA). Images were taken and analyzed using the Nikon AX confocal microscope system.

3.9 *In-vitro* phagocytosis and killing assays

In-vitro phagocytosis assay was performed using Vybrant Phagocytosis Assay Kit (ThermoFisher Scientific) according to the manufacturer's protocol. Briefly, BMDM plated at a density of 100,000 cells per well in a 96 well plate (Corning) in complete RPMI-1640 medium were pre- treated in groups with Cuc. B., IPI-549 and IL-4 overnight then media was replaced by 100 µL of fluorescent BioParticle suspension containing fluorescent E. coli bioparticles. Cells were then incubated at 37°C 5% CO2 for two hours, washed twice with 1X PBS to remove non-phagocytosed particles, resuspended in 1X PBS and fluorescence was analyzed using FITC channel on EVOS m7000 Cell Imaging (Thermo Fisher Scientific).

3.10 Statistical Methods

All data obtained during this study were presented as Mean ±Standard error of mean (SEM). Statistical analyses were done using unpaired, two-tailed student's *t*-test and one-way analysis of variance (ANOVA) with the level of significance set at *p*-values < 0.05 with the levels of significance represented respectively as follows *p< 0.05, **p<0.01, ***p<0.001. All graphs were generated using GraphPad Prism (5.03, GraphPad Software, Inc., La Jolla, CA, USA).

CHAPTER IV

RESULTS

4.1 Cuc. B exhibits a dose-dependent antiproliferative effect on Raw 264.7 cells

To assess the effect of Cuc. B on the viability of Raw 264.7 cells, cells were treated with Cuc. B at different concentrations for 24 and 48 hours respectively in the presence of IL-4 (20ng/mL) and cell viability was measured using MTT at O.D. 570nM. As shown in Figure. 1, Cuc. B produced a significant ($p<0.05$) dosage dependent decrease in the viability of Raw 264.7 cells (Figure 3.1) confirming the cytotoxic effect of Cuc. B on M2 polarized macrophages. IC_{50} was determined to be 500 nM and 250 nM at 24 and 48 hours respectively.

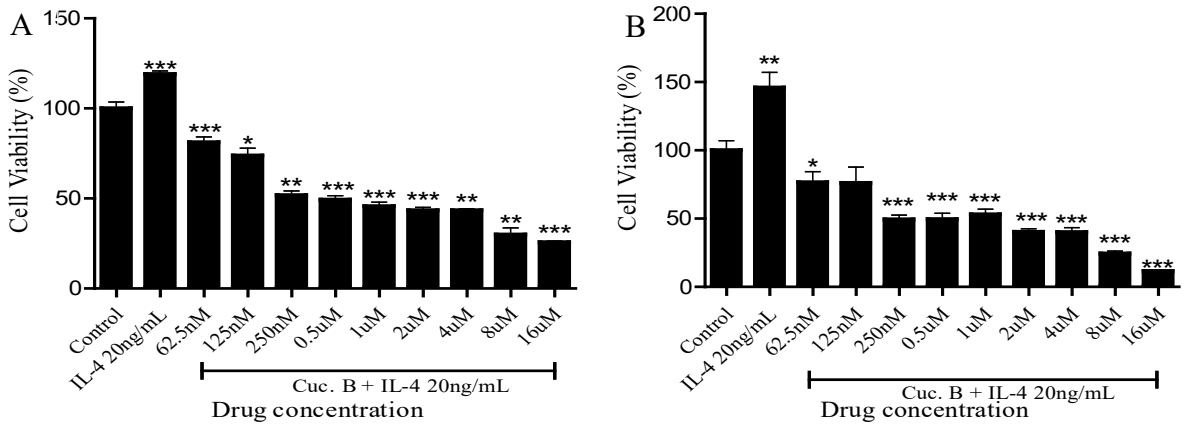

Figure 3.1 Cuc. B treatment exhibits cytotoxic effect on Raw 264.7 cells:
Raw 264.7 cells were treated with IL-4 (20ng/mL) or indicated concentrations of Cuc.B for24 hours (A) and 48 hours (B) Cell cytotoxicity was determined by MTT assay. Data are expressed in terms of percentage of control (t 100%) ±SEM (n=8). Comparisons were made by applying ANOVA followed by Dunnett's post hoc test; *p< 0.05, **p<0.01, ***p<0.001 were considered significant as compared to control group.

4.2 Cuc. B batch used was devoid of endotoxin contamination

To determine if the effect of Cuc. B is not attributed to any form of endotoxin contamination, endotoxin concentration in Cuc. B determined and endotoxin levels were found to be 0.004 eu/mL for Cuc. B. (Figure 3.2) which is below the minimum allowed endotoxin concentration (0.01eu/mL). From this, we can infer that all the results produced from Cuc. B treatment is solely due to the effect of Cuc. B.

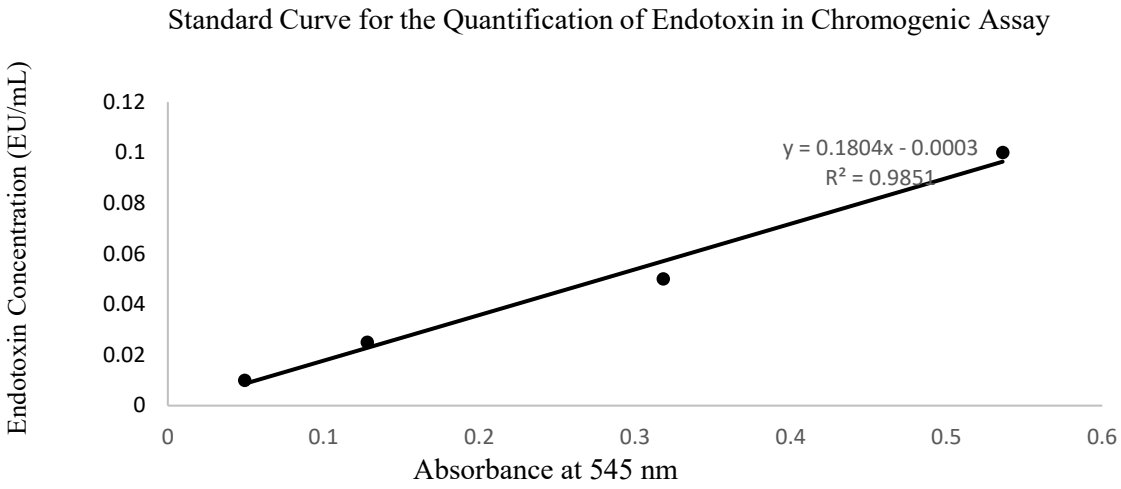

Figure 3.2: Cuc. B has no endotoxin contamination:
Endotoxin concentration in Cuc. B was determined using endotoxin kit by GeneScript. Results indicated shows endotoxin concentration of Cuc. B was found to be to be 0.004 EU/mL

Table 1: Table showing the respective absorbances and corresponding endotoxin concentrations of LAL standard samples and Cuc. B

Sample	Average Absorbance	Change in Absorbance	Conc. in EU/ml
LAL reagent water (Blank)	0.051	0	-
0.1 EU/ml Standard	0.587	0.536	0.1
0.05 EU/ml Standard	0.369	0.318	0.05
0.025 EU/ml Standard	0.179	0.128	0.025
0.01 EU/ml Standard	0.100333	0.049333	0.01
Cuc. B	0.073667	0.022667	0.0038

4.3 BMDMs and MDSCs were successfully generated form murine one marrow

In-vitro isolation of BMDMs and MDSCs was successfully performed after growing isolated murine bone marrows in BMDM complete media for 7 days and MDSCs complete media for 5 days respectively. Our results demonstrated that these cells were differentiated (Figure. 3.4). These cells were then used for our experiments.

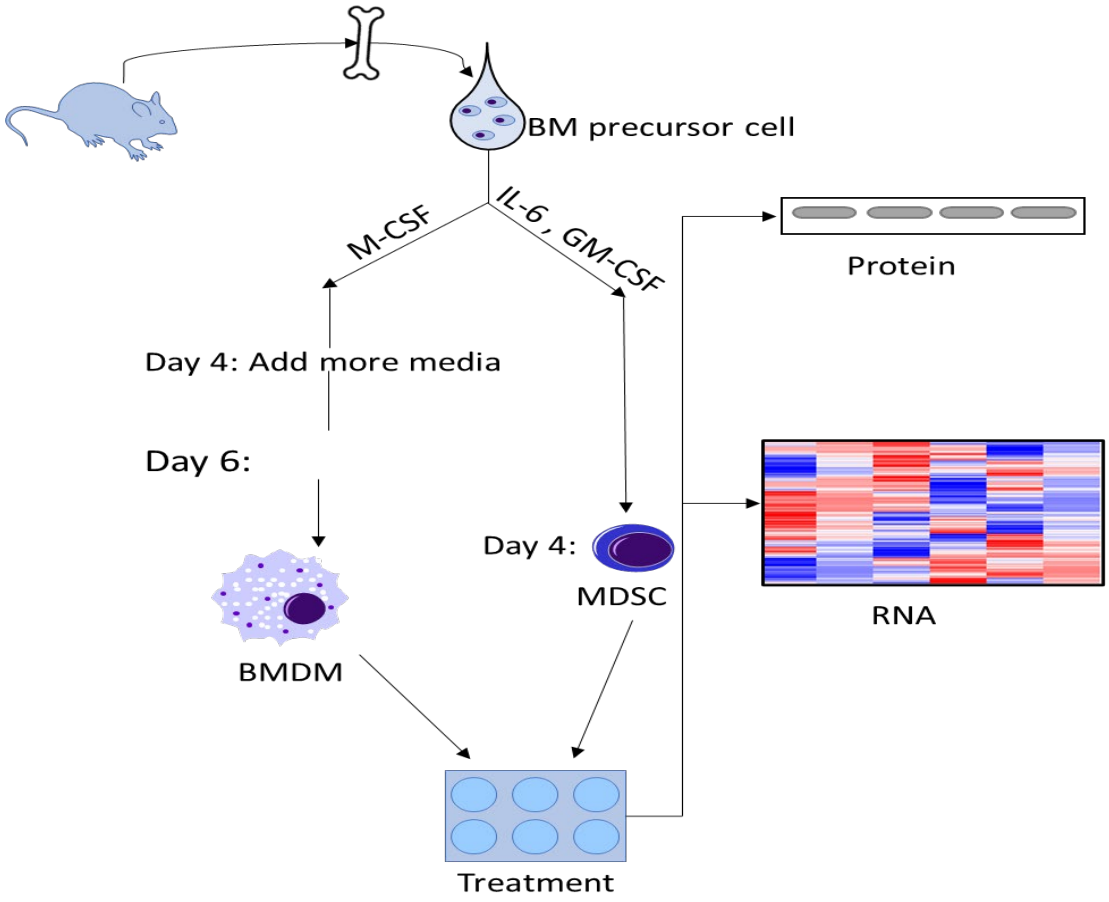

Figure 3.3: Schematic representation of steps for the *in-vitro* generation of BMDM and MDSC from murine macrophages

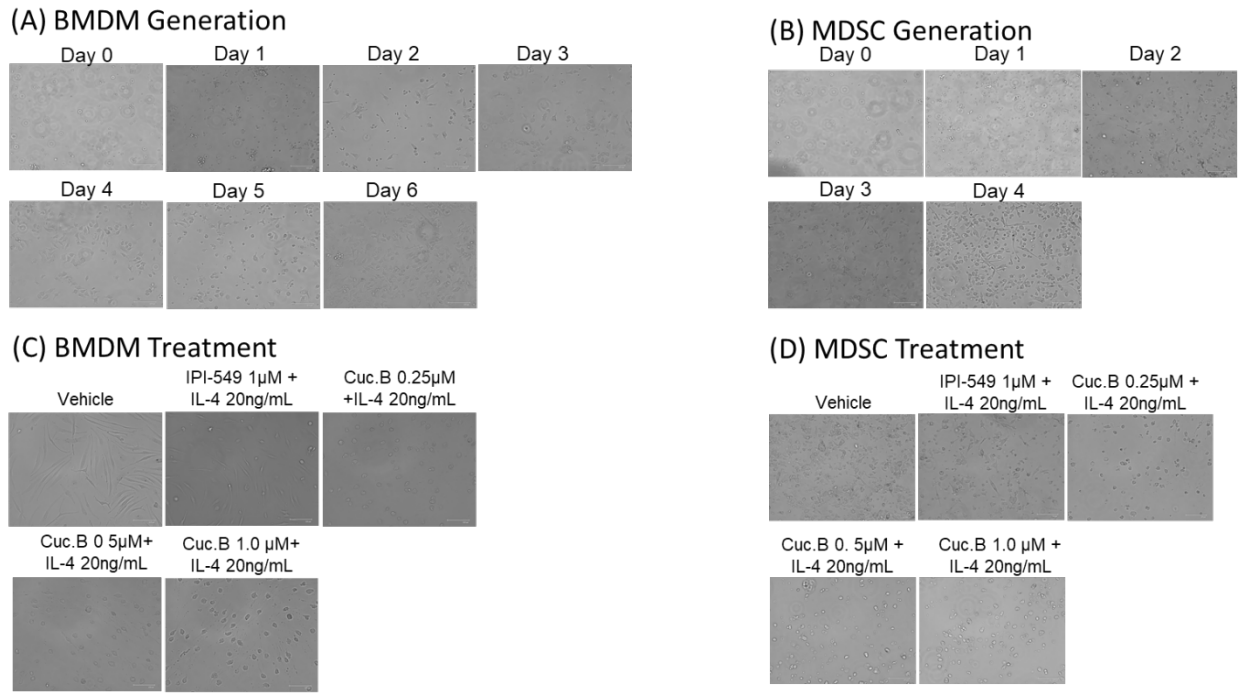

Figure 3.4: Representative images of *in-vitro* generation and treatment of BMDMs and MDSCs from mouse bone marrow.

Representative images are showing the phenotypic changes of isolated macrophages during *in-vitro* generation of BMDMs (A) and MDSCs (B) and images after 24 hours treatments (C&D) of PI3Kγ inhibitor (IPI-549 1μM) and Cuc. B (0.25 - 1.0μM) in the presence of IL-4 20 ng/mL or vehicle for BMDMs and without IL-4 for MDSCs

4.4 Cuc. B treatment restores the phagocytic capacity of TAMs

The main function of macrophages is to phagocytize foreign pathogens, and apoptotic bodies generated after apoptosis induction or killing tumor cells. To investigate the effect of Cuc. B on phagocytic capacity, we first polarized BMDMs into TAMs with the treatment of IL-4 (20 ng/mL) for 24 hours and then treated with PI3Kγ inhibitor (IPI-549 1µM) and Cuc. B (0.25µM) in the presence of IL-4 (20 ng/mL). Control group cells were treated with the vehicle. The phagocytic capacity of BMDMs was determined by quantification of bioparticles within macrophages after washing off non-ingested particles. We observed a significant ($P<0.001$) reduction of phagocytosis of bioparticles by IL-4 treated BMDMs, which was significantly recovered by Cuc B treatment at a lower concentration. However, IPI-549 was unable to significantly recover this phagocytic effect of BMDMs (Fig. 3.5). These results strongly suggest that Cuc. B treatment has the potential to enhance the innate immune response to kill tumor cells.

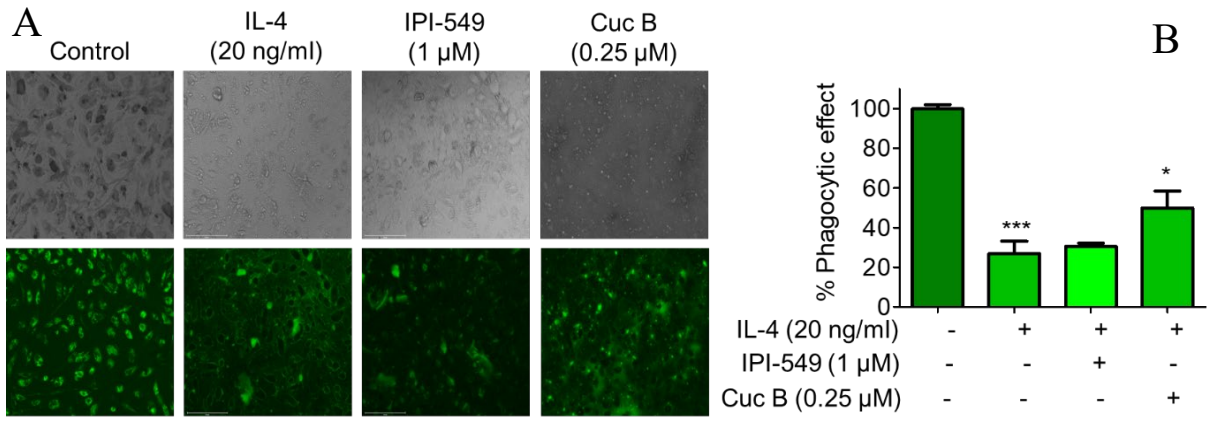

Figure 3.5: Cuc. B promotes the phagocytic capacity in macrophages *in-vitro:*

(A) Representative image of phagocytosis of fluorescent *E. coli* bioparticles by BMDMs with the treatment of PI3Kγ inhibitor (IPI-549 (1µM)) and Cuc. B 0.25µM, in the presence of IL-4 (20 ng/mL) or vehicle (control) as determined by fluorescent microscopy imaging on the FITC channel. B. Quantification of phagocytosis BMDMs in indicated groups. Data shown in the bar graph represents Mean ± SEM of triplicate well. *$p< 0.05$, **$p<0.01$, ***$p<0.001$ values were considered as significant as compared to control group.

4.5 Cuc. B abrogates M2 polarization of macrophages and promotes M1-like phenotype

To investigate whether Cuc B has the potential to inhibit M2 polarization and increase of M1 macrophage phenotype, We initially performed these experiments in polarized M2-TAMs murine macrophage cell line (Raw 264.7) and then in polarized BMDMs. In this experiment, RAW264.7 and BMDMs were treated with IL-4 (20 ng/ml). Control and treatment group cells were treated with vehicle(DMSO) and Cuc. B (0.25-1.0 μM) or IPI-549 (1μM) for 24 hours. In our first experiment, Cuc B treatment inhibited the expression of M2 markers (Arg I and IL-10) in Raw 264.7 cells (Fig. 3.7 B). It has been demonstrated that TAMs secrete PDL-1 which neutralizes the function of T-cells. Therefore, we determined the effect of Cuc B on the expression of PDL-1 in RAW264.7 cells. Results demonstrated a significant ($P<0.001$) decrease in PDL-1 expression in RAW264.7 cells. We further determine the effect of Cuc B on the expression of PI3Kγ and iNOS. Surprisingly, we observed that Cuc B treatment abolished the protein levels of PI3Kγ in RAW264.7 cells as determined by confocal microscopy (Fig.3.7A) and Western blot analysis (Fig. 3.7C).

In-vitro generated MDSCs cells were also cultured and treated with vehicle (DMSO), Cuc. B or IPI-549 for 24 hours. We further performed detailed experiments in M2-polarized BMDMs. We first determined the expression of various markers of M1 and M2 by qPCR. Cuc B treatment showed a dose-dependent significant ($P<0.01$-0.001) decrease expression of various M2 markers (Arg1, Ym1, FIZZ1, PPARγ, and TGFβ) and a significant ($P<0.01$) increase in M1 markers (NOS2, IL-6 and CD11C) compared to IL-4 alone treatment group (Figure 3.6 A-B) Cuc B also inhibited the protein level of CD206 in BMDMs (Fig.3.6 D) as determined by Western blot analysis. We observed a significant decrease of MCSFR, AKT, PI3K p110γ and p-

STAT3) in IL-4 polarized BMDMs treated with Cuc B when compared with IL-4 alone treatment (Figure 3.6 (D). These results strongly suggest that Cuc. B can inhibit the pro-tumorigenic potential of TAMs by targeting CSF/CSF1R, Stat3, PI3Kγ, and Akt signaling pathways.

4.6 Cuc. B has more potential in targeting TAMs compared to IPI-549

A comparative study was performed using IPI-549, a known potent inhibitor of PI3Kγ (p110γ) (Evans et al. 2016). From the results obtained, a marked decrease in protein expression of MCSFR was observed in IL-4 + Cuc. B treated BMDMs compared to BMDMs -treated with IL-4+ IPI-549, as determined by confocal microscopy (Figure. 3.8 A). Cuc B treatment also showed a marked decrease in the expression of STAT3, CSF1, CSFR, and PI3Kγ in IL-4 polarized BMDMs. This effect was more significant as compared to IPI-549 (Figure 3.8 B)

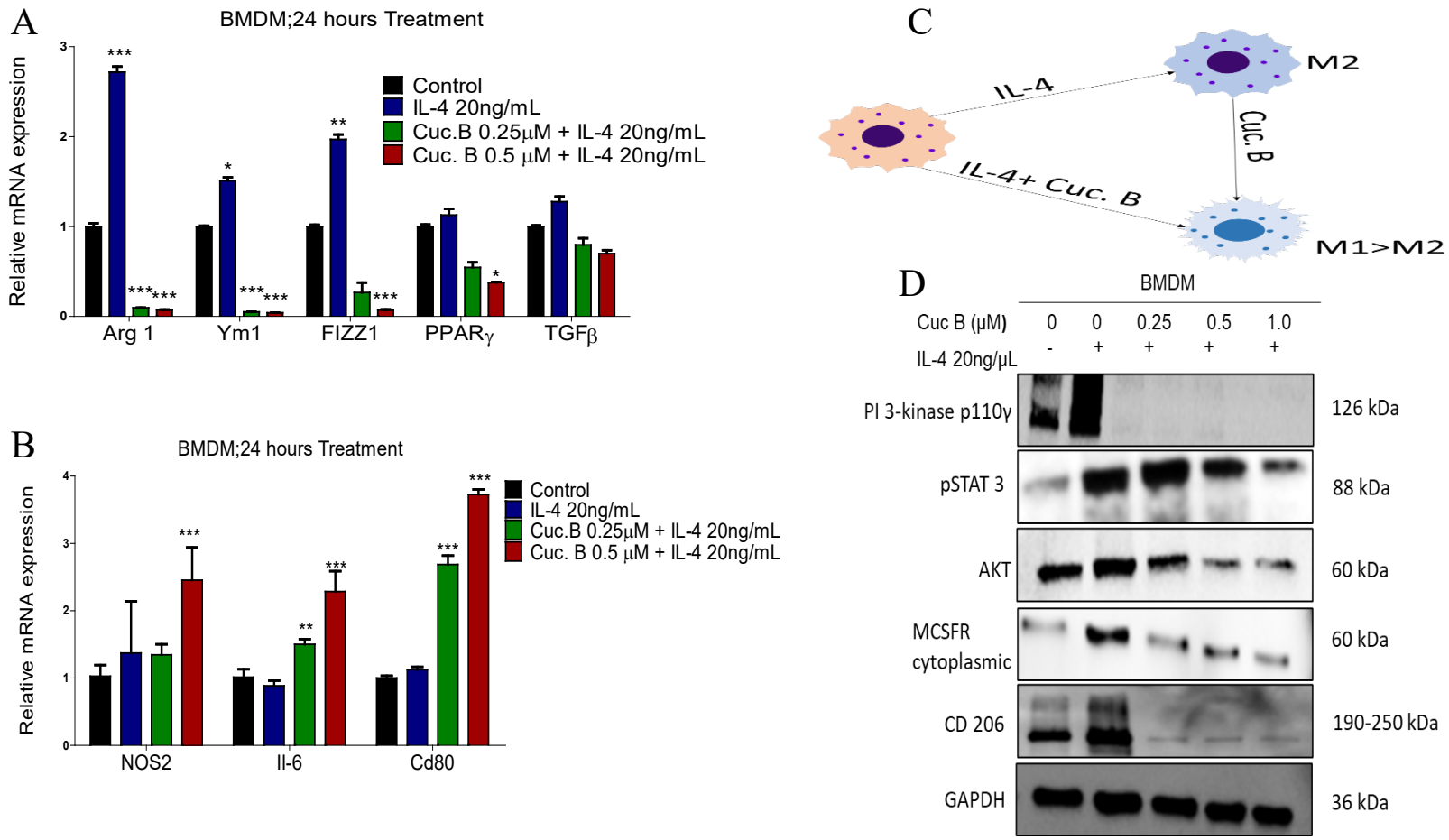

Figure 3.6: Cuc. B inhibits M2 polarization of BMDM and promotes M1-like phenotype *in-vitro*:

Relative mRNA expression of M1 markers (A) and M2 markers (B) in BMDM by p-PCR analysis and protein levels by Western blot analysis (D) in BMDMs after 24-hour treatment with Cuc. B (0.25 -1.0 μM) in the presence of IL-4 (20 ng/mL) or vehicle (control). Schematic representation of inhibitory effect off Cuc. B on IL-4 induce polarization of BMDM (C). Values in bar graph shows Mean ± SEM of biological triplicate. *p< 0.05, **p<0.01, ***p<0.001 were considered as significant compared to IL-4 treated group.

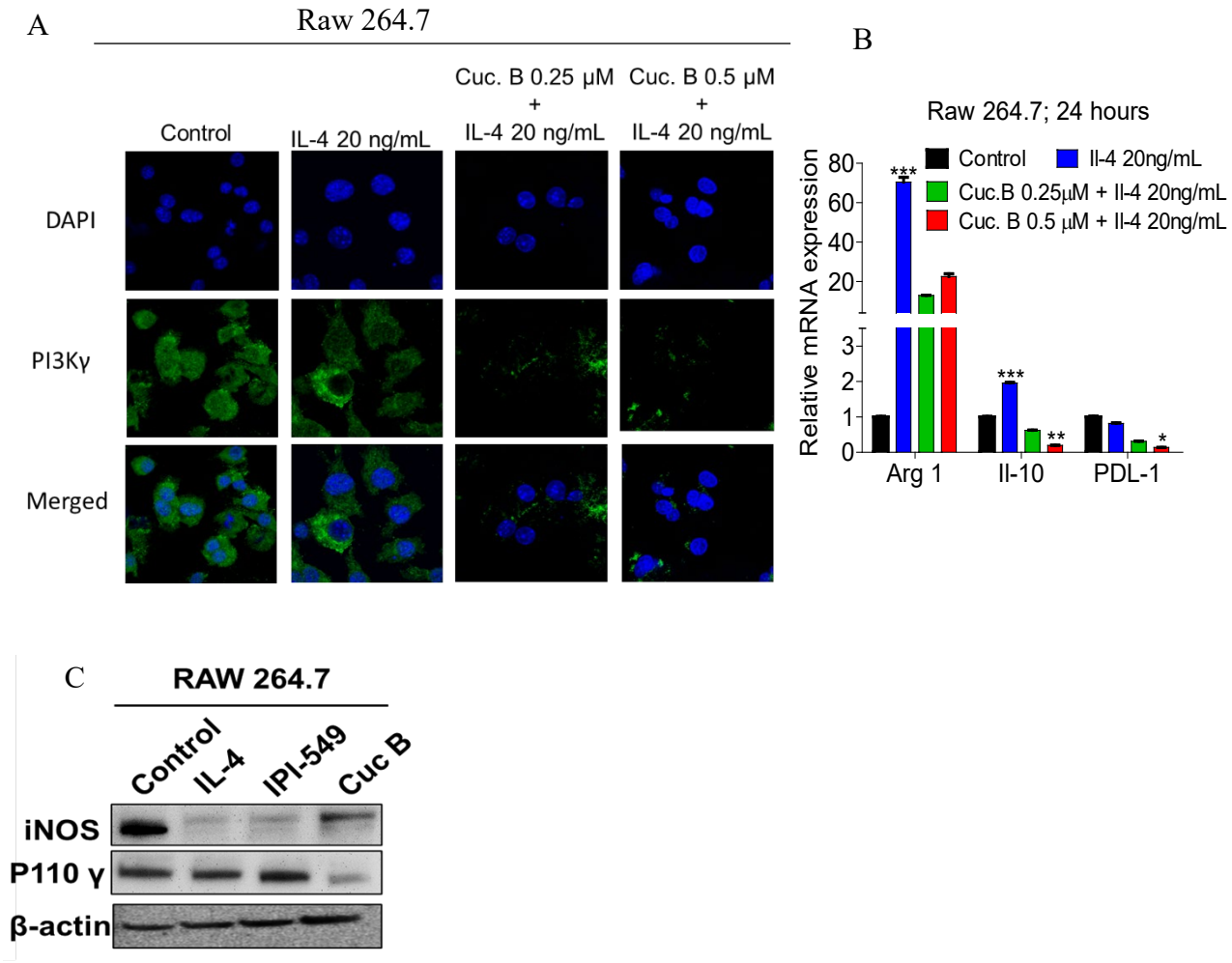

Figure 3.7: Cuc. B inhibits M2 polarization of Raw 264.7 M1-like phenotype *in-vitro*:

Confocal microscopy images (A) Relative mRNA expression (B) by RT-PCR analysis and protein expression by western blot (C) in Raw 264.7 cells after 24-hour treatment with PI3Kγ inhibitor (IPI-549 (1µM)), Cuc. B (0.25µM and 0.5µM) in the presence of IL-4 20ng/mL or vehicle (control). Mean ± SEM shown. *p< 0.05, **p<0.01, ***p<0.001 compared with control

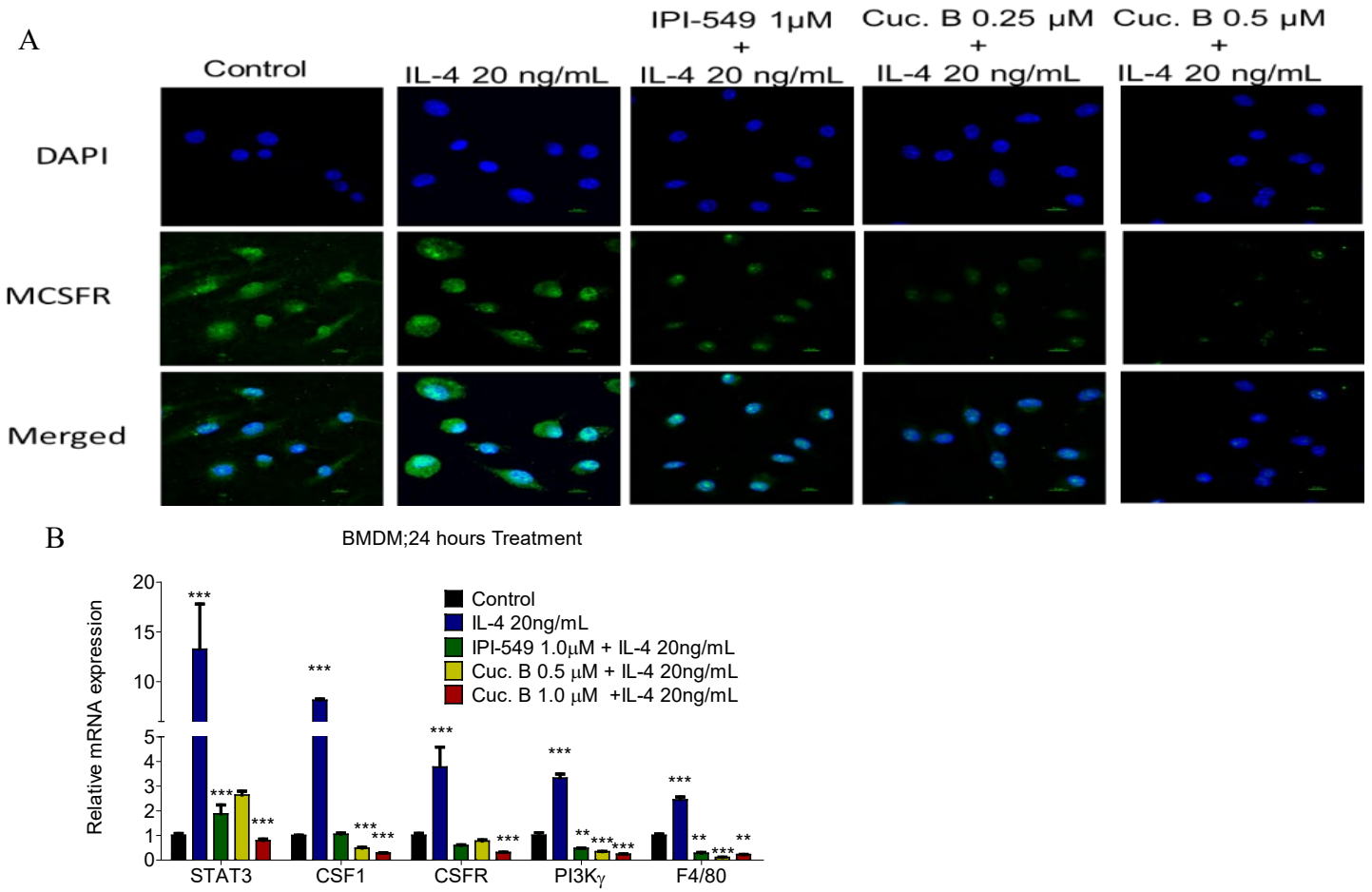

Figure 3.8: Cuc. B shows TAM suppressive effect comparable to PI3Kγ inhibitor, IPI-549:

Representative confocal microscopy images showing the expression of MCSFR (A) and relative mRNA expression of TAM markers (B) in BMDM by RT-PCR analysis after 24-hour treatment with PI3Kγ inhibitor (IPI-549 (1μM)), Cuc. B (0.25μM and 0.5μM) in the presence of IL-4 20ng/mL or vehicle (control). Mean ± SEM shown. *p< 0.05, **p<0.01, ***p<0.001 compared with control.

4.7 Cuc. B suppresses bone marrow derived MDSCs activity *in-vitro*

MDSCs are one of the key tumor immune cells which create immunosuppressive TME to facilitate the growth and metastasis of tumor. Thus, targeting these cells help in suppressing the growth and metastatic phenotypes of cancer. We further investigate the effect of Cuc. B on the bone marrow derived MDSCs. In this experiment, MDSCs were treated with Cuc. B (0.25 - 1.0μM) or IPI-549 (1μM) or vehicle (control). Protein expression by western blot analysis showed a dose-dependent decrease in expression of Arg-1, Stat3, and CD206 when compared to control. We also observed a marked decrease in the protein levels of Stat3, pAKT and CD206 compared to control. These results strongly suggest the potential suppressive effect of Cuc. B on MDSCs.

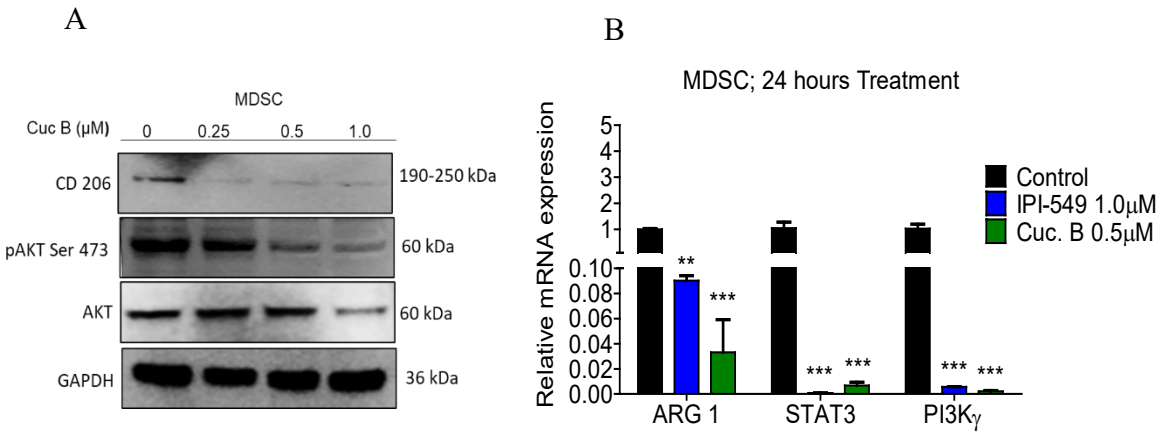

Figure 3.9: Cuc. Suppresses MDSC activity *in-vitro*:
Protein expression by western blot (A) and relative mRNA by pRT-PCR analysis (B) showing a decrease in the expression of various MDSC markers after 24-hour treatment with Cuc. B (0.25μM, 0.5μM and 1.0μM) or IPI-549 1μM in the presence of IL-4 20ng/mL or vehicle (control). Mean ± SEM shown. *p< 0.05, **p<0.01, ***p<0.001 compared with control

CHAPTER V

DISCUSSION

The tumor microenvironment remains a very important component that influences tumor development, growth and progression (Denton et al. 2018; Hanahan and Weinberg 2011). Various immune components such as TAMs, CAFs, MDSCs, neutrophils, dendritic cells, T-cells that are transformed in the tumor milieu during tumorigenesis promote tumor progression and spread of tumors (Kalluri & Zeisberg, 2006; Junttila & de Sauvage, 2013). Additionally, response to therapy is greatly influenced by the components of the TME (Shree et al. 2011; Vitale et al. 2019). This presents targeting of the TME as an attractive treatment modality in cancer therapeutics. Clinically, targeted therapies that focus on ceasing the progression of changes as well as restoring alterations in the tumor immune landscape during tumorigenesis are producing good clinical outcomes. Notable amongst these agents include checkpoint blockade inhibitors anti-CTLA4 and PD1 antibody (Hamid *et al*., 2013; Sharma *et al*., 2011).

Cuc. B., a triterpenoid of the Cucurbitaceae family has extensive evidence supporting its anti-tumor effect in various solid tumors *via* a myriad of suggested mechanisms such as induction of apoptosis and cell cycle arrest (Jayaprakasam *et al*., 2003;Tannin-Spitz *et al*., 2007). Multiple studies have reported on the inhibitory effect of Cuc. B on various pathways that promote tumor progression and metastasis such as the Janus kinase2 (JAK2)/STAT3 and the PI3K/Akt/mTOR pathways in various tumors (Xie *et al*., 2016; J. Zhou *et al*., 2017).

Interestingly, these pathways have also been reported to mediate immune checkpoint blockade immunotherapy resistance against tumors (Cannarile et al. 2017; O'Donnell et al. 2018; Zou et al. 2020). In this study, we investigated the Cuc. B potential in targeting key tumor immune cell populations (TAMs and MDSCs) which are linked to generating immunosuppressive TME and checkpoint blockade immunotherapy resistance and failure against various tumor types.

Macrophages are undoubtedly a major component of the innate immune system that opposes the progression and spread of cancer and any treatment modality that promises to improve macrophage immune function promises to be essential artillery in the battle against cancer (Mantovani and Sica 2010). Our findings strongly suggest that Cuc. B has the potential of targeting both TAMs and MDSCs which was evidenced by a significant decrease in various markers of M2-TAMs and MDSCs. Cuc B treatment restored the phagocytic potential of M2 polarized BMDM. These results suggest the likely restoration of the functionality of macrophages upon repolarization of TAMs into M1 phenotype after Cuc. B treatment. Cuc. B produced results comparable to, IPI-549, an inhibitor of PI3Kγ, which is also producing significant preclinical results in cancer immunotherapy (De Henau et al. 2016). This study provides convincing evidence that paves the way for further studies into the role of Cuc. B in tumor immune surveillance and its role in enhancing tumor immune checkpoint blockade therapy.

CHAPTER VI

CONCLUSION

TAMs and MDSCs are the major immune cells generating tumor immunosuppressive environment and checkpoint blockade immunotherapy resistance. PI3Kγ, MCSFR and Stat3 signaling are activated in these cells and targeting these pathways have been shown to enhance checkpoint blockade immunotherapy response against cancer. Our results strongly demonstrated that Cuc. B is a potential natural agent which has strong potential in simultaneously targeting Stat3/CSF-1R and PI3Kγ in both TAMs and MDSCs. Therefore, we strongly believe that Cuc B treatment will work as an adjuvant to enhance checkpoint blockade immunotherapy response in less responsive tumors. Further studies are required to prove this hypothesis in appropriate pre-clinical mouse models of cancer.

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APPENDIX A

APPENDIX A

Table 2: List of items with their respective manufacturers and catalogue numbers

Item	**Manufacturer**	**Catalogue/Item No.**
Cucurbitacin B	Cayman Chemical	14820
GMCSF	Sigma	SRP3201
MCSF	Sigma	M9170
Mouse Il-6 Recombinant protein	Invitrogen	RMIL61
Mouse Il-4 Recombinant protein	Sigma	SRP3211
TRIzol Reagent	Life technologies	399612
RIPA Lysis Buffer System	Sant cruz Biotechnology	24948A
FBS	Life Technologies	10437-028
RPMI Media	Life Technologies	1187-093
Antibiotic-Antimycotic	Life Technologies	15240-062
0.25% Trypsin-EDTA	Life Technologies	25200-056
PBS	Life Technologies	10010-023
Accutase Solution	Sigma	A6964
HEPES Solution	Sigma	SRE 0065
RBC lysing buffer	Sigma	R7757
L-Glutamine	Sigma	TMS-002-C
TRIzol	Life Technologies	15596018
Vybrant Phagocytosis Assay	ThermoFischer Scientific	V6694

Abbreviations

TAM	Tumor Associated Macrophages
MDSC	Myeloid Derived Suppressor Cells
Cuc. B	Cucurbitacin B
PDL-1	Programmed death ligand 1
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTL	Cytotoxic T lymphocyte
TME	Tumor microenvironment
CTLA-4	The cytotoxic T-lymphocyte-associated antigen-4
PD1	Programmed Death 1
PDL1	Programmed death ligand1
GM-CSF	granulocyte–macrophage colony stimulating factor
ROS	Reactive oxygen species
CAF	Cancer-associated Fibroblasts
VEGF	Vascular endothelial growth factor
EGF	Epidermal growth factors

BIOGRAPHICAL SKETCH

Emmanuel Anning (Pharm.D.) is a Graduate of the University of Texas Rio Grande Valley (UTRGV) with a degree in Biochemistry and Molecular Biology (July 2022). He worked as a graduate research assistant in the department of Immunology and Microbiology, School of Medicine at the University of Texas Rio Grande Valley in Dr. Bilal Hafeez's lab during his graduate studies at UTRGV. He had his 6-year undergraduate professional Doctor of Pharmacy (Pharm.D.) degree at the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana in June 2019. Prior to his Master's degree, he was a Graduate Teaching Assistant at the Department of Pharmacology at the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, where he also worked as a hospital pharmacist at the University's hospital.

His keen interest in translational clinical research propelled him to pursue a 2-year Master's degree in Biochemistry and Molecular Science at the University of Texas, Rio Grande Valley, after which he gained admission into Baylor College of Medicine to pursue a PhD in Biomedical Sciences with a concentration in Chemical, Physical and Structural Biology. Dr Anning's areas of research interest includes cancer therapy, natural product drug discovery, pharmacology and toxicology. He can be contacted *via* email at anningemmanuel18@gmail.com