MODULATING ER AND PPAR PATHWAYS IN TUMOR CELLS: THE EFFECT ON MACROPHAGE ACTIVATION STATE AND PRO- TUMOR FUNCTION.

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OSHIN J. MIRANDA

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ABSTRACT:

Owing to its aggressive resistance to therapies, lung cancer is the leading cause of cancer deaths with 5-year survival rate of 17.5%. Non-small cell lung cancer (NSCLC) is a common subtype of lung cancer. Almost 80% of lung cancer cases are NSCLC cases. There has been evidence that the estrogen receptor (ER) signaling pathway and peroxisome proliferative activation receptor (PPAR) signaling pathway have an important role to play in cancer and inflammation. Previous reports have shown that activation of PPAR provides an anti-proliferative effect on epithelial cells. However, there have been reports that PPAR activating agents like pioglitazone shift the macrophage paradigm towards the M2 like/ pro-tumorigenic phase, an effect that is undesirable. Estrogen can enhance the proliferation of lung cancer cells through activation of the human epidermal growth factor receptor (EGFR) pathway, by inducing EGFR ligand release. It also makes the tumor microenvironment more tumorigenic by enhancing activation of M2 macrophages. To curb the action of pioglitazone on M2 macrophages, an estrogen receptor inhibitor like Fulvestrant can be used to avoid ER signaling, shifting the macrophages more to the M1 like/ pro-inflammatory phase. It is still not clear how ER and PPAR pathways are modulated in macrophages and

lung tumor cells. We investigated the levels of M1 and M2 cytokines produced by macrophages and used conditioned medium from tumor cells to identify the ligands that were modulated by treatment with pioglitazone and fulvestrant. AREG, IL-10, VEGF and IL-1b were detected as the M2 prominent ligands modulating the crosstalk between tumor cells and macrophages. These ligands were expressed in reduced amounts in presence of the combination of fulvestrant and pioglitazone. We also showed that combination treatment leads to downregulation of COX-2/PGE2 in tumor cells and macrophages. The combination also targets proliferative pathways. Estradiol levels were curbed in presence of the combination. There was also a significant downregulation of COX-2 in the presence of neutralizing antibodies to AREG and IL-1β. This suggests that AREG and IL-1β, the prominent ligands that are modulated by the drug combination, are likely responsible for the COX-2 downregulation. Pharmacologically, the combination shifts the pro-tumorigenic macrophages towards the pro-inflammatory macrophages phenotype, which could make the tumor microenvironment less supportive of tumor progression. Analysis of cell proliferation via culturing of macrophages media with tumor cells showed a significant decrease in tumor cell proliferation when macrophages were pre-treated with pioglitazone and fulvestrant. Interestingly, we also observed that

VEGF could be one of the cytokines involved in maintenance of M2-like/ pro-tumorigenic microenvironment. Dual therapy also strongly affected the cell migration of tumor cells in presence of macrophage conditioned media. From the evidence gathered above, we believe that the drugs could worked synergistically to affect cell proliferation, cell migration and shift the M1/M2 balance in the NSCLC tumor microenvironment. Therefore, we suggest a new combination therapy that targets both tumor cells and macrophages in NSCLC. It would be worthwhile to further investigate its effect in xenograft models of lung cancer.

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INTRODUCTION

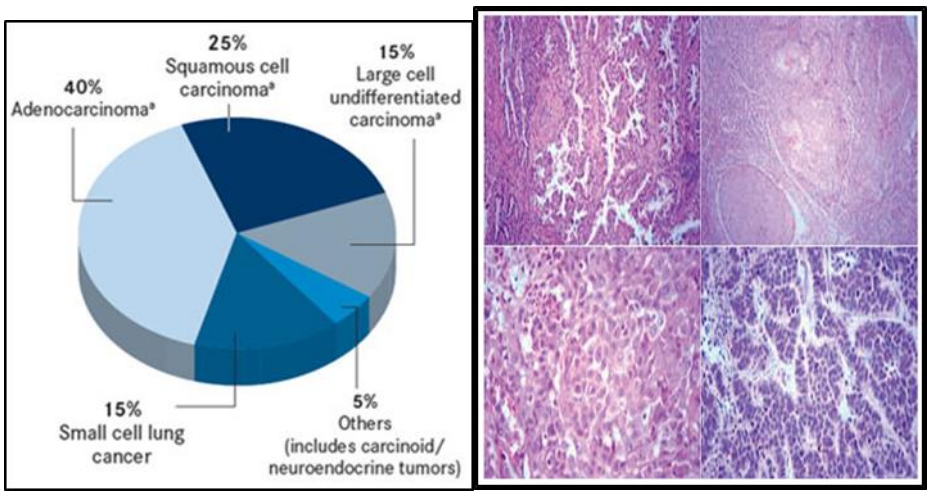
LUNG CANCER: EPIDEMIOLOGY AND RISKS

Lung cancer is one of the most dangerous cancers in both the sexes and is known to have a very low prognosis [1]. It has been observed that more than half of patients diagnosed with lung cancer die during their diagnosis period. The 5- year survival is found to be 17.8% [2]. The average age of the patients at the time of diagnosis is found to be 70. The number of deaths are far more in lung cancer in comparison to the deaths caused by colon cancer, pancreatic cancer and breast cancer [2]. According to American Cancer Society, the estimates for lung cancer cases in both men and women in United States for 2018 are- 234,030 new cases of lung cancer and 154,050 deaths from lung cancer [2]. Overall, the chance of a man and a woman to develop lung cancer in their lifetime is 1:15 and 1:17 respectively [2]. Lung cancer or lung carcinoma is a tumor malignancy that can be characterized by uncontrolled cell growth. The tumor can metastasize into nearby tissues or organs which is one of the hallmarks of cancer. These malignancies generally arise from the epithelial cells and can be distinguished based on their size and appearance. The common symptoms are dry and bloody cough, shortness of breath, sudden weight loss and severe chest pains. There are three main types of lung cancer (**Fig 1A**) - small-cell lung carcinoma (SCLC), non-small-cell lung carcinoma (NSCLC) and lung carcinoid tumor. Small cell lung cancer or oat cell cancer comprises of 10-15% of lung cancer cases. This type of cancer is known to be highly aggressive and metastasizing. Non- small cell lung cancer is the most common type of cancer. About 85% of lung cancer cases are non-small cell lung cancer. The subtypes of non- small cell lung cancer based on their histology are (**Fig 1B**) - squamous cell carcinoma (40% of cases), adenocarcinoma (50% of cases) and

large cell carcinoma (10% of cases). ADC arise in the distal airways and are less likely to be associated with smoking and chronic inflammation while. Squamous cell carcinomas are formed in proximal airways and are more related with smoking and chronic inflammation. SCC appear to be more aggressive as compared to ADC, the latter being a slower-growing tumor although capable of metastasizing early. The third subtype, LCC, is identified if the tumor cells appear to be neither glandular nor squamous or do not express the biomarkers for ADC and SCC [3].Lung carcinoid tumors or lung endocrine tumors are very rare and they grow very slowly. Lung carcinomas are histologically divided into two major subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter being relatively chemo-resist [2]. Because, most patients are detected at the advanced stages of lung cancer, surgery is no longer the best curative option. Based on the current scenarios patients undergo platinum chemotherapy. The survival rates are found to be above average [3]. Due to the lack of better treatments for advanced lung cancer, research has been more focused on the importance of targeted therapy coupled with individualized sequencing. Many small molecule inhibitors of receptor tyrosine kinases have proven to be successful in becoming the standard first line treatment for lung adenocarcinoma patients with mutations (Epidermal growth factor receptor (EFGR) and ALK) [4] [5]. However, it has been observed that a majority of these tumors lack these mutations making these treatments less effective. Another serious problem that gives rise to ineffective treatment is resistance. Recently, a lot of research has been focusing on the role of immune system in tumorigenesis. Invasion of the immune system is now being considered as a hallmark of cancer [6]. Researchers started focusing on developing immunotherapies that could maintain and develop patient’s anti-tumor defenses. Recent

findings from various immunotherapy trials that target immune checkpoints like- cytotoxic T-lymphocyte–associated protein-4 [CTLA-4] and programmed cell death protein-1 [PDL-1], suggests that lung cancer is certainly immunogenic [7]. Inflammation is paramount for wound healing and infection. However, if the balance is not maintained it could lead to uncontrolled tissue damage. Risk factors of chronic inflammation like smoking could lead to lung cancer [8]. Inflammation actively participates in all the stages of tumorigenesis. Tumor related deaths (approximately 15%) can be linked to inflammatory responses caused by underlying infections [6]. Certain tumors that are caused by chronic inflammation are known to exhibit a process called as smoldering inflammation. It is characterized by a concoction of infiltrating leucocytes, matrix disintegrating enzymes, growth factors, chemokines and cytokines [9]. Depending upon the nature of the tumor microenvironment – tumor infiltrating leucocytes can be either tumorigenic or antitumorigenic [10]. These infiltrating leucocytes comprise of a mixture of macrophages, T-cells and dendritic cells. Macrophages comprise of the majority of the tumor infiltrating leucocytes and exert different effects based upon their phenotype with the tumor microenvironment [11]. Even though a solid mechanism via which inflammation can modulate tumor progression is not fully deduced, there are two proposed hypothesis that can be taken into consideration – intrinsic pathway and extrinsic pathway. Genetic mutations and inflammation conditions are the supposed causes of tumorigenesis in the former and latter hypothesis respectively [12].

A



B

FIG 1: (A) Lung cancer subtypes. (B) Histological subtypes of Lung cancer**: Top left**: Adenocarcinoma; **Top right:** Squamous cell carcinoma; **Bottom left:** Large cell carcinoma; **Bottom right:** Small cell carcinoma. *(Lung Cancer: Peter J. Mazzone, Humberrto K. Choi, Duc Ha; Published: March 2014; Cleveland Clinic.)*

PATHWAYS LINKING INFLAMMATION AND CANCER:

The two pathways which link inflammation and cancer together are intrinsic and extrinsic pathways (**Fig 2**). Extrinsic pathway or the tumor microenvironment- Chronic inflammation is known to cause an increase in cancer risk and also lead to promotion of tumorigenesis. Chronic inflammation leads to approximately 15-20% of cancer deaths worldwide [13]. Tumors that arise from chronic inflammation sites tend to have infiltrating leucocytes (mixture of macrophages, T cells and other immune cells), chemokines, cytokines, growth factors, and matrix disintegrating enzymes [14]. M2 macrophages are typically pro-tumorigenic, however M1 macrophages support tumorigenesis via the generation of reactive oxygen species, nitrogen intermediates. These species induce DNA

damage in proliferating cells and other epithelial cells causing them to undergo a tumorigenic transformation. In response to this tissue damage inflammatory cytokines are released. These cytokines initiate the recruitment of cells to generate repair, however it has been noticed that these cytokines promote tumorigenesis by inhibiting enzymes [13]. Promotion of tumor growth leads to accumulation of DNA damaging agents and subsequently affects the integrity of the genome thus supporting tumorigenesis (14). This clearly demonstrates that how microenvironment caused by chronic inflammation promotes tumor progression. The intrinsic pathway (Genetic)- Alterations in genes leads to inactivation of tumor suppressor genes and activation of tumor oncogenes by dysregulation of pathways that lead to the hallmarks of cancer [3]. There are various tumor suppressor genes and tumor oncogenes that have implications in inflammation. These genes are known to have a role in the regulation of neovascularization in tumorigenesis, restructuring of the tumor microenvironment, induction of metastasis and suppression of adaptive immune response [15]. The innate immunity and inflammation pathways together are paramount in promoting tumorigenesis by reducing tumor suppressive immunity. Macrophages are believed to be involved in tumor progression via inflammation and extrinsic pathway. This leads to activation of oncogenes, crosstalk between tumor cells and immune cells. Tumor cells recruit monocyte precursors from the blood to differentiate them into macrophages. Different factors which leads to differentiation are- chemokine (C-C) motif ligand 2 (CCL2), macrophage colony–stimulating factor (M-CSF), IL-4,IL-10,and IL-13. Activation of STAT3 and nuclear factor (NF)-kB activation, leads to secretion of these cytokines. These cytokines allow monocytes to be differentiated into M2 macrophages [16].

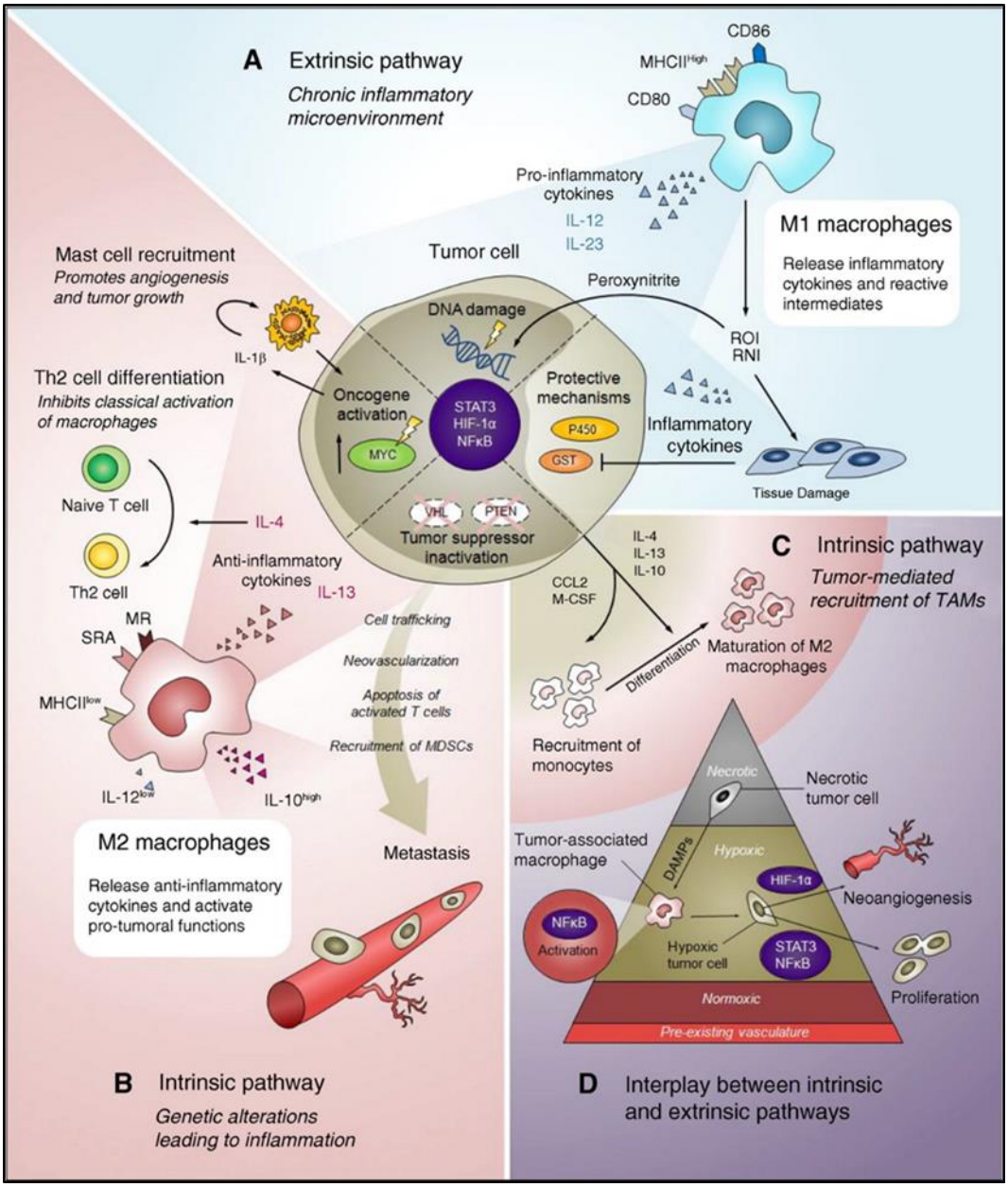


FIG 2: Pathways connecting inflammation and cancer. (*Sica A and Bronte V 2007).*

MACROPHAGES IN CANCER:

Macrophages are immune cells that are paramount for maintaining immunological responses against foreign cells. However they are also necessary for mediation of wound

healing. Macrophages are plastic and are diverse in their functionality. Based upon their phenotypes which depend upon a combination of number of factors like various signaling pathways in the tumor microenvironment they can be either tumorigenic or anti-tumorigenic [17]. An array of macrophage activation states exists, where M1 and M2 are the major extremes of this array. M1 or the classically activated macrophages are pro-inflammatory in function. They are characterized by high production of pro-inflammatory cytokines. They also lead to immune-stimulation because they are antigen presenting cells [18]. M2 or the alternatively activated macrophages are scavengers of debris, promoters of angiogenesis and also have a role to play in tissue remodeling and repairing. Apart from that the most important function is that they are found to be protumorigenic in nature [19]. They are characterized by high IL-10, low IL-12, high IL-1RA, high IL-1decoyR, high CCL-17, high CCL-22, high expression of vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), metalloproteinases (MMP) and cyclooxygenase-2 (COX-2) [20]. M2 macrophages can be further distinguished into M2a, M2b, and M2c subsets based on their gene expression profiles (21). Macrophages can certainly be elicited based on the amount of different cytokines, immune complexes and their surface markers. However, what remains a challenge is to understand the functional link between the macrophages in vivo. Macrophage polarization totally depends upon the combination of signals and cytokines levels in the tumor microenvironment. This plasticity is very evident during an infection, where M1 macrophages are recruited and eliminate the bacteria and activate an adaptive immune response, this process is continued with the action of M2 macrophages. The M2 macrophages modulate inflammation and tissue repair [22]. Tumor-associated macrophages (TAMs) mimic the sequence of activation states

observed during a normal situation. TAMs are known to be more skewed towards the M2 phase, it is known to promote tumor progression, survival, angiogenesis, suppression of immune system and epithelial to mesenchymal transition [20]. In colorectal carcinomas, M1 macrophages serve a better prognosis as compared to M2 macrophages [21]. There is evidence suggesting that the stage of the tumor development, interaction between pathways and tumor microenvironment might determine the phenotype of TAMs. TAMs contribute to tumor progression and aggressiveness by secreting factors that promote cell survival and proliferation. They cause DNA damage and immune escape through migration inhibitory factor (MIF), which suppresses p53 activity. They accumulate in the hypoxic regions to express hypoxia-inducible factor-1α (HIF1α), vascular endothelial growth factor (VEGF), IL-8, cyclooxygenase-2 (COX-2), and matrix metalloprotease (MMP)-9 to promote angiogenesis. They also cause invasion and metastasis. There is recruitment of T-cell subsets devoid of cytotoxic functions (T-helper type 2 [Th2] cells and regulatory T cells [Tregs]) to further promoting an anti-inflammatory environment [18]. Another important effect of TAMs is immune suppression it targets the expression of PDL-1. (**Fig 3**)

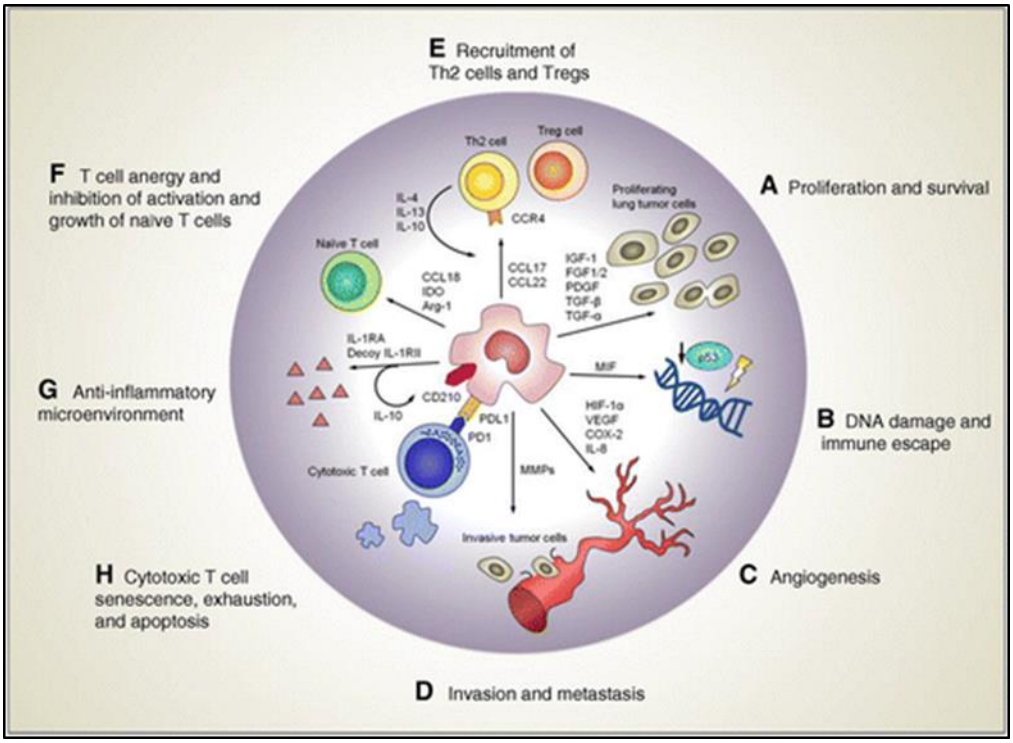


FIG 3: Tumor-promoting functions of M2 macrophages. (*Sica A and Bronte V 2007).*

A network of transcription factors and post-transcriptional regulators are involved in M1/M2 polarization. Interferon regulatory factor (IRF), signal transducers and activators of transcription (STAT) and suppressor of cytokine signaling (SOCS) proteins all play a role in skewing macrophage function towards either the M1 or M2 phenotype [23]. The IRF/STAT pathways, activated by IFNs and toll-like receptor (TLR) signaling, polarize macrophages to the M1 activation state via STAT1 [24]. On the other hand, IL-4 and IL-13 skew macrophages toward the M2 activation state via STAT 6 (**Fig 4**). Arginase 1 production is a distinct hallmark of M2 macrophages and is transcribed by STAT6, which is downstream of IL-4/IL-13 receptor signaling. Krüppel-like factor 4 (KLF-4) coordinates with STAT6 to induce M2 genes such as *Arg-1, Mrc1, Fizz1* and *PPARγ*, and inhibit M1 genes such as *TNFalpha, Cox-2, CCL5* and *NOS2* [25]. In addition, the nuclear receptor,

peroxisome proliferator-activated receptor γ (PPARγ), has been shown to regulate genes involved in oxidative metabolism and activation of the M2 phenotype (**Fig 5**).

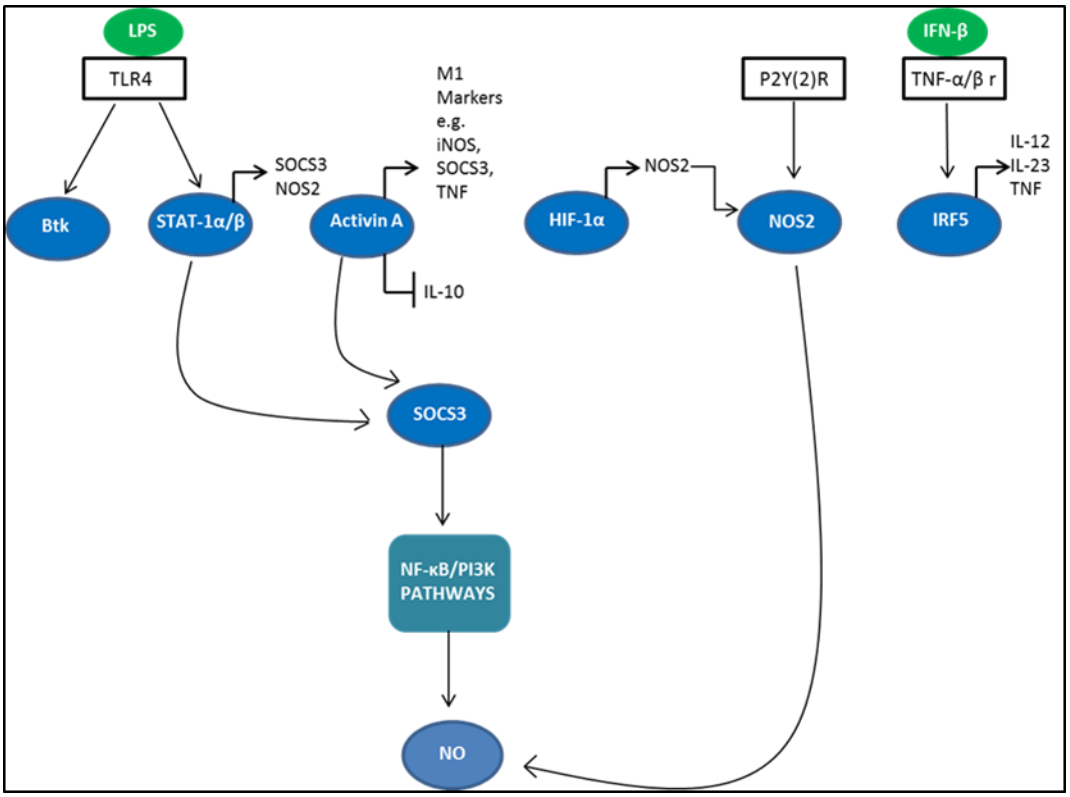


FIG 4: *Signaling molecules involved in M1 polarization. (https://www.bio-rad-antibodies.com/macrophage-polarization-minireview.html)*

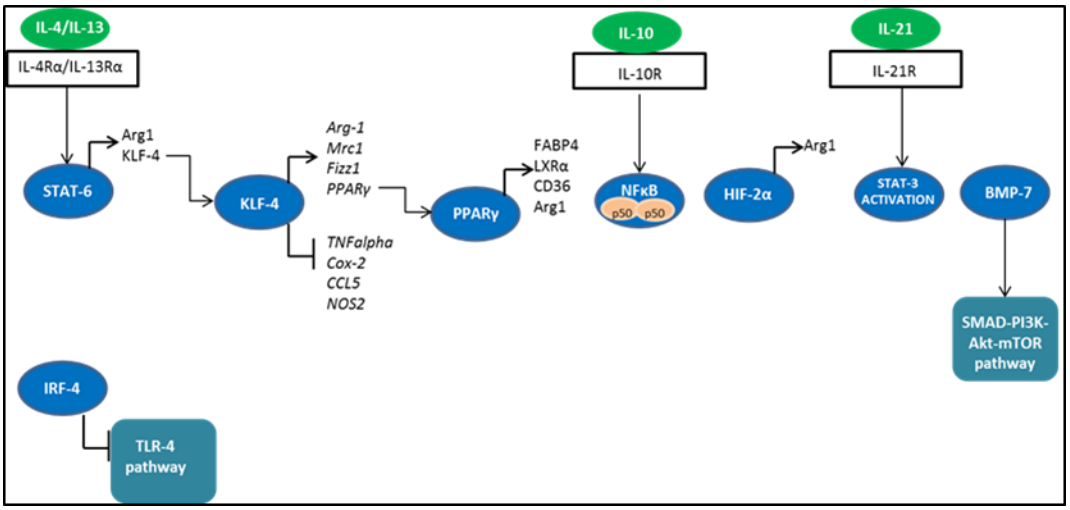


FIG 5: *Signaling molecules involved in M2 polarization. (https://www.bio-rad-antibodies.com/macrophage-polarization-minireview.html)*

ER SIGNALING PATHWAY:

Estrogens are steroidal hormones that have a regulatory function in various physiological processes. 17-β-Estradiol (E2) is the primary reproductive hormone synthesized in the ovary. The follicular stimulating hormone and the luteinizing hormone are responsible for stimulating its production [26]. Estrone and estriol are two forms that are synthesized in the liver from E2. Estrogens have genomic and non- genomic biological effects and are mediated by interactions with either, estrogen receptor α (ERα) or estrogen receptor β (ERβ). The ER isoforms are exhibited by different genes, but they have similar structural and functional organizations [27]. Both the receptors react in a similar manner with endogenous estrogens, mainly 17β-estradiol (E2) [28]. Apart from maintenance of homeostasis, E2 also has an important role in the maintenance of malignant conditions in

cancers. ERs are present in abundance in the nucleus and the cytoplasm of tumor cells. This enables the regulation of pro- tumorigenesis related transcription of genes. These genes are involved in cell survival, proliferation and differentiation [29]. There is also a crosstalk between various non genomic growth factor pathways like- epidermal growth factors (EGF) and fibroblast growth factors (FGF) [30]. Apart from the tumor cells it has been observed that non- cancerous cells like immune cells which comprise the tumor microenvironment have a critical role in tumor progression. E2 mediates immune responses like regulation of lymphopoiesis, differentiation of various immune cells and regulation of immune genes that have certain sequences [31]. E2 modulation has a role to play in both adaptive and innate immunity. Their role has several implications in the tumor microenvironment, tumor progression and metastasis. The main cause of disrupted homeostasis and potentiated tumorigenesis is the cross talk between tumor cells and the tumor microenvironment [32]. The cellular components of the tumor microenvironment include- cancer associated fibroblasts (CAFs), tumor associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs), immune T and B cells, natural killer (NK) cells, and endothelial cells [33]. TAMs can promote progressive tumor cell proliferation, imbalanced inflammatory conditions and metastasis [34]. Aromatase and ERβ are expressed in TAMs in NSCLC patients [35]. Recent evidence showed that E2 lead to induction of M2 polarization, infiltration of tumor cells and increased secretion of vascular endothelial growth factor (VEGF) [36]. E2 has been shown to cause increased expression of VEGF and M2 polarization in the lungs of mice exposed to carcinogens [37]. Current literature suggests E2 may facilitate an immunosuppressive TME by shifting the balance in favor of Th2 responses, production of tumor-promoting cytokines (IL-6, IL-4, TNFα,

and IL-17A). E2 may further promote tumor immune evasion through proliferation of Treg and MDSC populations, increased tumor cell PD-L1 expression, and inhibition of CD8+ T cell and NK cell induced apoptosis (**Fig 6**). Therefore, targeted inhibition of the E2 pathway may act as a novel strategy to enhance the effects of immunotherapies and reverse this immune imbalance within the TME.

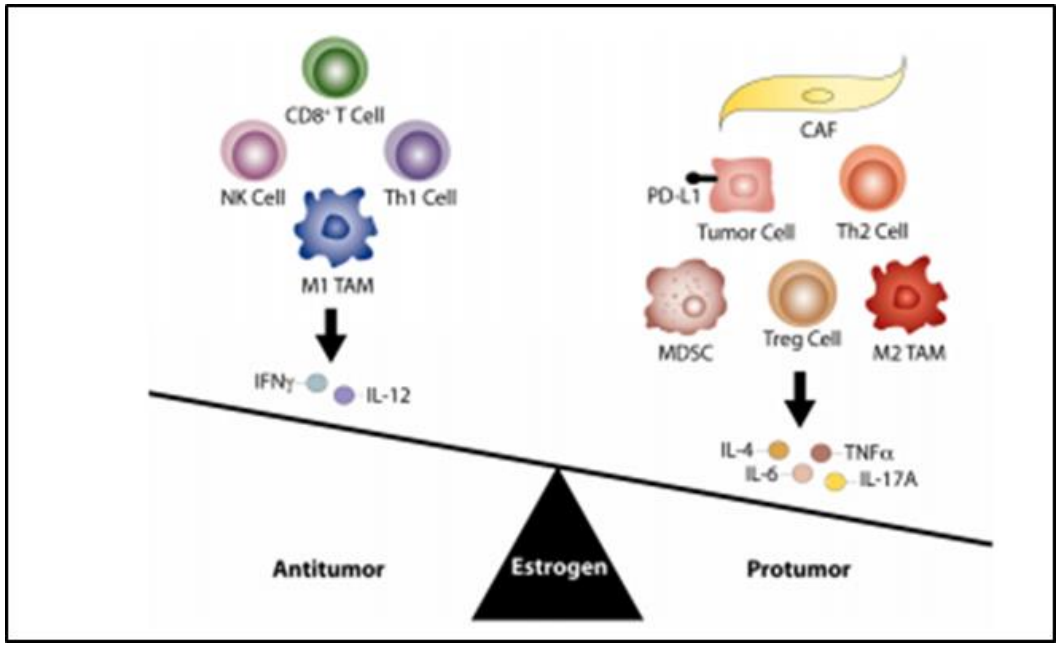


Figure 6: Maintenance of tumor microenvironment by E2 (*Natalie J R, Ashwin S, Laura P. S, The Role of the Estrogen Pathway in the Tumor Microenvironment, Int. J. Mol. Sci. 2018, 19, 611; doi:10.3390/ijms19020611).*

PPAR/COX-2 SIGNALING PATHWAY:

An approach that has gained a lot of attention because it has shown a lot of therapeutic potential in many human diseases is activation of nuclear receptors [38]. One such example

of nuclear hormone receptors is peroxisome proliferator activated receptors (PPARs). PPARs are known to play a role in glucose and lipid metabolism [38]. PPARs are also known to play a role in cancer cell differentiation, proliferation, survival, angiogenesis and apoptosis [39]. These cancer effects are mediated by either repression or activation of PPAR target genes [38]. The three PPAR members include, PPARα, PPARβ/δ, and PPARγ. All these are encoded by different genes and are known to have a different structure and function [38]. These receptors are found in abundance all over the body but they have different expression levels and expression patterns depending upon each of their receptor types [40]. PPARγ is known to have an important role as a tumor suppressor in comparison to PPARα or PPARβ/δ [41]. PPARγ is transcribed from one of three mRNAs—γ1, γ2, and γ3. They are different based on their transcriptional sites of initiation and splicing [40]. PPARγ1 mRNA is found abundantly in humans and there is restricted expression of PPARγ2 and PPARγ3 mRNAs in humans [42]. γ1 and γ3 mRNAs are translated into indistinguishable proteins [41]. This results in detection of only two protein isoforms—PPARγ1 and PPARγ2—in humans. PPARγ1 is more abundant than PPARγ2 [39]. PPARγ has shown to be involved lung cancer and also in a wide variety of other cancers. PPARγ is expressed in both small cell lung cancer and in non- small cell lung cancer [41]. According to past lung cancer research the receptor is inactive due to either modifications in the functional domains of the receptors, absence of activation ligands or accumulation in the cytoplasm [43]. However, the receptor is believed to be inactive in lung cancer cells, as suggested by cytoplasmic accumulation that reflects activation failure, perhaps due to some modification in its functional domains or to the absence of ligands in these cells [43]. PPARγ expression can be correlated with the stage of cancer and the

histology of cancer. A well differentiated adenocarcinoma has higher expression in comparison to squamous cell carcinoma [44]. PPARγ ligands can be naturally and synthetically occurring. Various saturated and unsaturated fatty acids, eicosanoid derivatives such as 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), and nitrated fatty acids activate PPARγ [40]. Thiazolidinediones (TZDs) or PPARγ agonists such as pioglitazone, troglitazone, rosiglitazone and ciglitazone bind to the receptors [38]. There has been a lot of evidence suggesting that these drugs are antitumorigenic. They hinder the modulation of tumor cell proliferation, tumor growth and progression and differentiation, apoptosis, motility and the hospitality of tumor microenvironment in lung cancer [39]. Apart from the immune cells other components of the tumor microenvironment include- adipocytes, fibroblasts, lymph and blood vessels, growth factors, cytokines, chemokines and extracellular matrix [45]. PPARγ is a key regulator of cellular differentiation, a crucial factor in the receptor's antitumor potential. An animal study involving troglitazone and pioglitazone as well as sulindac sulfide significantly reduce primary tumor growth of A549 NSCLC cells in a xenograft mouse model [46]. Certain other mouse studies, where mice were injected with tobacco carcinogens underwent rosiglitazone or pioglitazone treatment showed significant decrease in tumor growth and progression [40]. Angiogenesis is an important physiological process necessary for establishing primary tumors and metastasis [42]. Newer blood vessels allow tumors to grow and surpass complications like lesser oxygen supply and nutritional diffusion at sites [41]. It is also an easy access way for cancer cells to metastasize and reach other secondary organs. Under physiological conditions, angiogenesis is regulated by a balance between pro-angiogenic and anti-angiogenic factors. However, during tumorigenesis the pro-angiogenic factors are more favored, thus leading

to formation of tumor associated blood vessels. [42]. These blood vessels are highly permeable facilitating tumor cell trafficking and metastasis. Activation of PPARγ, which is highly expressed in tumor-associated endothelial cells, can also block angiogenesis by directly suppressing endothelial cell growth [45]. The role of PPARγ in lung cancer extends beyond the regulation of primary tumor formation; mounting evidence suggests that PPARγ activation suppresses tumor metastasis. According to a study using a mouse xenograft model, there was significant reduction of A549 cell metastasis in response to PPARγ agonists [47]. There was fewer and smaller tumors in comparison to the placebo treated groups. Hence, activation of PPARγ leads to prodifferentiation, anti-proliferative and pro-apoptotic effects in lung cancer. Several studies have demonstrated elevated constitutive expression of the inducible proinflammatory enzyme, cyclooxygenase-2 (COX-2) in human lung cancer [48]. Mounting evidence from investigations into the molecular effects of COX-2 over-expression in lung tumor cells indicates that this enzyme has a multifaceted role in conferring the malignant and metastatic phenotypes. The COX-2 enzymatic product prostaglandin E2 (PGE2) has been implicated in apoptosis resistance [48], angiogenesis [49], decreased host immunity [40], and enhanced invasion and metastasis [38]. The COX metabolite 15d-PGJ2 is a natural PPARγ ligand. It is a strong negative regulator of inflammatory and immune responses [42]. Cyclooxygenase is the rate-limiting enzyme for production of prostaglandins and thromboxanes from free arachidonic acid [50]. The most studied COX isoforms are COX-1 and COX-2. COX-1 is mainly expressed in most cells and tissues. COX-2 is an enzyme. Once induced, it produces prostaglandins and/or thromboxanes during an acute inflammatory response. The direct enzymatic product of COX-2 and PGH2 is converted to prostaglandins or thromboxanes

by individual isomerases or prostaglandin synthases. The production of other products depends upon the levels of metabolic and catabolic enzymes present in the cells. In NSCLC, prostaglandin E2 (PGE2) is the major eicosanoid product that is produced. PGE2 is produced via microsomal PGE2 synthase (mPGES). The nicotinamide adenine dinucleotide positive-dependent catabolic enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) metabolizes PGE2 to various biologically inactive 15-keto derivatives. The final PGE2 concentration produced by NSCLC cells depends upon expression of PGES and 15-PGDH. According to a lot of evidence, increased PGE2 production is paramount to tumorigenesis. An overexpression of COX-2 is observed in NSCLC. This leads to increased cell proliferation, invasion, angiogenesis, and resistance to apoptosis in the tumor microenvironment. This shows that COX-2 and its other downstream signaling pathways are potential targets for lung cancer prevention therapies. Certain studies show that COX-2 and PPARγ signaling pathways can be entwined. In macrophages, astrocytes and other epithelial cells, PPARγ ligands can suppress LPS and PMA- induced expression of COX-2 [51]. The COX-2 metabolite 15d-PGJ2 is an endogenous ligand for PPARγ [50]. During an inflammatory response there is an elevation of 15d-PGJ2 production which downregulates COX-2 via a negative feedback loop involving PPARγ [44]. Synthetic and endogenous PPARγ ligands decrease the high COX-2 expression associated with several malignancies [49]. Over expression of PPAR decreases COX-2 levels in lung cancer cells [48]. PPARγ agonists decrease COX-2 expression or prevent COX-2 induction in most cancers **(Figure 7).** However, there have been studies showing increased expression of COX-2 [51]. An example, Ikawa et al. reported that rosiglitazone increases expression of COX-2 in human colorectal carcinoma

cells [50]. PPARγ ligands also have been shown to induce COX-2 expression in mammary epithelial cells, monocytes, and fibroblasts [50]. One must take into consideration, the cell type, specific molecules used and presence of inflammatory cytokines while understanding the effect of PPARγ agonists on COX-2 expression. High levels of Tumor associated macrophages (TAMs) are often correlate to increased cell proliferation and metastasis. TAMs exhibit a more M2-like phenotype [49]. In general, the hallmarks of M2-macrophages are production of IL-10 (high), IL-12 (low), IL-1RA (high), IL-1decoyR (high), CCL17 (high), CCL22 (high), high expression of mannose, scavenger and galactose-type receptors, poor antigen-presenting capability, wound healing promotion, debris scavenging, angiogenesis, and tissue remodeling through high expression of VEGF, cyclooxygenase-2 (COX2), epidermal growth factor receptor (EGFR), and metalloproteinases (MMPs) [38]. According to an epidemiological study, regular intake of nonsteroidal anti-inflammatory drugs (NSAIDs)/ cyclooxygenase-2 (COX-2) inhibitor such as aspirin, could lead to the decrease in the development of different cancers [42]. When macrophages are activated for synthesis of inflammatory mediators, COX is key. Various mediators include- prostaglandin E2, prostacyclin I2, thromboxane A2, VEGF-A and VEGF-C [45]. There is evidence that shows that COX-2 is necessary for macrophage polarization. Macrophages are related to tumor growth, metastasis, and relapse. Macrophage-mediated immune suppression is correlated with increased CD4+ CD25+ regulatory T cell infiltration and reduced CD8+ cytotoxic T cell function. COX-2 inhibition blocks M-CSF-induced M2 macrophage differentiation and drives pro-inflammatory

activities in human and murine macrophages. This may suppress tumorigenesis

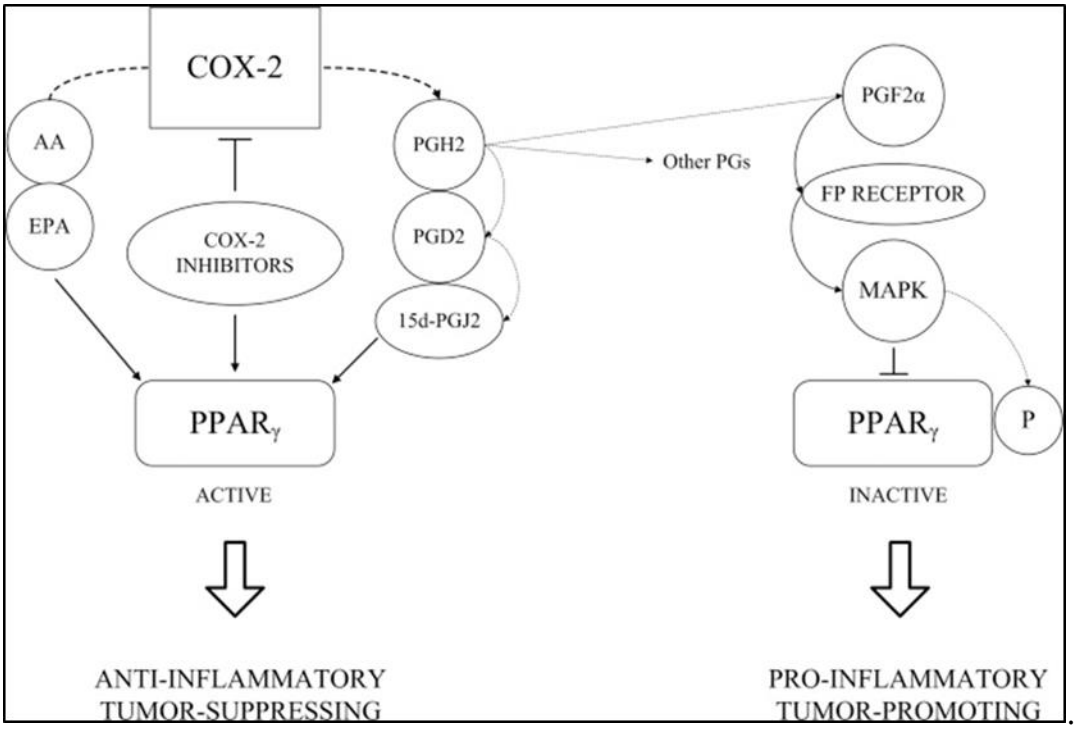


Fig 7: Cox-2 regulates the activity of PPARγ *(https://www.spandidos-publications.com/etm/1/2/257.*

INTERLEUKIN 1 β (IL-1 β) -:

The IL-1 family consist of three proteins, where two are agonists- IL-1α and IL-1β and the third one is an antagonist -IL-1ra. The agonists are functionally similar but have been obtained from a different set of genes. They bind to the same receptor and carry out similar biological activities. However, there is a difference in the way they are secreted and processed. The agonist IL-1β is first cleaved by the interleukin converting enzyme (ICE) to be converted into their active form [51]. Patients with inflammatory conditions show elevated plasma concentrations of IL-1β only. This might explain that IL-1β has a systemic role **(Figure 8)** [52]. IL-1β leads to induction of vascular permeability, fever, increased

secretion of additional cytokines in autoimmune disease, release of prostaglandins, pituitary hormones, and collagenases [53]. Cancer cells can directly produce IL-1β. It has been shown that there can be a cross-talk between cancer cells producing IL-1β and the immune cells in the tumor microenvironment [54]. Patients with solid tumors in which IL-1β has been shown to be up regulated can the cause of bad prognosis [52]. IL-1β can exhibit both autocrine and paracrine behavior. It induces expression of metastatic genes such as matrix metalloproteinases (MMP) and stimulates nearby cells to produce angiogenic proteins and growth factors such as VEGF, IL-8, IL-6 and TNFα [52]. These angiogenic factors are responsible for M2 activation and proliferation in the tumor microenvironment. IL-1β is important for tumor growth, proliferation, metastasis, and angiogenesis [53].

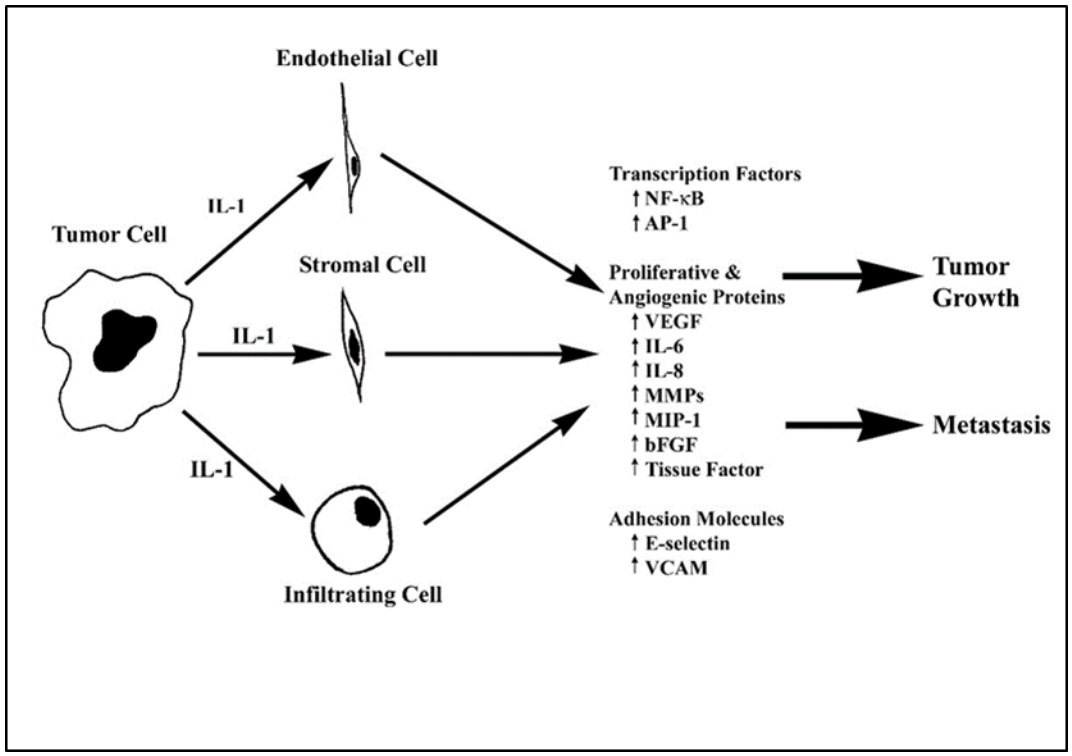


FIG 8: Role of IL-1 β on tumor growth and development. (*Lewis et al; licensee BioMed Central Ltd. 2006, Journal of Translational Medicine20064:48)*

AMPHIREGULIN:

AREG is the member of the epidermal growth factor (EGF) family. AREG is synthesized as transmembrane precursor that needs to undergo proteolytic cleavage to be released and binds to the EGFR **(Figure 9).** During development and homeostasis, it is expressed by a number of epithelial and mesenchymal cell types. Apart from other physiological processes, it is paramount in mammary gland and lung development [55]. AREG is required to restore tissue integrity after a severe injury or infection. It has been studied that the immune system may crosstalk with epidermal growth factor receptor (EGFR) via amphiregulin. Multiple immune modulators like prostaglandin E2, cAMP, and insulin-like growth factor-1 (IGF-1) can lead to the induction of AREG. Certain studies show that AREG levels are proportional to various immune activities in an immune response.

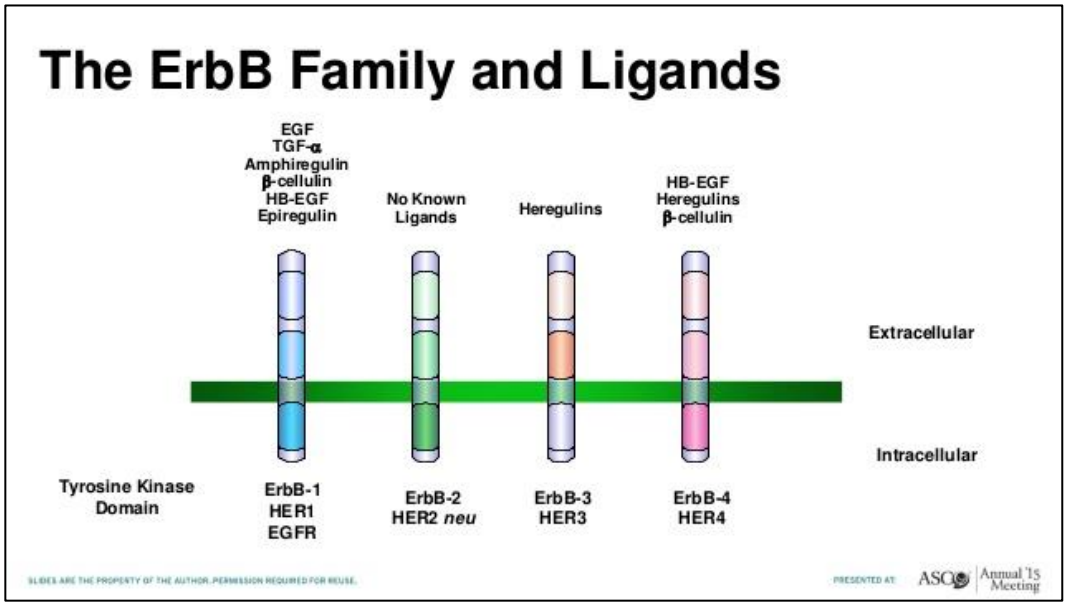


FIG 9: EGFR family and their respective ligands
(https://www.slideshare.net/OSUCCCJames/carbone-dt-discussion-share.

FULVESTRANT:

Fulvestrant is a selective estrogen receptor degrader (SERD) or a novel estrogen receptor antagonist **(Figure 10).** It blocks the activity of both ERα and ERβ. It was first approved by United States in 2002 for medical use. It is the first from its class to be approved. It binds to the estrogen receptor and destabilizes it. This leads to a protein degradation process by the cell. This medication is used to treat hormone receptor (HR)-positive metastatic breast cancer in postmenopausal women with disease progression. It is generally administered via the muscular route of administration.

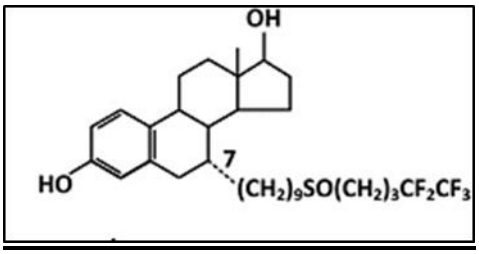


FIG 10: Fulvestrant, a selective estrogen receptor degrader.

PIOGLITAZONE:

Pioglitazone is a type 2 diabetes therapy and it can be used as a chemotherapeutic agent. It belongs to the thiazolidinedione class of drugs **(Figure11).** The mechanism of action is as follows- it is a proliferator-activated receptor gamma (PPARγ) activator. It has both preclinical and clinical effects in head, neck and lung cancer. It is administered orally.

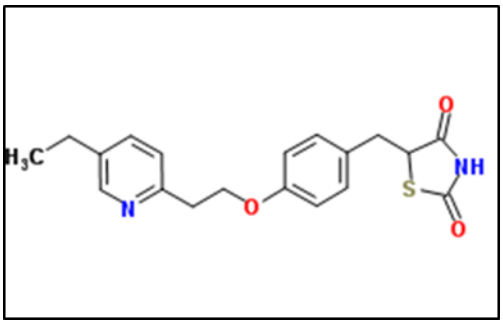


FIG 11: Pioglitazone, a PPAR-γ activator.

Our first goal was to examine the expression of ERβ and PPAR receptors in tumor cells and macrophages at basal levels. We used two different adenocarcinoma NSCLC cell lines- 201T (wild type EGFR) and A549 (KRAS mutant) and a human leukemia monocytic cell line- THP-1. We set up a model of macrophage polarization, starting from THP-1 monocytes differentiated into macrophages using PMA (Phorbol 12-myristate 13-acetate). Once differentiated (M0 macrophages), they were incubated with IL-4 and IL-13 in order to obtain M2 polarized macrophages or with lipopolysaccharide (LPS) for M1 polarized macrophages. Western blots showed that all three adenocarcinoma cell lines and all three macrophage polarizations had the presence of ERß and PPARγ. To mimic the communication between cancer cells and M0, M1 or M2 macrophages and 201T or A549 cancer cells the conditioned medium from the macrophages or tumor cells was placed on the other cell type. There were two aims- first was to modulate ERß and PPARγ pathways in cancer cells and test the resulting conditioned medium on macropahges, and the second was to modulate ERß and PPARγ pathways in macrophages and test the resulting conditioned medium on tumor cells. In order to understand the effect of activating PPAR receptors and inhibiting ER receptors in macrophages and tumor cells, the impact of conditioned media from tumor cells pre-treated with Fulvestrant and/ or Pioglitazone on macrophage polarization was studied and the effect of macrophage pre-treated conditioned medium on cancer cell proliferation was assessed. We were able to demonstrate that there were increased levels of M1 macrophage markers like IL-1b and TNF-alpha with pioglitazone alone and with the combination (pioglitazone and fulvestrant). Based on our expectations there were decreased levels of M2 macrophage markers like VEGF, IL-10 and PGE-2. We observed complete inhibition of COX-2 relative to the control in the

adenocarcinoma NSCLC cell lines. We were able to show that Pioglitazone alone reduces the expression of COX-2. However, we were also able to show that Pioglitazone alone shifts the macrophage balance more to the M2 polarization sate and also leads to increased secretion of M2 macrophage markers in the media. Amphiregulin, an EGFR ligand that is expressed in macrophages had lower levels when the tumor cells were incubated with pioglitazone as compared to fulvestrant. AREG is a known driver of carcinogenesis and we observed a decrease in its levels when the combination was used. We expected to see an increase in M1 polarization state markers when fulvestrant was administered alone. Lack of M2 polarization state markers lead us to believe that fulvestrant shifts the balance of macrophages more towards the M1 phase. The experiments with macrophage conditioned medium were further carried out only using M2 polarized macrophages because the best effects of tumor cell conditioned medium were observed with acropahges in the M2 state. In the 201T and A549 cell line we observed that on treatment with Fulvestrant there was an increased expression of COX-2, this was accompanied by an increased level of PGE2 and estradiol suggesting that induction of COX-2 and estrogen can function as a compensatory mechanism in both cell lines. However, treatment with pioglitazone decreased the expression of COX-2 and was accompanied by decreased level of PGE2 and estradiol in both cells. We have been able to show that the combination shows a complete inhibition of COX-2 and further decrease of PGE2 and estradiol in both the cell lines. Of interest, there was higher sensitivity towards the KRAS mutant- A549 cell line. We were able to show that by using a recombinant antibody for AREG and/ or IL-1β there was overexpression of COX-2 and huge PGE2 levels in the samples obtained from the co-culture experiments. We were also able to block COX-2 by using a neutralizing antibody

for AREG and/ or IL-1β. There was also decreased levels of PGE2 levels in the samples, which suggests that AREG and IL-1β are the possible cytokines that modulate the pathways in tumor cells, and have an effect on the macrophage activation, polarization state and pro-tumor function. MTS assay performed post treatment with Fulvestrant and pioglitazone showed a decrease in proliferation rate after 48 hours. A scratch assay showed a considerable reduction in the migration of cancer cells in presence of the combination treated conditioned media from macrophages.

RESULTS:

To measure basal receptor expression levels in both adenocarcinoma cell lines (201T and A549) and the macrophage polarized states (M0, M1 and M2).

The two receptors characterized were ER β and PPAR γ, these receptors are commonly involved in the progression of cancer. ER β and PPAR γ were expressed in all of cell lines used. We then quantified the expression levels in all cell lines while using MCF-7 (a human breast adenocarcinoma cell line) and THP-1 (a human leukemia monocytic cell line) as a control for NSCLC adenocarcinoma cell lines and macrophage polarization states respectively, using immunoblotting **(Fig 12)**. Thus we concluded that targeting the ER β and PPAR γ pathways in a combination would have effects on both cell types since both were expressed in adenocarcinoma cell lines and in all the macrophage polarization states.

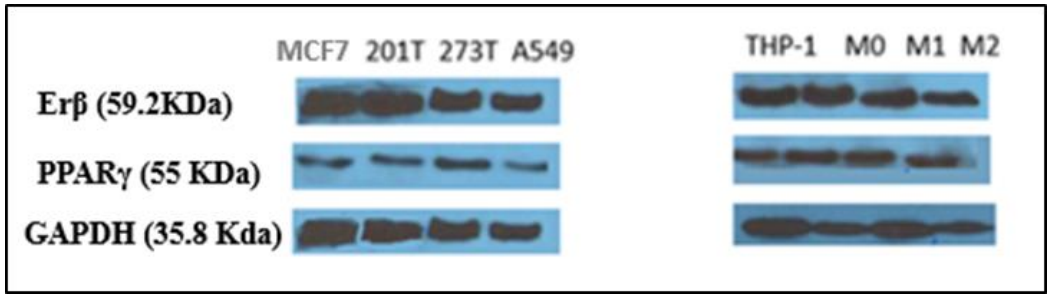


FIG 12: Basal expression of ER-β and PPAR γ in 201T, 273T, A549, M0, M1, M2 macrophages.

Conditioned media from lung tumor cells treated with Fulvestrant and/or Pioglitazone causes higher levels of M1 macrophage markers.

In order to understand how modulating the *ER* β *and PPAR* γ pathways in tumor cells affects macrophages we decided to carry out experiments using tumor cell conditioned media to establish our main hypothesis. We tried to mimic the possible communications that could be taking place by subjecting the macrophages to the conditioned media from cancer cells, after the tumor cells were treated with the drugs for 6 hours. The medium was changed with fresh serum free media. The conditioned media was collected after 24 hours. Then the medium was placed on to THP-1 cells that were differentiated into M0, M1 and M2 macrophages and also in empty wells as a control (**Figure 13**). The macrophage medium was collected and used to analyze the expression of several pro-inflammatory and pro-tumorigenic cytokines at the protein level using ELISA. TNF-α and IL-12 are prominent M1 biomarkers. They were present in all the macrophage polarization states. However, their levels were more in macrophages incubated with the A549 conditioned media. This could be due to the KRAS mutation present in A549. In all experiments, the amount of each cytokine in the tumor cell medium alone (background), was much lower than that found in the macrophage cultures, and was either unchanged by drug treatment or was modulated in the same direction as in macrophages. In F**igure 14** shows a significant increase of TNF-a levels by M0 macrophages in presence of the conditioned medium of 201T cells treated with combination (right). There was a slight increase in TNF-a levels by

M0 macrophages in presence of the conditioned medium of A549 treated with combination (left). This helps us to understand that the combination drives the polarization of macrophages towards the M1 state more effectively in the presence of 201T conditioned medium in M0 macrophages. **Figure 15** shows a significant increase of TNF-a levels by M1 macrophages in the presence of the conditioned medium of A549 treated with the combination (left). This significant increase can be contributed to the individual effect of tumor conditioned medium treated with fulvestrant on the M1 macrophages. There was a significant increase of TNF-a levels by M1 macrophages in presence of the 201T conditioned medium treated with fulvestrant and pioglitazone (right). There was also a significant increase of TNF-a in M1 macrophages when they were incubated with 201t conditioned medium treated with fulvestrant. **Figure 16** shows the significant increase in TNF-a levels when M2 macrophages were subjected to a 24 hour incubation with A549 (left) and 201T (right) conditioned medium treated with combination. Although there were increase in TNF-a levels in M2 macrophages in presence of conditioned medium treated with fulvestrant, this effect was maximized when the macrophages were incubated with conditioned medium treated with the combination. In F**igure 17** shows a significant increase of IL-12 levels by M0 macrophages in presence of the conditioned medium of 201tTtreated with combination (right) and A549 treated with combination (left). There was a significant decrease in IL-12 levels by M0 macrophages in presence of the conditioned medium of A549 treated with pioglitazone (left). This effect is rescued when the M0 macrophages subjected to the tumor conditioned medium pretreated with the combination. Based on the result obtained, fulvestrant shifts the balance to the M1 polarization state. **Figure 18** shows a significant increase of IL-12 levels by M1 macrophages in the presence

of the conditioned medium of 201T treated with the combination (right). This significant increase can be contributed to the individual effect of tumor conditioned medium treated with fulvestrant on the M1 macrophages. There was an increase of IL-12 levels by M1 macrophages in presence of the A549 conditioned medium treated with fulvestrant and pioglitazone (left). **Figure 19** shows the significant increase in IL-12 levels when M2 macrophages were subjected to a 24 hour incubation with 201T (right) conditioned medium treated with combination. There was an increase of IL-12 levels by M2 macrophages in presence of the A549 conditioned medium treated with fulvestrant and pioglitazone (left). This explains that the combination probably modulates the pathways in tumor cells which promotes the anti-tumorigenic phase of macrophages.

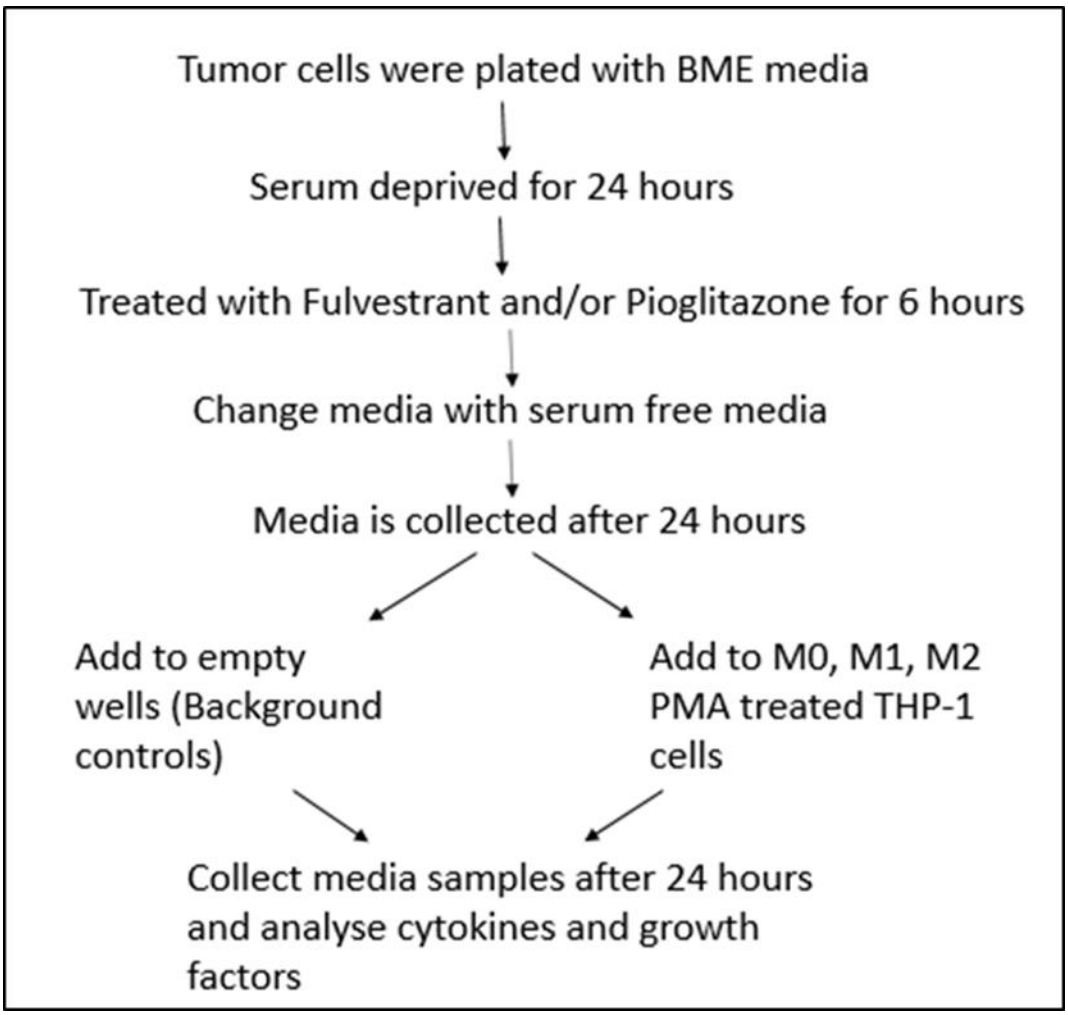


FIG 13: Experimental design for understanding expression levels of TNF-a, IL-10, IL-12, VEGF, AREG and IL-1b.

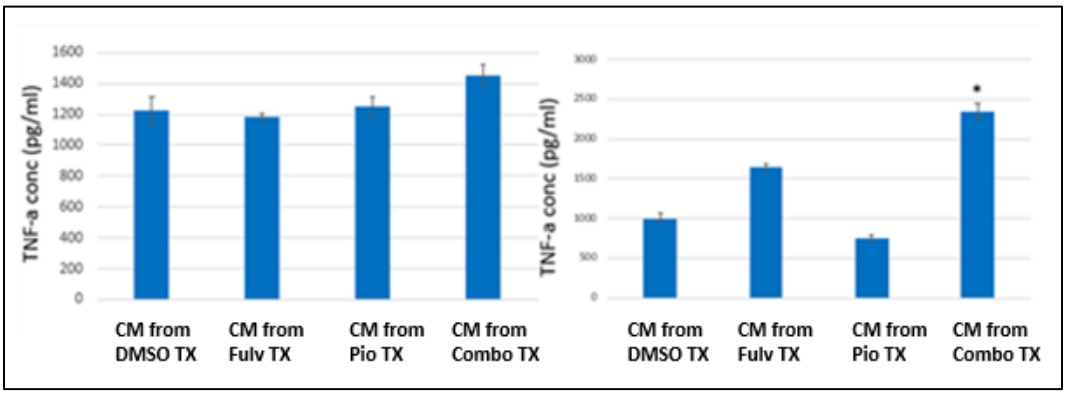


FIG 14: Release of TNF-a by M0 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of TNF-a in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.001).

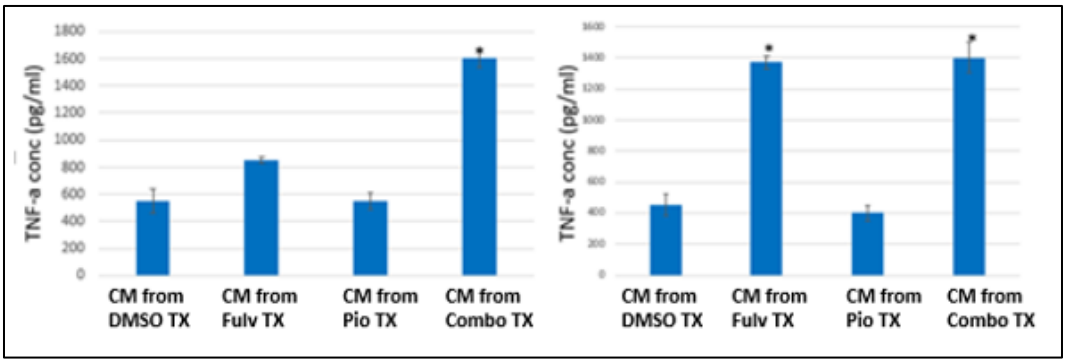


FIG 15: Release of TNF-a by M1 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of TNF-a in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.001).

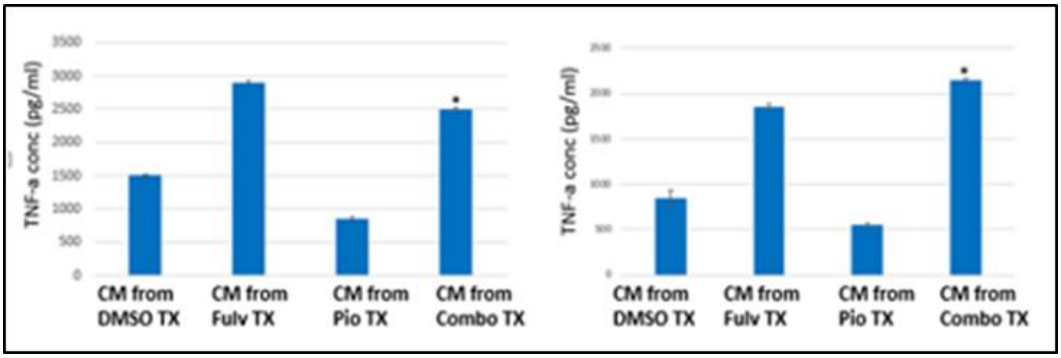


FIG 16: Release of TNF-a by M2 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of TNF-a in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.001).

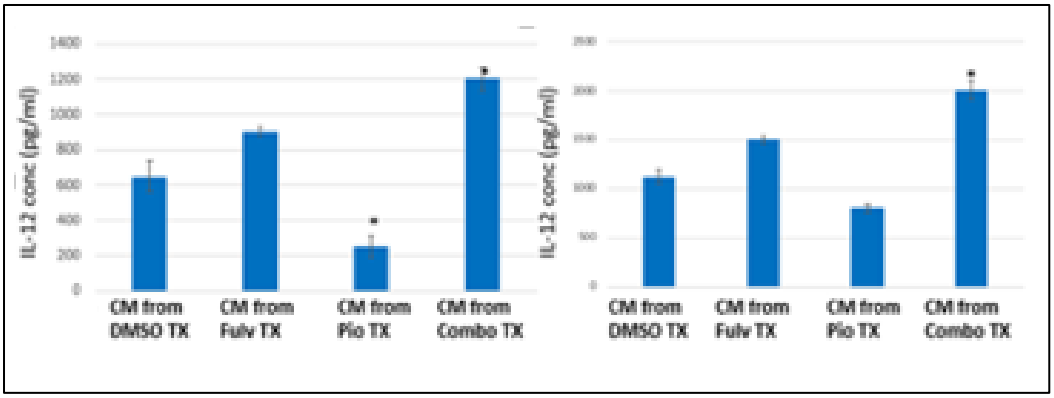


FIG 17: Release of IL-12 by M0 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-12 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

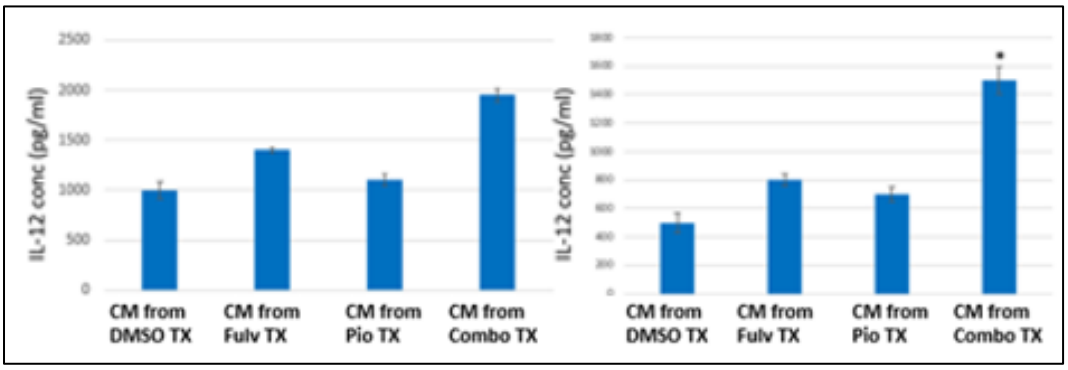


FIG 18: Release of IL-12 by M1 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-12 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.001).

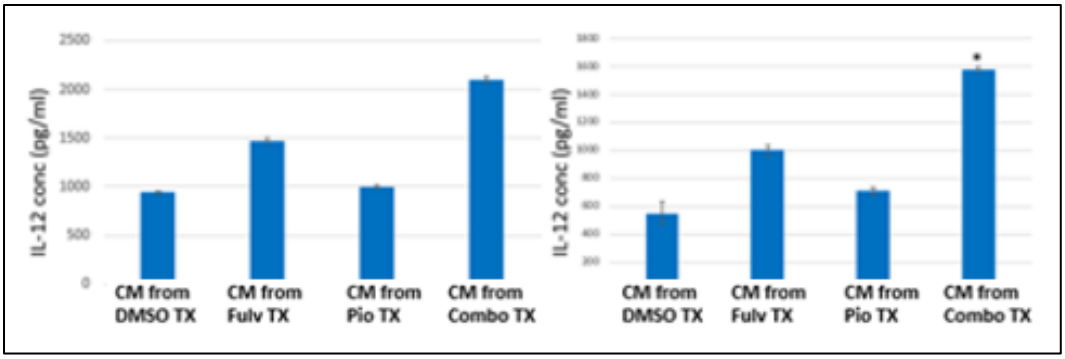


FIG 19: Release of IL-12 by M2 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-12 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.001).

Conclusion- For TNF-a release, in M1 and M2 macrophages, the conditioned medium from 201T and A549 cells pretreated with the combination stimulated the levels significantly, while pioglitazone could not. Fulvestrant alone could stimulate TNF-a release, sometimes to the same extrent as combination. In M0 macrophages, this effect was significantly only seen using 201T cell conditioned medium. For IL-12 release, in M0, M1 and M2 macrophages, the conditioned medium from 201T cells pretreated with the combination stimulated the levels significantly. M0 macrophages incubated with A549 conditioned medium treated with combination showed significant increase in levels, but these effects did not reach significance in the M1 and M2 state. Overall the combination of fulvestrant and pioglitazone was more effective in maximizing the release of M1 biomarkers by macrophages.

Significantly low release of M2 macrophage markers, when the conditioned medium of tumor cells pretreated with drugs was placed on macrophages at different polarization states.

We expected to see a decreased level of M2 cytokine expression using tumor cell conditioned medium pretreated with combination and incubated with macrophages. The macrophages treated with the pretreated conditioned medium from tumor cells collected underwent an ELISA analysis for expression of pro-tumorigenic cytokines (**Figure 13**). F**igure 20** shows a significant decrease of IL-10 levels by M0 macrophages in presence of the conditioned medium of 201T treated with combination and fulvestrant (right). The anti-tumorigenic effect can be contributed to the effect of fulvestrant. According to the hypothesis fulvestrant maintains an anti-tumorigenic environment by blocking estrogen. This result shows that the combination attains the required effect in M0 macrophages. There was also a significant decrease in IL-10 levels by M0 macrophages in presence of the conditioned medium of A549 treated with combination (left). **Figure 21** shows a significant decrease of IL-10 levels by M1 macrophages in the presence of the conditioned medium of A549 treated with the combination and fulvestrant (left). This significant decrease can be contributed to the individual effect of tumor conditioned medium treated with fulvestrant on the M1 macrophages. There was also a similar significant decrease of IL-10 levels by M1 macrophages in presence of the 201T conditioned medium treated with combination and fulvestrant (right). **Figure 22** shows the significant decrease in IL-10 levels when M2 macrophages were subjected to a 24 hour incubation with A549 (left) conditioned medium treated with combination and pioglitazone. In 201T (right), there was a slight decrease in IL-10 levels in M2 macrophages in presence of conditioned medium

treated with combination, this effect could be compared with the placebo effect. It was observed that the pioglitazone rescued the effect obtained by fulvestrant alone. Overall the anti-tumorigenic effect was maintained when the M2 macrophages were incubated with conditioned medium treated with the combination. In F**igure 23** shows a significant decrease of VEGF levels by M0 macrophages in presence of the conditioned medium of 201t treated with combination (right) and A549 treated with combination (left). **Figure 24** shows a significant decrease of VEGF levels by M1 macrophages in the presence of the conditioned medium of 201T treated with the combination (right). This significant decrease can be contributed to the individual effect of tumor conditioned medium treated with fulvestrant on the M1 macrophages. There was a significant decrease of VEGF levels by M1 macrophages in presence of the A549 conditioned medium treated with fulvestrant and combination (left). **Figure 25** shows the significant decrease in VEGF levels when M2 macrophages were subjected to a 24 hour incubation with 201T (right) conditioned medium treated with combination and fulvestrant. There was a significant decrease of VEGF levels by M2 macrophages in presence of the A549 conditioned medium treated with fulvestrant and combination (left). There was significant effect observed in presence of fulvestrant alone, however the anti-tumorigenic effect was maximized when 201T (right) and A549 (left) cell lines were pretreated with the combination and the M2 macrophages were incubated with this conditioned medium. This explains that the combination could effectively modulate the crosstalk between the cells.

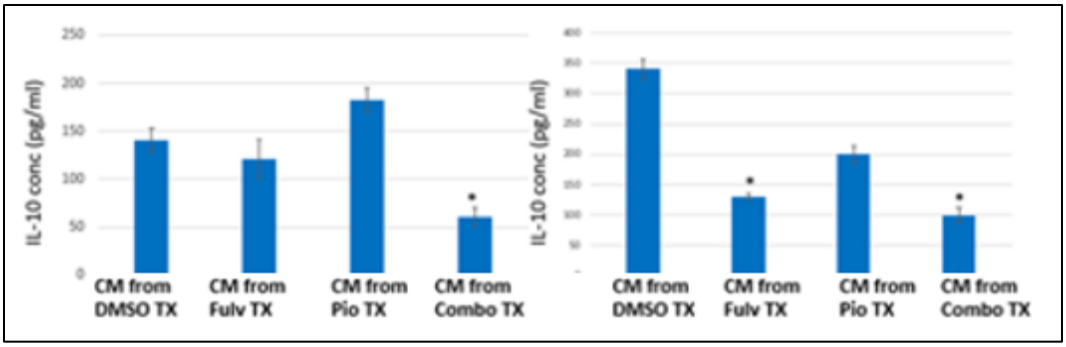


FIG 20: Release of IL-10 by M0 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-10 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

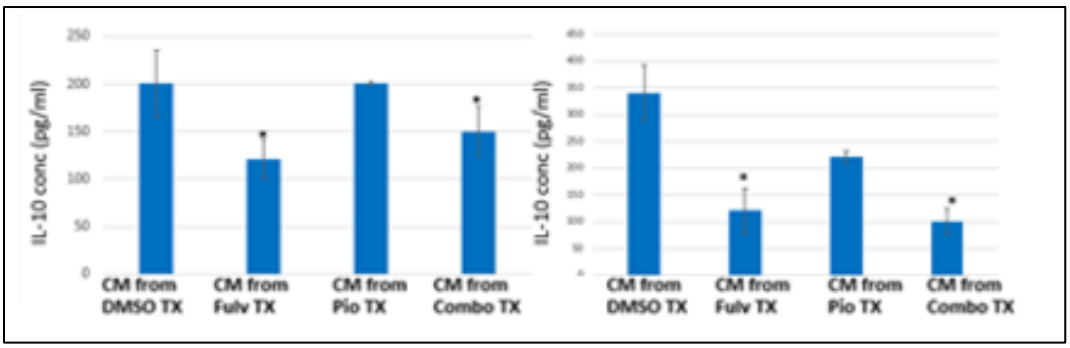


FIG 21: Release of IL-10 by M1 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-10 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

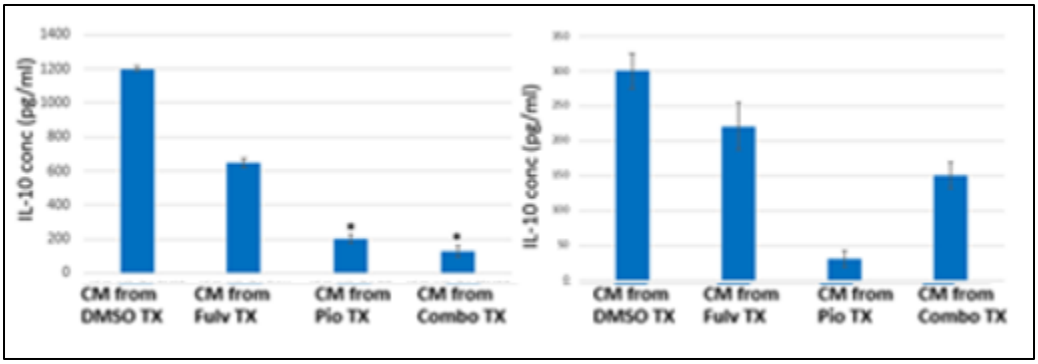


FIG 22: Release of IL-10 by M2 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-10 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

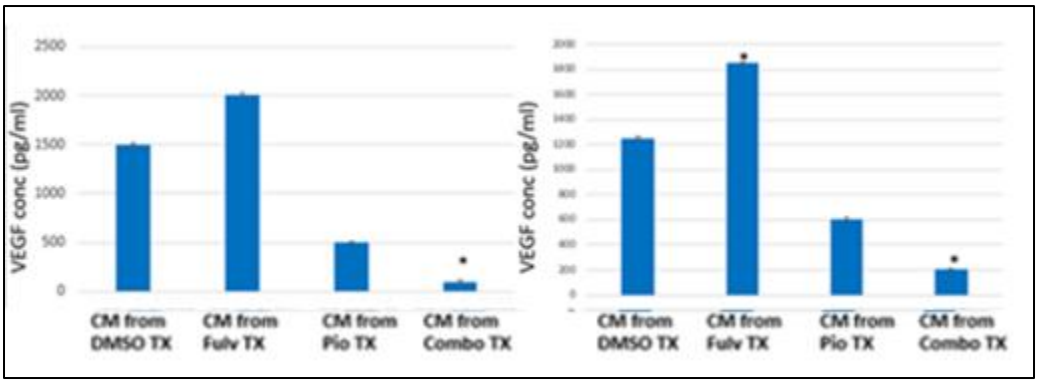


FIG 23: Release of VEGF by M0 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of VEGF in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.001).

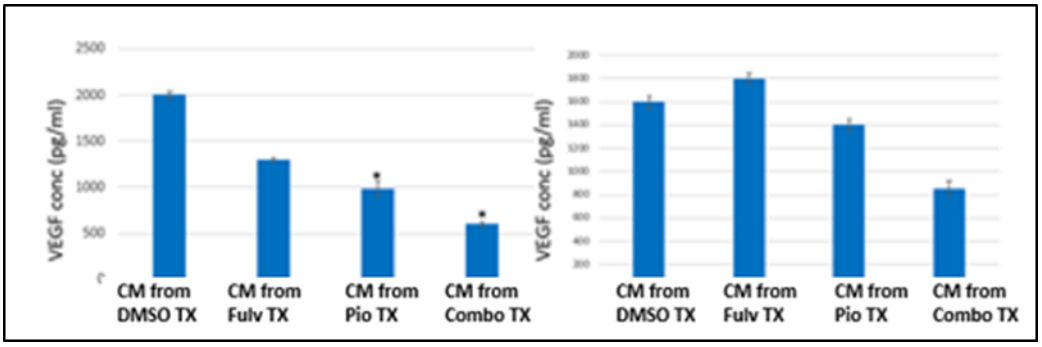


FIG 24: Release of VEGF by M1 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of VEGF in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.005).

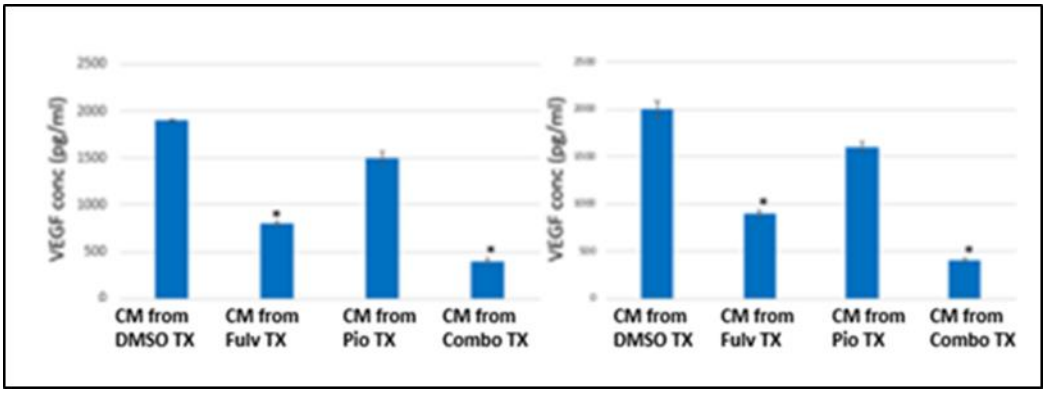


FIG 25: Release of VEGF by M2 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of VEGF in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.005).

Conclusion- For IL-10 release, in M0 and M1 macrophages, the conditioned medium from 201T and A549 cells pretreated with the combination decreased the levels significantly. In M2 macrophages, this effect was significantly only seen in A549 cells. For VEGF release, in M0 and M2 macrophages, the conditioned medium from 201T and A549 cells pretreated

with the combination decreased the levels significantly. While in M1 macrophages incubated with A549 conditioned medium treated with combination showed significant decrease in levels. Overall the combinationof fulvestrant and pioglitazone was more effective in minimizing the release of M2 biomarkers by M2 macrophages.

Decreased tumor cell proliferation observed using macrophage conditioned medium pretreated with combination.

The tumor cells were plated in a 96 well plate and incubated with the pretreated (fulvestrant and/or pioglitazone) macrophage (M0/M1/M2) conditioned media for 48 hours. The analysis was done after 2 days of exposure of the conditioned media **(Figure 26).** The MTS assay showed a significant decrease in tumor cell proliferation when the tumor cells were subjected to incubation with the macrophage conditioned medium from combination treatment. The pioglitazone group showed less proliferation in comparison to the fulvestrant group. F**igure 27** shows a decrease in cell proliferation by A549 (left) and 201T (right) in presence of the conditioned medium of M0 macrophages treated with combination. F**igure 27** shows a similar trend, decrease in cell proliferation by A549 (left) and 201T (right) in presence of the conditioned medium of M1 macrophages treated with combination. Figure 16 shows the significant decrease in tumor cell proliferation levels when A549 (left) and 201T (right) were subjected to a 24 hour incubation with M2 macrophage conditioned medium treated with combination. Although there was a decrease in cell proliferation in both adenocarcinoma cells in presence of M2 macrophages conditioned medium treated with pioglitazone, this effect was maximized when the

adenocarcinoma cells were incubated with conditioned medium treated with the combination. This experiment explains the importance of using pioglitazone in the combination. The results from the previous experiments explain that pioglitazone as a monotherapy shifts the macrophage paradigm towards the pro-tumorigenic phase. But we can conclude that the results obtained via the MTS assay show us that pioglitazone alone acts on cancer cells in presence of the macrophage conditioned media by decreasing cell proliferation. This shows that pioglitazone has a dual functionality.

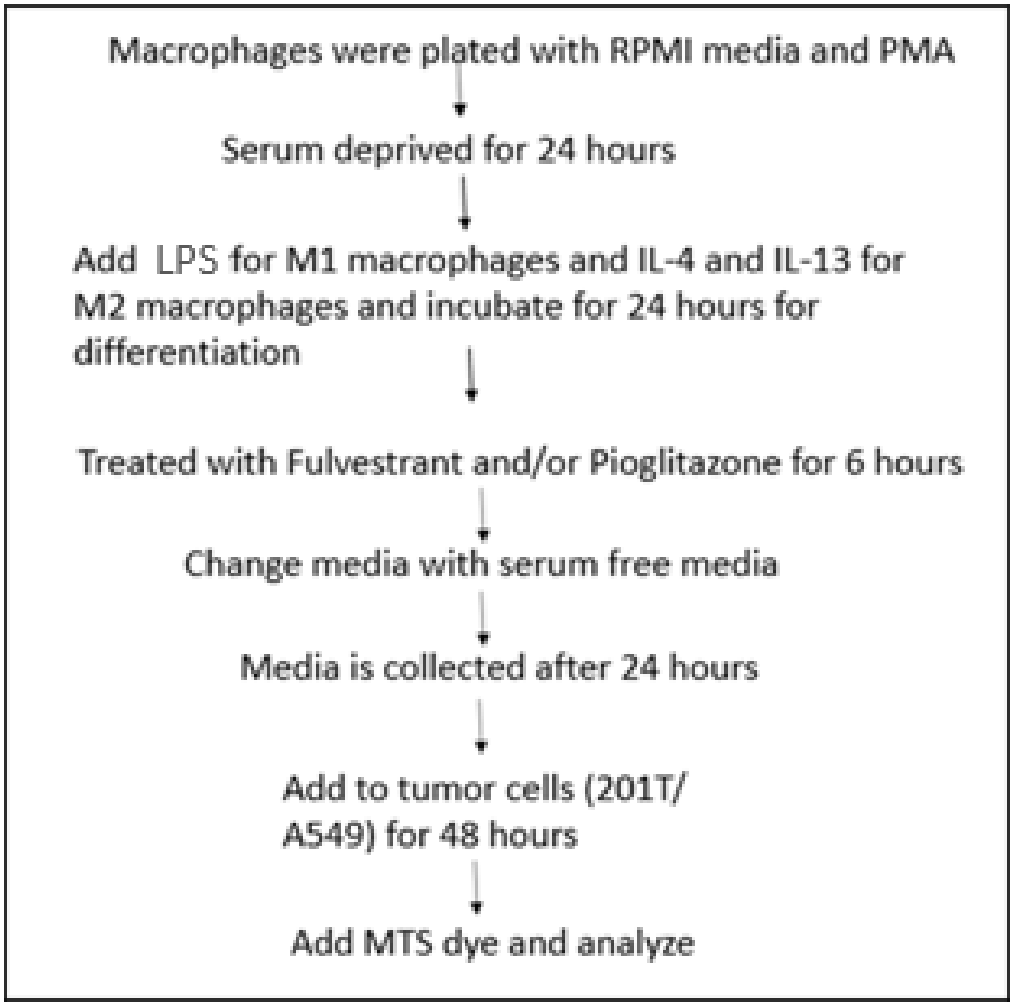


FIG 26: Experimental design for determination of tumor cell proliferation

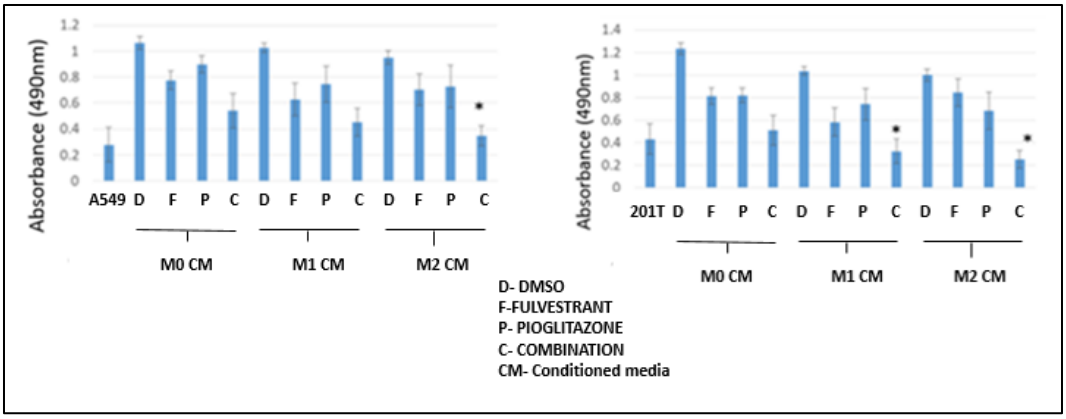


FIG 27: MTS assay performed on A549 (Left) and 201T (Right) after 48 hours of incubation with macrophage conditioned media showed significant decrease in cell growth by combination treatment (M2 conditioned media) in both cell lines. (* P value < 0.05.

Conclusion- In 201T, M1 and M2 conditioned medium treated with combination reduced cell proliferation significantly. However, this effect was seen mainly in M2 macrophages in A549 cells.

Effect of tumor conditioned medium pretreated with combination on E2 levels and ERβ signaling in different macrophage states.

Fulvestrant an anti-estrogen, binds to the estrogen receptor and destabilizes it. Estrogen is known as a prominent M2 marker and its role is well known in lung cancer. Based on our hypothesis, fulvestrant provides an anti-tumorigenic effect by targeting the estrogen receptor and thus shifting the macrophage paradigm more towards the M1 (anti-tumorigenic) phase. In order to substantiate our hypothesis, we ought to determine the effect of estrogen in presence of tumor conditioned medium pretreated with the combination in macrophages. We treated 201T and A549 NSCLC cell lines with the

fulvestrant and/or pioglitazone for 6 hours. The macrophages in their respective polarized states were treated with Androstenedione (E2 precursor) at 300uM for 24 hours in serum free media. The macrophages were then subjected to incubation with the pretreated tumor cell conditioned medium along with the precursor for 24 hours **(Figure 28).** We observed a similar trend in both adenocarcinoma cell lines. F**igure 29** shows a similar significant trend, decrease in E2 levels by M0 macrophages in presence of the combination treated tumor conditioned medium of A549 (left) and 201T (right). **Figure 30** shows a significant decrease in E2 levels by M1 macrophages in presence of the combination treated tumor conditioned medium of A549 (left) and 201T (right). Although there was a decrease in E2 levels by M2 macrophages in presence of the pioglitazone treated tumor conditioned medium in A549 (left) and 201T (right), this effect was maximized when the M2 macrophages in presence of the combination treated tumor conditioned medium in A549 (left) and 201T (right) **(Figure 31).** Fulvestrant induces E2 level in both cell lines suggesting that an induction of E2 could be a compensatory mechanism by which the tumor cells survive. However, we expected that the treatment with pioglitazone will rescue the E2 induction caused by fulvestrant. In this experiment the combination decreased the E2 levels and shifted the macrophage paradigm away from the pro-tumorigenic phase.

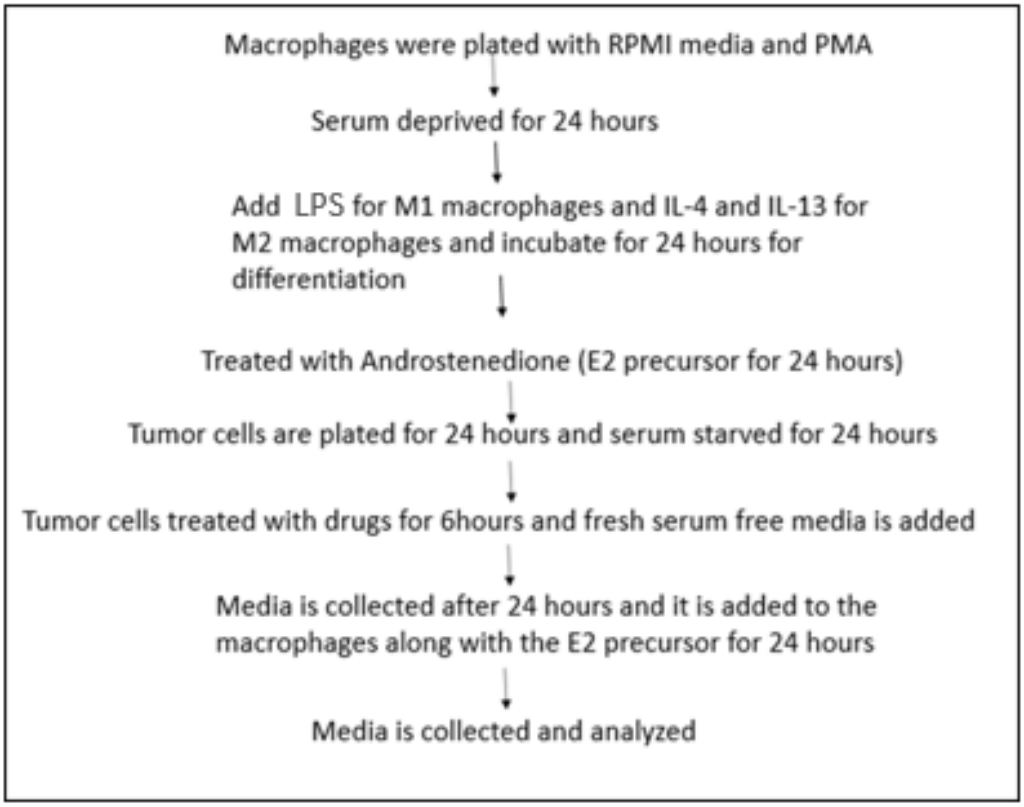


FIG 28: Experimental design to determine E2 levels

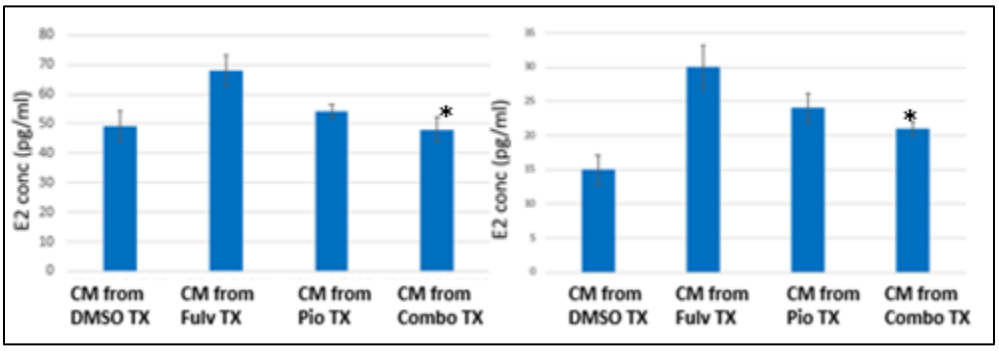


FIG 29: Release of E2 by M0 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of E2 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

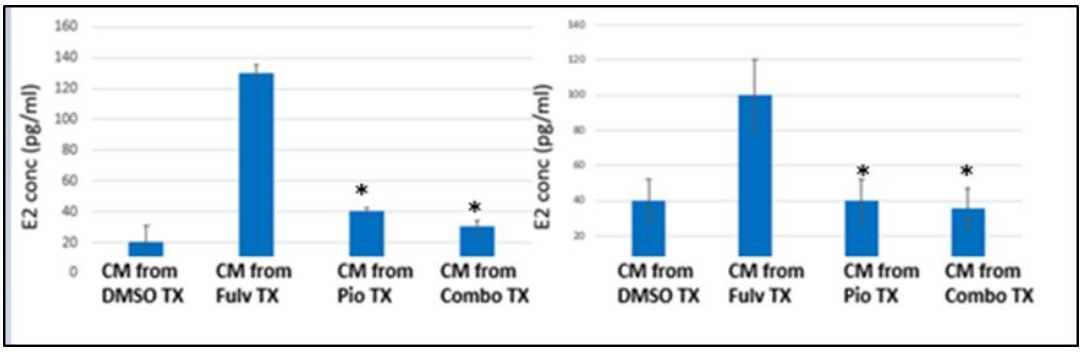


FIG 30: Release of E2 by M1 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of E2 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

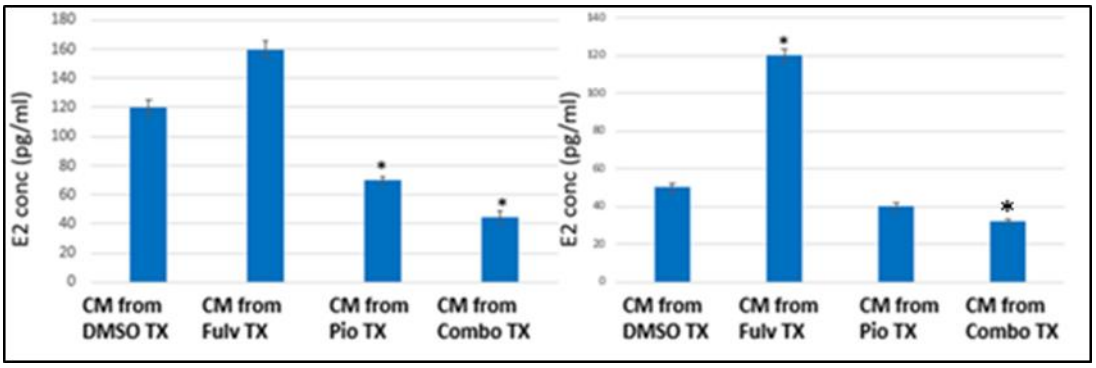


FIG 31: Release of E2 by M2 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of E2 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

Conclusion- For E2 levels, the M0, M1 and M2 macrophages were incubated with the tumor conditioned media of A549 and 201T cells pretreated with the combination showed

significant decrease in E2 levels. This effect was seen the best in when M2 macrophages were incubated with the pretreated tumor conditioned media of A549 and 201T.

Decreased expression levels of Amphiregulin could be one of the effects of dual therapy.

Amphiregulin is a prominent EGFR ligand and it is widely expressed in various tissues and in carcinogenesis. AREG plays a bi-functional role in inflammation. It can regulate secretion of pro-inflammatory cytokines. In order to understand the role of AREG in macrophages, we carried out an experiment where we treated macrophages with fulvestrant and/or pioglitazone and used that conditioned media to incubate it with different adenocarcinoma cells **(Figure 13).** We observed a significant decrease in AREG levels when M0 macrophages were pretreated with the combination and used to incubate A549 (left) and 201T (right) cells **(Figure 32).** In **Figure 33,** we saw a significant decrease in AREG levels when M1 macrophages were pretreated with the combination and it was subjected to incubation with 201T (right) cells. In **Figure 34**, there was a significant decrease in AREG levels observed when M2 macrophages were pretreated with the drugs and subjected to incubation with 201T (right) cells and A549 (left). We believe that by blocking E2 in the tumor microenvironment, the fulvestrant could shift the macrophage paradigm more towards the M1 phase but an increase in AREG levels is believed to be a compensatory mechanism of the fulvestrant driven ER blockade. Overall, to provide a promising effect the AREG levels should be below the placebo and this was taken care by pioglitazone. The 201T adenocarcinoma cell lines were more susceptible to the combination compared to the KRAS mutant A549**.** However, similar results i.e. a significant decrease in the AREG levels were seen.

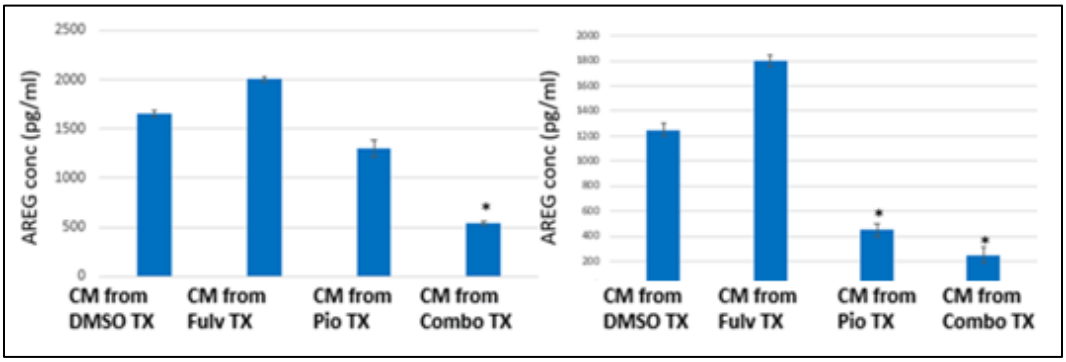


FIG 32: Release of AREG by M0 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of AREG in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.005).

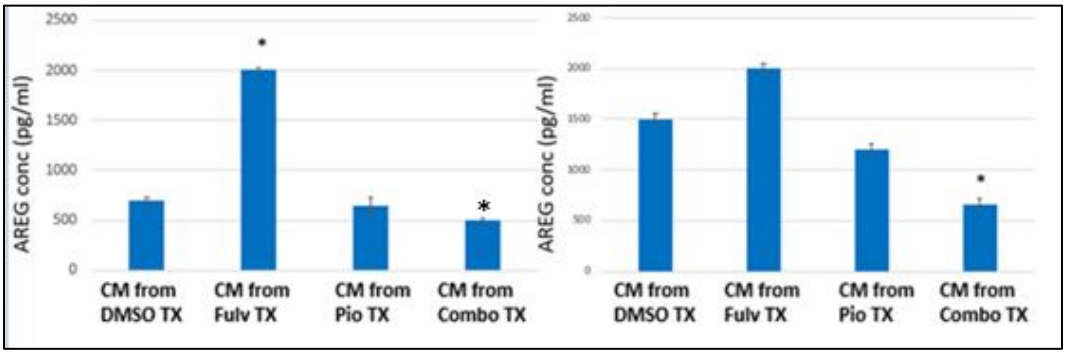


FIG 33: Release of AREG by M1 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of AREG in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

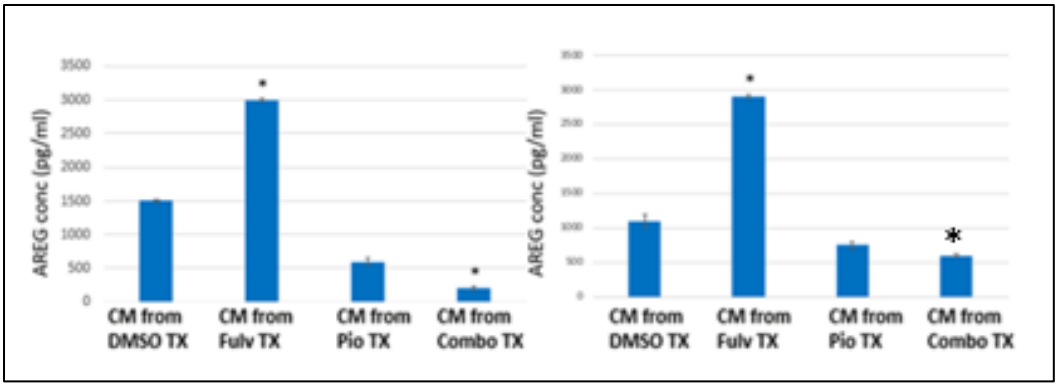


FIG 34: Release of AREG by M2 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of AREG in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.005).

Conclusion- For AREG levels, the M0, M1 and M2 macrophages were incubated with the tumor conditioned media of A549 and 201T cells pretreated with the combination showed significant decrease in AREG levels. This effect was seen the best in when M2 macrophages were incubated with the pretreated tumor conditioned media of A549 and 201T.

Decreased expression levels of IL-1 β could be another effects of dual therapy.

Cancer cells can directly produce IL-1β. It has been shown that there can be a cross-talk between cancer cells producing IL-1β and the immune cells in the tumor microenvironment. Patients with solid tumors in which IL-1β has been shown to be up regulated can the cause of bad prognosis. IL-1β can exhibit both autocrine and paracrine behavior. It induces expression of metastatic genes such as matrix metalloproteinases

(MMP) and stimulates nearby cells to produce angiogenic proteins and growth factors such as VEGF, IL-8, IL-6 and TNFα. These angiogenic factors are responsible for M2 activation and proliferation in the tumor microenvironment. IL-1β is important for tumor growth, proliferation, metastasis, and angiogenesis. In order to understand the role of IL-1 β in macrophages, we decided to pretreat the tumor cells with fulvestrant and/or pioglitazone and incubate macrophages (MO, M1 and M2) with the tumor conditioned media. We observed a significant decrease in levels when A549 (left) and 201T (right) cells were pretreated with the combination and M0 macrophage were incubated with the conditioned media **(Figure 35)**. In **Figure 36,** we saw a significant decrease in IL-1b levels when 201T (right) cells were pretreated with the combination and it was subjected to incubation with M1 macrophages. In **Figure 37,** there was a significant decrease in IL-1b levels observed when 201T (right) cells and A549 (left) were pretreated with the drugs and subjected to incubation with M2 macrophages. We believe that by blocking IL-1b in the tumor microenvironment by pioglitazone, there could be a connection with activation of PPAR and decreased COX-2 expression levels.

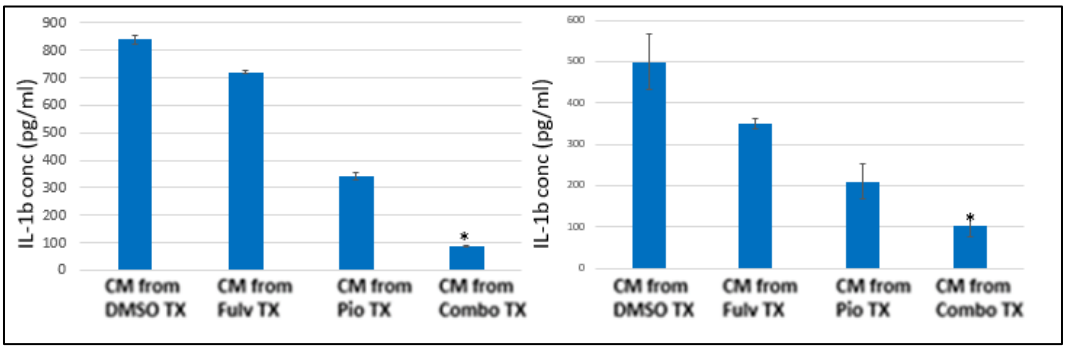


FIG 35: Release of IL-1b by M0 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-1b

in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.005).

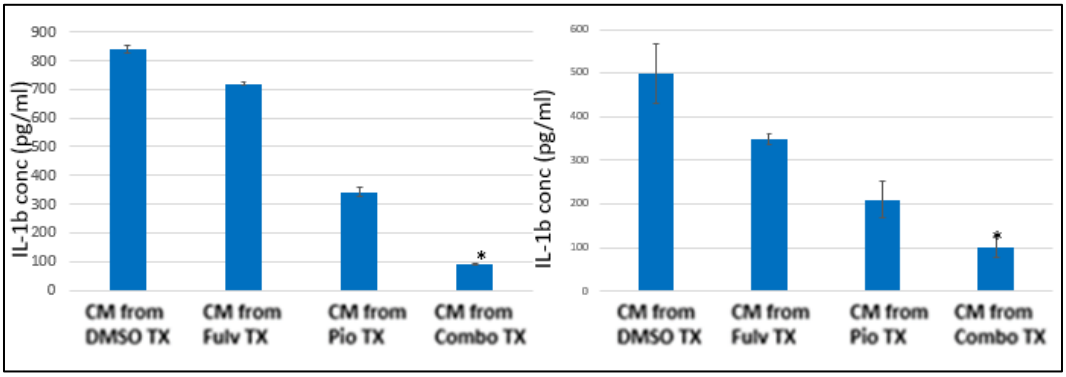


FIG 36: Release of IL-1b by M1 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-1b in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

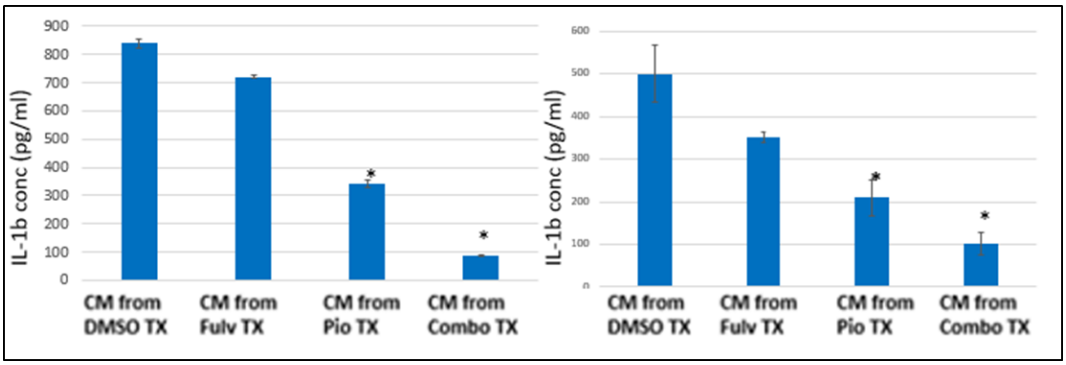


FIG 37: Release of IL-1b by M2 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of IL-1b in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.005).

Conclusion- For IL-1b levels, when 201T and A549 cells were pretreated with combination and then their conditioned media was incubated with M0, M1, M2 macrophages there was significant decrease in release of IL-1b observed. This effect was seen the best in when M2 macrophages were incubated with the pretreated tumor conditioned media of A549 and 201T.

Treatment with dual therapy inhibits expression of COX-2 and PGE2

Based on the above results we observed that the combination acts on IL-1B which is the major inducer of COX-2 and PGE2. We treated the macrophages with fulvestrant and/or pioglitazone for 6 hours. The tumor cells were then incubated with the treated macrophage conditioned media for 24 hours. The media samples and the lysate samples were collected after 24 hours for analysis **(Figure 38).** In **Figure 39** there was no substantial difference in the regulation of COX-2, pAKT, pMAPK, cyclin- D1 and PGE2 in tumor cells incubated with the media from M0 macrophages. However, we observed a different trend with M1 macrophages. There was a downregulation of the COX-2 protein shown in the western blot below. The blot was also probed for other downstream signaling targets like- pAKT, pMAPK and cyclin D1. The combination showed a decrease in phosphorylation of pAKT, pMAPK and cyclin D1 in 201T and A549 cell lines subjected to incubation by pretreated M1 macrophage conditioned medium. We believe that the combination might arrest the cell proliferation and cell survival cycle. We did see a better result with M2 macrophages. There was a complete downregulation of COX-2, pAKT, pMAPK and cyclin D-1 when the membranes were probed in 201T and A549 cell lines subjected to incubation by pretreated M2 macrophage conditioned medium**.** We expected to see overlapping results

of COX-2 with PGE2 levels in the samples. There was significant decrease of PGE2 levels when the M0 and M2 macrophages were pretreated with the combination and incubated with 201T (right) and A549 (left) **(Figure 40 and 42).** However, there was not much of a difference in the PGE2 levels when the M1 macrophages were pretreated with the combination and incubated with 201T (right) and A549 (left) **(Figure 41).** We believe that the combination targets the COX-2 pathway and that IL-1b is one of the major growth factors responsible for the downregulation of COX-2.

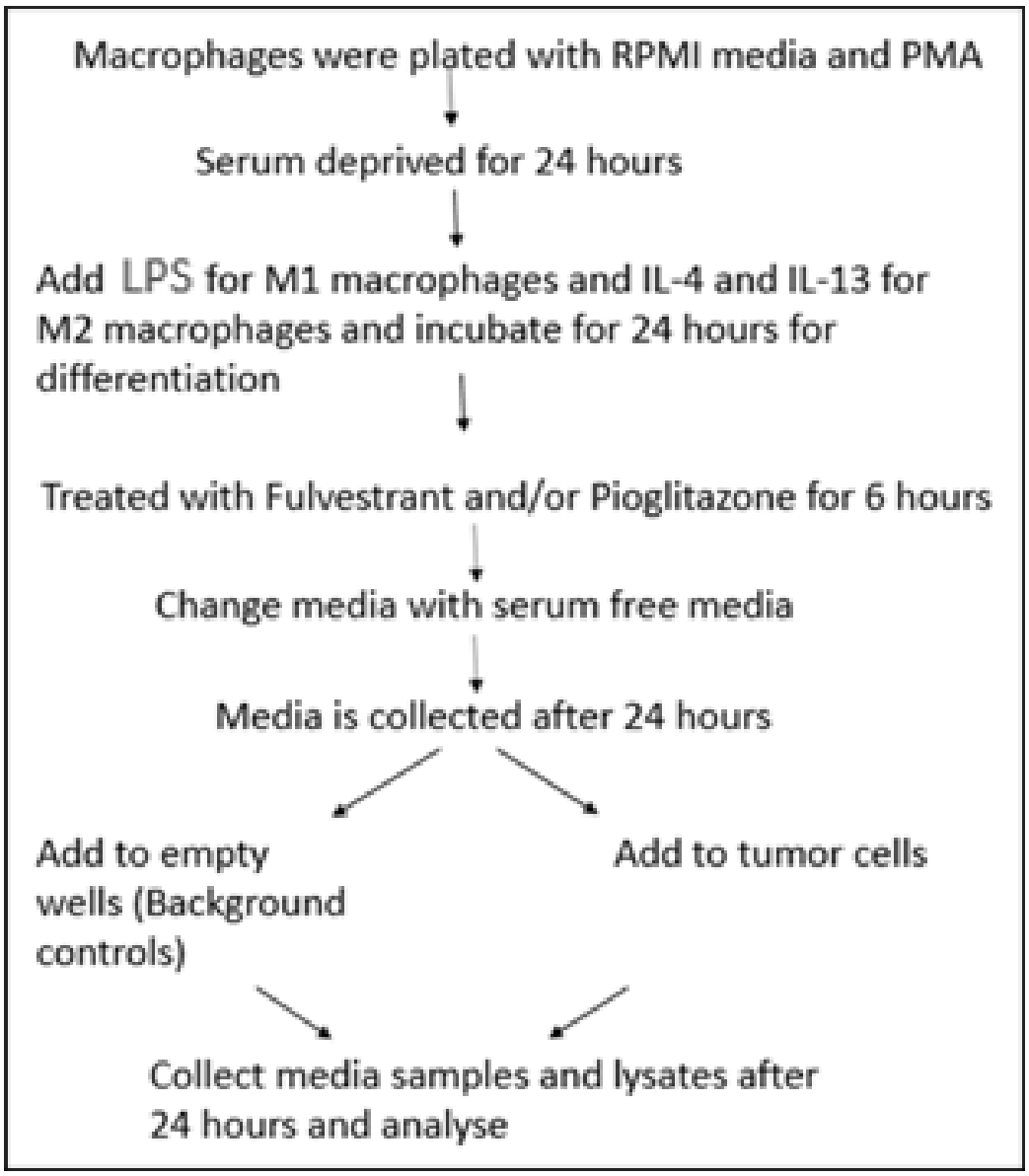


FIG 38: Experimental design for determining COX-2 and PGE-2 levels

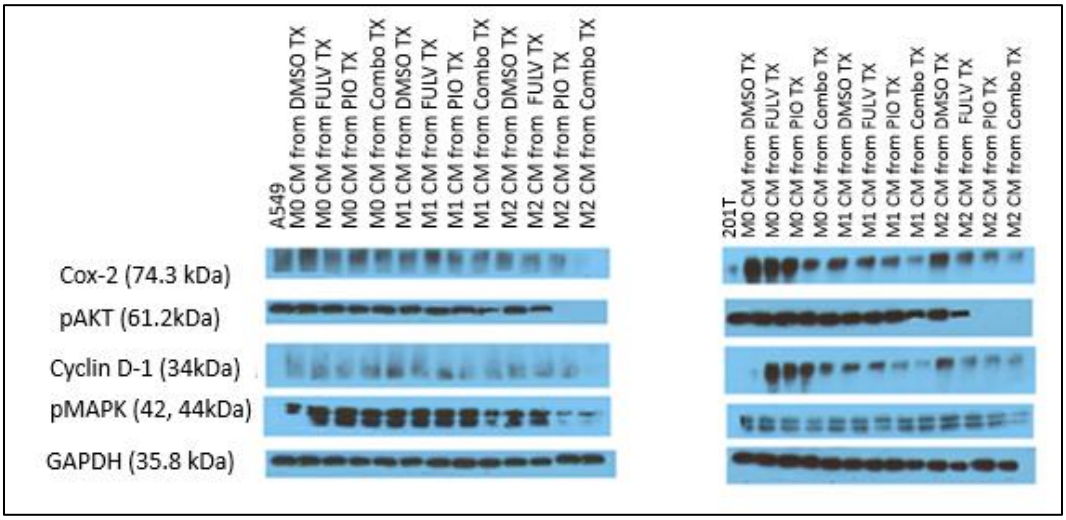


FIG 39: COX-2 levels are inhibited when A549 (Left) and 201T (Right) were incubated with combination treated M1 as well as M2 conditioned media for 24 hours. Decreased phosphorylation of pAKT, pMAPK and cyclin D1 in both the cell lines.

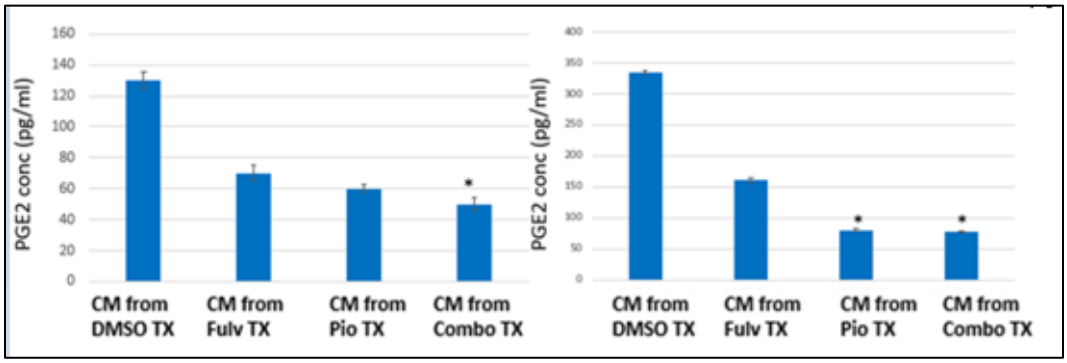


FIG 40: Release of PGE2 by M0 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of PGE2 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.05).

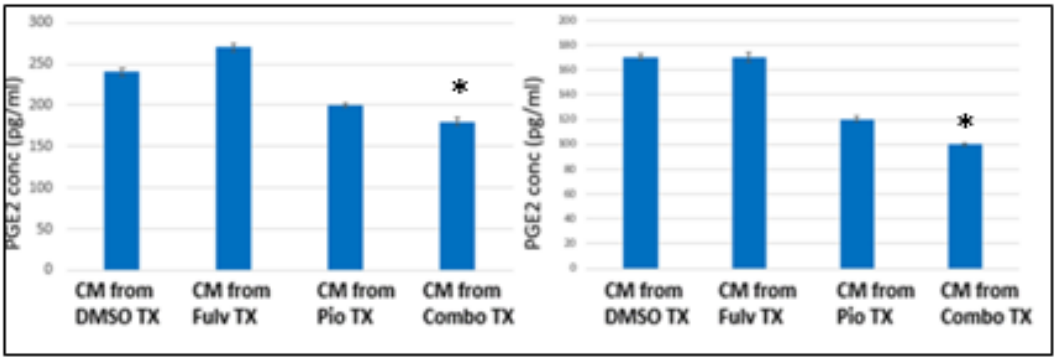


FIG 41: Release of PGE2 by M1 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of PGE2 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.005).

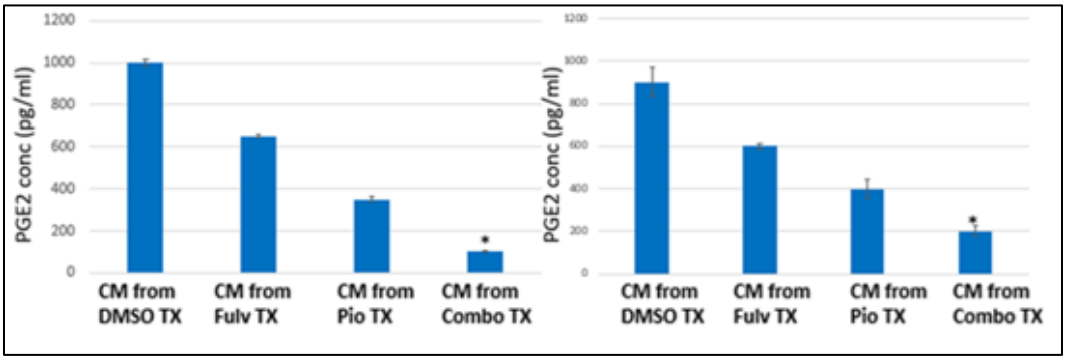


FIG 42: Release of PGE2 by M2 macrophages incubated with lung tumor conditioned medium A549 (left) cells and 201T (right) cells after drug treatment. The amount of PGE2 in conditioned media alone was subtracted from the levels detected in macrophage medium. TX- treatment (* P value < 0.005).

Conclusion- For COX-2 expression, there was highest inhibition of COX-2 expression observed when the 201T and A549 cells were incubated with pretreated combination M2 conditioned media. These results were mimicked by the PGE2 results as well.

Incubation with AREG neutralizing antibody and macrophage conditioned medium pre-treated with drug combination showed decreased COX-2 expression in 201T and A549

Based on the AREG ELISA results, we decided to use a neutralizing AREG antibody. We hoped to see a similar effect when we use the neutralizing AREG antibody or the medium pre-treated with the drug combination. We incubated the tumor cells with recombinant AREG (10ng/ml), control antibody (1ug/ml) or AREG neutralizing antibody (0.8ug/ml) for 2 hours. The tumor cells were then subjected to drug-pretreated macrophage conditioned media incubation for 24 hours. Recombiant AREG (positive control) induced COX-2. In DMSO treatments, AREG neutralizing antibody partially blocked the expression of COX-2, suggesting AREG is one of the factors that controls COX2 expression. With reduced COX2 expression seen with drug treatments, the neutralizing antibody could block it further, suggesting the lower levels of AREG found previously are one of the main factors that control COX-2 expression after drug treatment. . Treatment with conditioned medium from macrophages pre-treated with the drug combination itself did directly block COX-2 regulation as seen by the COX-2 blot, so no further effect was observed with the neutralizing AREG antibody. Since conditioned medium from double treated macrophages had the lowest AREG levels, the is consistent with the hypothesis that AREG could be one of the prominent ligands responsible for downstream signaling and a possible mediator for crosstalk between macrophages and tumor cells. **(Fig 43)**.

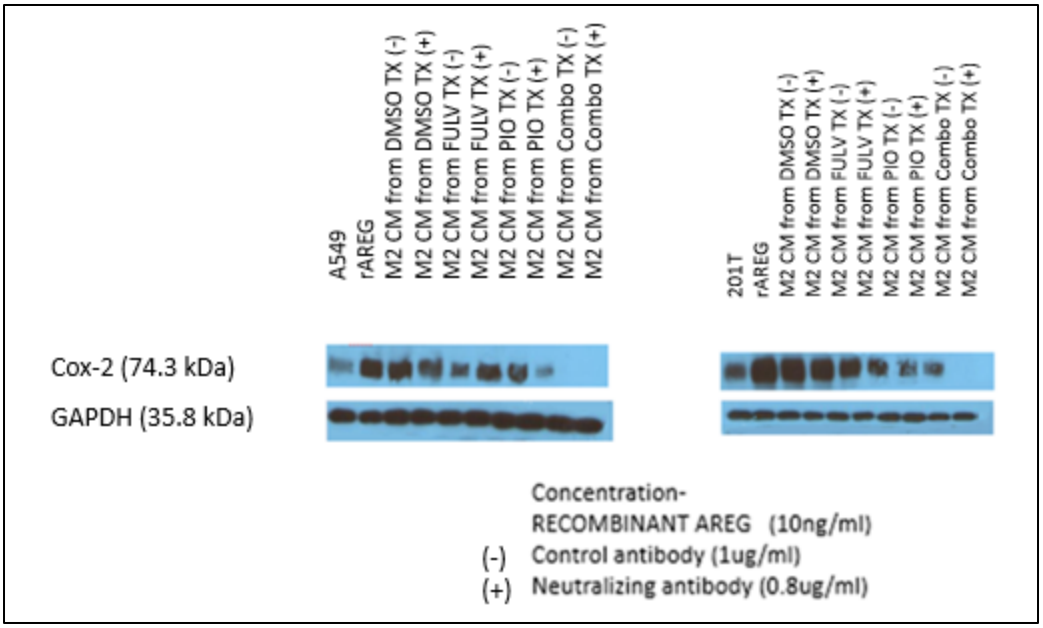


FIG 43: Inhibition of COX-2 by the macrophage conditioned medium pretreated with drugs with and without the preincubation of neutralizing AREG Ab in A549 (Left) and 201T (Right) in presence of M2 conditioned media.

Conclusion- There was highest inhibition of COX-2 expression in 201T/ A549 cells with the pretreated combination conditioned media from M2 macrophages in presence or absence of the neutralizing AREG antibody, most likely because AREG levels are suppressed aftere dual drug treatment (see Fig. 34). In the single treatments and DMSO treatment, AREG neutralizing antibody partially blocked the effects. This shows that AREG is a prominent ligand responsible for COX-2 downregulation.

Decreased regulation of COX-2 by blocking IL-1β using a neutralizing antibody.

Observing a fall in COX-2 expression status on treatment with combination- treated conditioned medium, as well as AREG neutralizing antibody, we next looked at whether

the same decrease was observed by targeting IL-1β. IL-1β is a known inducer of COX-2. We incubated M2 macrophage conditioned medium with tumor cells that received pretreatment with neutralizing IL-1b antibody (2 hours). The tumor cells were also subjected to M2 macrophage conditioned medium pretreated with (i) DMSO and (ii) combination. We hoped to mimic the effects of the dual therapy by using an IL-1b neutralizing antibody (conc. 1.5ug/ml). There was a change in COX-2 levels in the dual treatment group with the A549 cells in comparison to the IL-1β neutralizing antibody treatment group. However, there wasn't much change observed in COX-2 expression with 201T cells. There could be a possibility that AREG levels are higher and that could have an impact on the effect of the neutralizing antibody on these cells **(Figure 44).** These above results were similar to the results obtained by the PGE2 ELISA analysis. DMSO pre-treated conditioned medium from M2 macrophages induced PGE2, and this effect could be blocked by neutralizing antibody. The amount of PGE2 produced in presence of neutralizing antibody was similar to that seen for conditioned medium pretreated with the drug combination, showing the actions were mimicked **(Figure 45 and 46).**

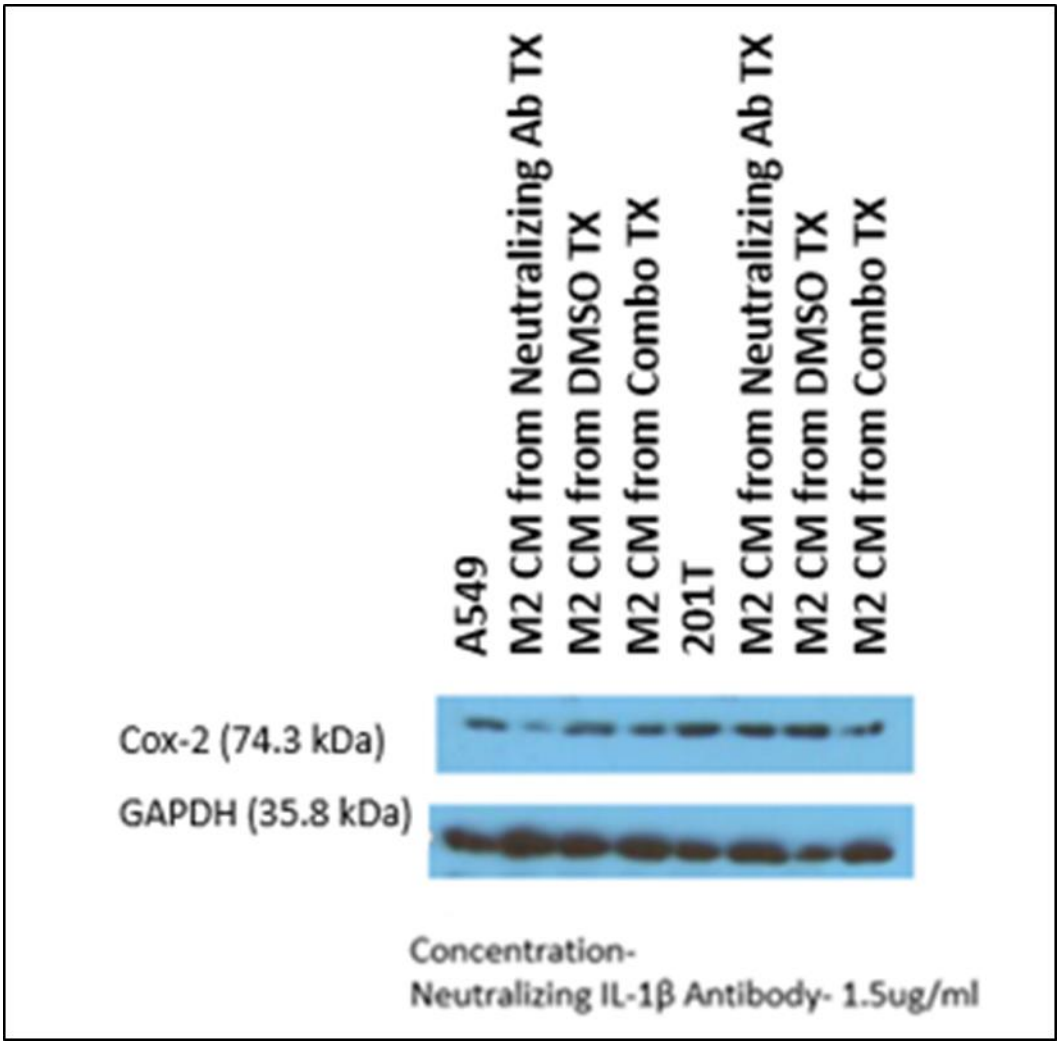


FIG 44: COX-2 levels decreased in presence of IL-I beta neutralizing antibody and conditioned medium pretreated with drug combination for 201T and A549.

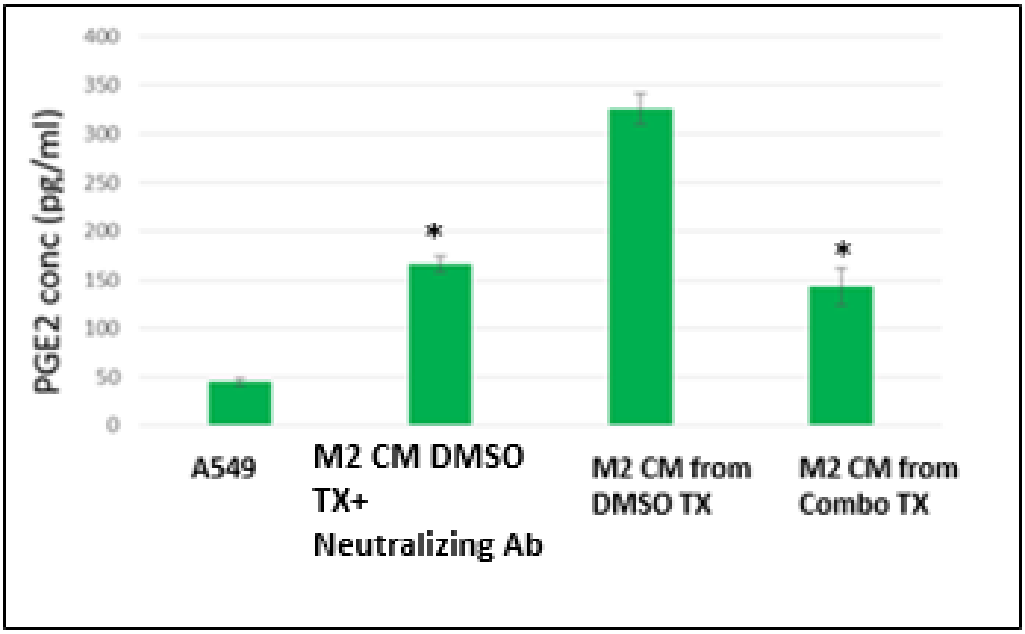


FIG 45: PGE2 levels when A549 was preincubated with IL-1 β neutralizing antibody in the presence of DMSO pre-treated conditioned medium, DMSO-pretreated conditioned medium or combination-pretreated M2 macrophage media (* P value < 0.005).

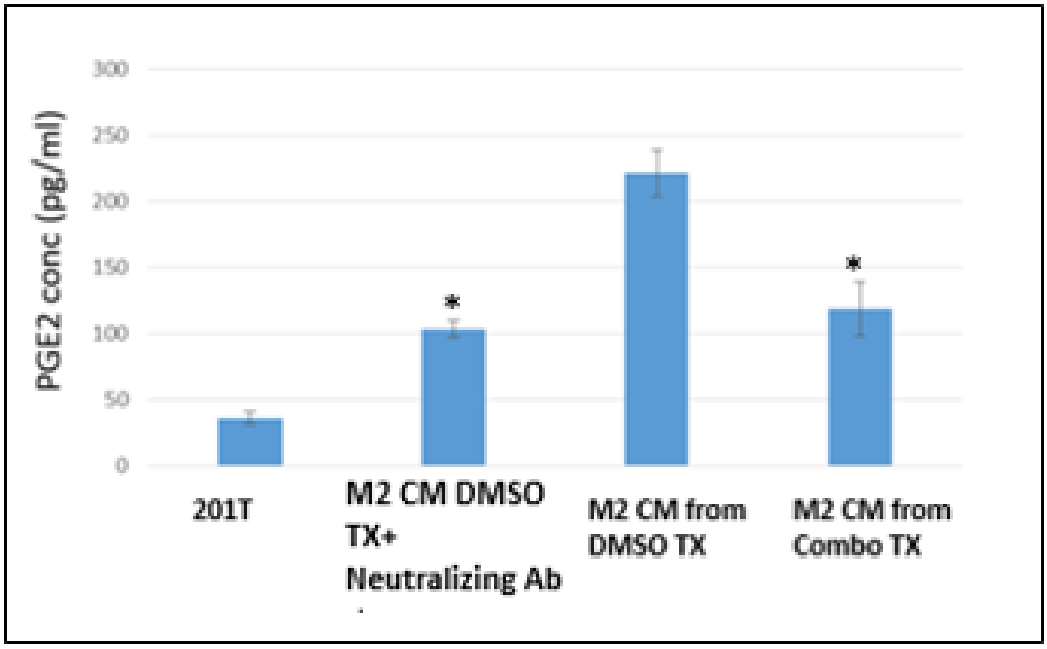


FIG 46: PGE2 levels when 201T was preincubated with IL-1 β neutralizing antibody in the presence of DMSO pre-treated conditioned medium, DMSO-pretreated conditioned medium or combination-pretreated M2 macrophage media, (* P value < 0.005).

Conclusion- There was inhibition of COX-2 expression and PGE2 release in 201T/ A549 cells in presence of the neutralizing IL-1b antibody with DMSO treated conditioned medium and in 201T/ A549 cells subjected to pretreated drug combination conditioned media from M2 macrophages. These results were replicated by ELISA experiments done using the media from the same experiment. Since the neurtalizing antibody condition mimicked the therapeutic effect, this shows that IL-1b is most likely one of the prominent ligands responsible for COX-2 downregulation.

Treatment with IL-1β neutralizing antibody and AREG neutralizing antibody lead to downregulation of COX-2 and PGE2 in two different NSCLC cells.

Based on the above results, we decided to block the prominent receptors responsible for macrophage control of COX-2 expression in tumor cells, that are responding to the ligands controlled by the PPAR and ER receptors in macrophages. In order to show that IL-β and AREG from the macrophages are paramount for the regulation of COX-2 signaling in cancer cells, we incubated the starved tumor cells with a recombinant IL-1b (rIL-1b) and recombinant AREG (rAREG) for 24 hours. According to our hypothesis we observed a cross-talk between the macrophages incubated with IL-4 and IL-13 and the tumor cells. We serum starved the tumor cells and incubated them with either rAREG (10ng/ml)/ rIL-

1b (0.1ng/ml)/ rAREG and rIL-1b for 2 hours and supplemented them with fresh serum free media. The main goal of this treatment group was to understand whether the AREG and IL-1β lead to specific upregulation of COX-2 and PGE2 levels in cancer cells. Based on our expectations, we saw an increase in expression of COX-2 when the cells were treated with the recombinant molecules. There was a higher expression when the two recombinant molecules were combined in comparison to the treatment from a single recombinant molecules. Once we established that AREG and IL-1β do have an effect on the regulation of the COX-2 signaling pathway, we decided to incubate the tumor cells with the following conditions- neutralizing AREG antibody (0.8ug/ml)/ neutralizing IL-1β antibody (1.5ug/ml)/ neutralizing AREG and IL-1β antibody for 2 hours. The above conditions were incubated with the M2 macrophage control (DMSO) conditioned media for 24 hours. This media had the same amount of AREG and IL-1β in all the conditions. The final condition involved the addition of the conditioned media from the macrophages pretreated with the combination of drugs onto starved tumor cells. We saw a decrease in the expression of COX-2 in presence of the neutralizing antibody. There was a complete inhibition of PGE2 and COX-2 when the combination of neutralizing antibodies were used **(Figure 48 and 49).** We hoped to see a similar action being mimicked by the cancer cells when they were treated with combination therapy pre-treated conditioned medium **(Figure 47).** This result fulfilled our expectations in both 201T and A549 adenocarcinoma cell lines. This further adds evidence that AREG and IL-1beta release have an effect on the downstream signaling of COX-2 via their regulation by PPAR signaling and ER signaling in macrophages. Other possible cytokines like VEGF could also be involved.

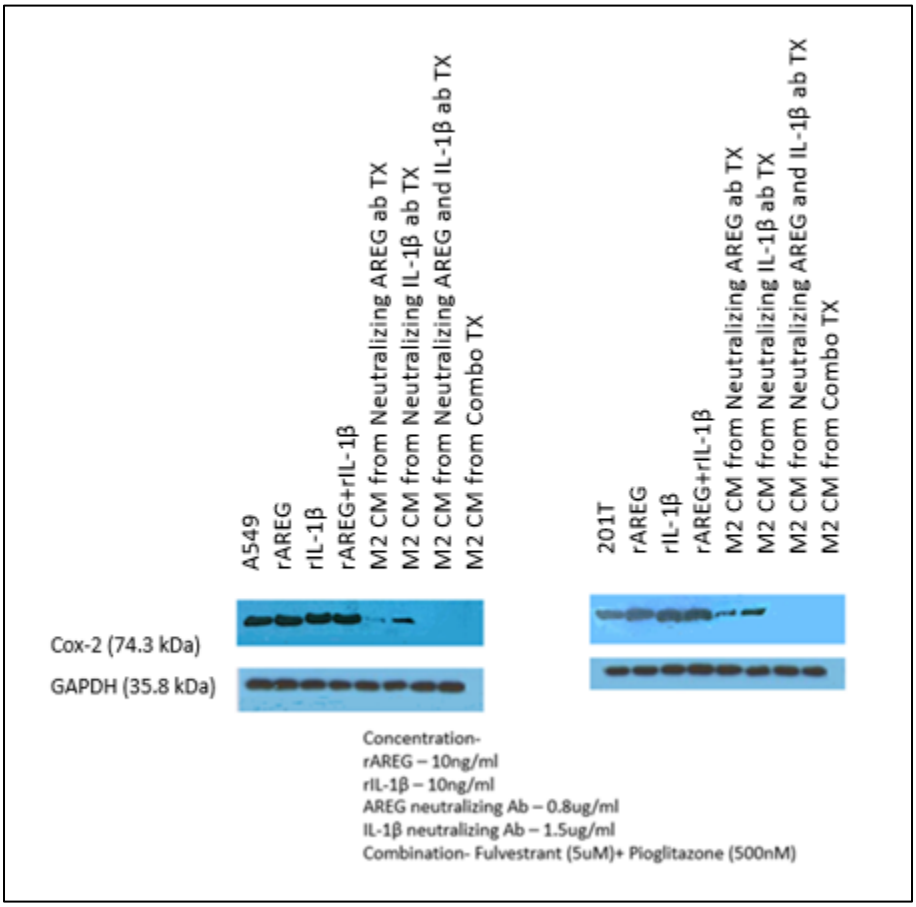


FIG 47: Inhibition of COX-2 when A549 (Left) and 201T (Right) were preincubated with a combination of neutralizing antibodies (IL-1 β and AREG) and was exposed by the M2 conditioned media. This effect was mimicked by the incubation with drug combination pre-treated M2 conditioned media on A549 (A) and 201T (Right).

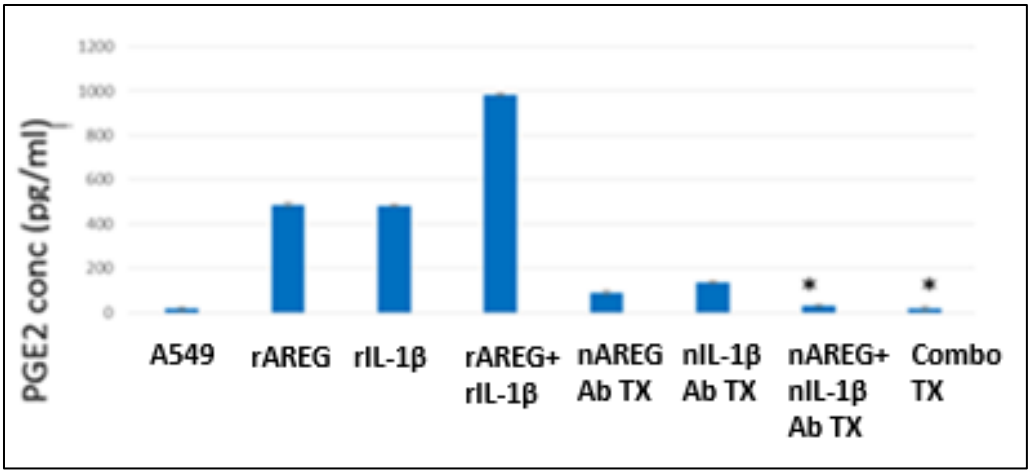


FIG 48: PGE2 levels were significantly decreased when A549 was preincubated with a combination of neutralizing antibodies (IL-1 β and AREG) and was exposed by the M2 conditioned media. This effect was mimicked by the incubation of combination treated M2 conditioned media on A549. (* P value < 0.005).

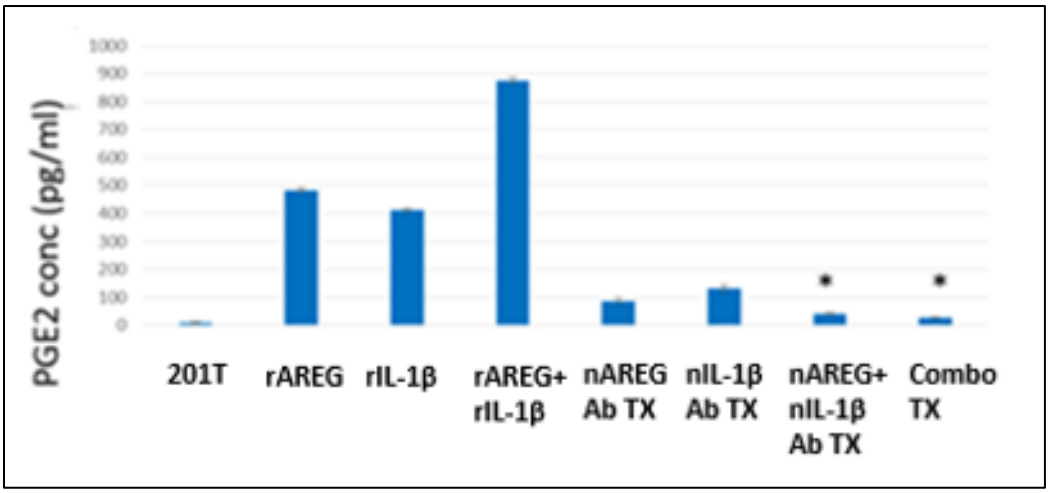


FIG 49: PGE2 levels were significantly decreased when 201T was preincubated with a combination of neutralizing antibodies (IL-1 β and AREG) and was exposed by the M2 conditioned media. This effect was mimicked by the incubation of combination treated M2 conditioned media on 201T (* P value < 0.005).

Conclusion- There was highest inhibition of COX-2 expression and PGE2 release in 201T/ A549 cells in presence of the combination of neutralizing IL-1b antibody and neutralizing AREG antibody. These results were mimicked when the 201T/A549 cells were subjected to incubation with pretreated combination M2 macrophage media. Both these conditions mimicked the same therapeutic effect. This shows that IL-1b and AREG are prominent ligands responsible for COX-2 downregulation and its subsequent activity.

Combination decreases possible targets involved in cell migration.

IL-1β levels can affect the crosstalk between macrophages and tumor cells. Evidence suggests that a decrease in IL-1β can arrest cell migration. In our results we showed that the combination decreased the levels of IL-1β generally. We expected to see a decrease in cell migration of tumor cells when they were incubated with treated macrophage conditioned media for 72 hours. The scratch was made using a 200ul pipette on starved tumor cells. The images were taken before and after the 72 hour incubation period **(Figure 50 and 51).** The images were then analyzed and the extent of the closure of the gap was calculated. The combination arrested the closure completely in comparison to the placebo as we had expected. There were better results obtained in the A549 cells in presence of the combination **(Figure 52).** Fulvestrant had more number of cells filing the central gap in

comparison to pioglitazone.

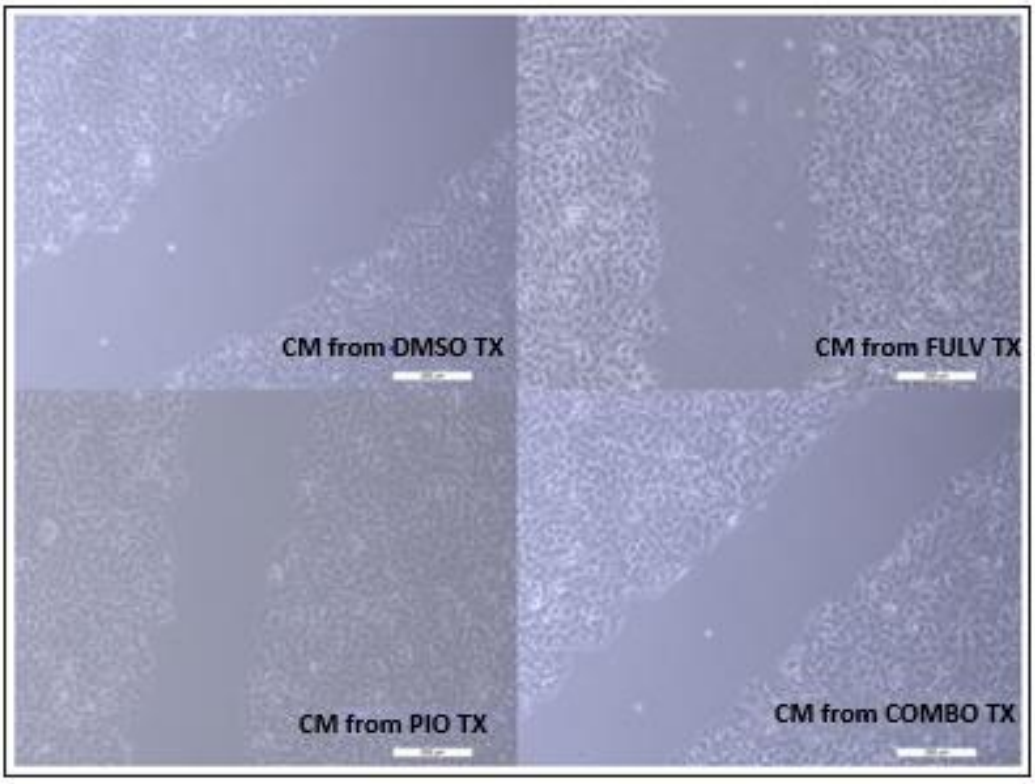


FIG 50: Scratch assay. Images taken after making a scratch using a 200ul pipette tip. The A549 cells were exposed to various treatments M2 .

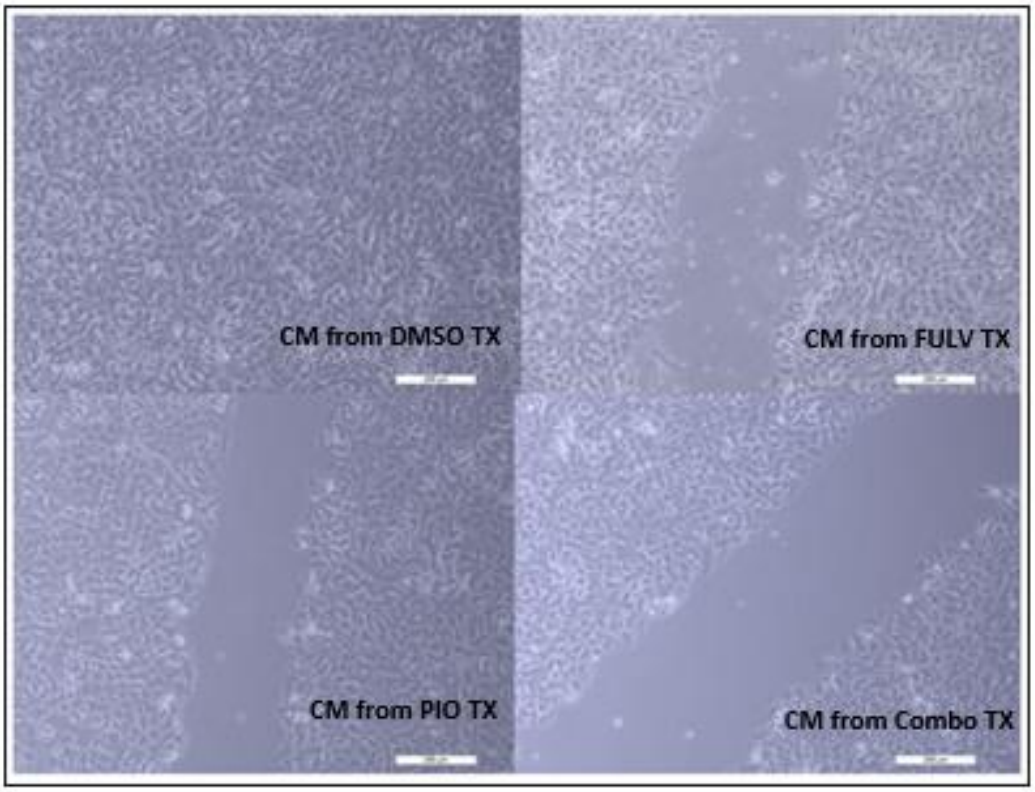


FIG 51: Tumor-promoting Scratch assay after 96 hour incubation. The placebo group showed highest wound healing capacity. However, the A549 cells were arrested when treated with combination. Therefore, no wound healing was seen.

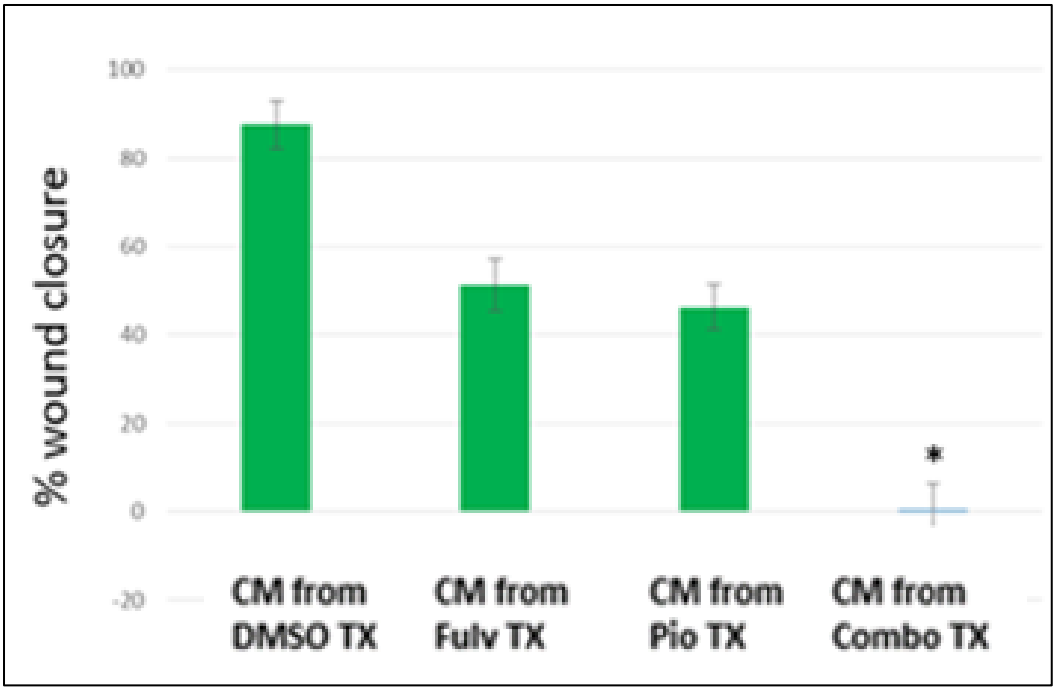


FIG 52: Highest wound healing observed when the A549 cells were incubated with the placebo (DMSO) for 96 hours in presence of M2 conditioned media. There was a significant reduction of wound healing capability by the cells in presence of the combination. (* P value < 0.005).

Conclusion- There was lowest percentage (8%) of wound healing when the A549 cells were incubated with pretreated combination M2 macrophage conditioned media incomparison to placebo. The combination might have an effect on migratory proteins or pathways which are responsible for crosstalk between macrophage and tumor cell migration.

DISCUSSION:

Here we have aimed at studying the mechanisms that modulate ER and PPAR pathways in tumor cells and macrophages. Overexpression of ER, increased levels of E2, AREG, IL-1B and VEGF, and infiltration of tumor associated M2 macrophages are common in several cancer subtypes including NSCLC. We used a selective estrogen receptor degrader Fulvestrant and a PPARy activator Pioglitazone to study the possible signaling pathways and downstream targets that could modulate the crosstalk between macrophages and tumor cells in the tumor microenvironment. Previous studies in our laboratory have suggested that Pioglitazone has an anti-proliferative effect on tumor cells making it a possible choice for treatment of NSCLC. However, it can be a driver for protumorigenic macrophage production in the tumor microenvironment. In order to maintain a balance between M1/M2 macrophages we decided to use fulvestrant. Fulvestrant is an ER degrader that blocks ER signaling in macrophage and we hoped to see a shift of the macrophage paradigm more towards the pro-inflammatory/ M1 phenotype. We observed that by incubating the macrophages with the dual therapy treated tumor conditioned media, there was a decrease in M2 cytokines and increase in M1 cytokines. We conclude that there is an interaction between the ER and PPAR pathways in both tumor cells and macrophages. We wanted to understand the workings of the two signaling pathways in macrophage in presence of the tumor conditioned media. We validated that dual therapy showed decreased cell proliferation of tumor cells when incubated with macrophage conditioned media. From the ELISA results, we examined ligands released by macrophages that could contribute to downstream signaling in these cells. The combination showed decreased IL-1β, AREG, VEGF, IL-10, PGE2 and E2 levels. Based on published evidence, IL-1β is a prominent

inducer of the PPAR/COX-2/PGE2 signaling pathway. We continued our experiments with M2 macrophages because we observed higher responsiveness to the combination. We were able to validate that the dual therapy downregulates COX-2, Cyclin D1 and dephosphorylates pAKT in tumor cells incubated with pretreated M1/ M2 macrophage conditioned media. We also saw decreased levels of PGE2. Tumor cells incubated with IL-1β neutralizing antibody showed an inhibition of COX-2 in tumor cells. This led us to believe that IL-1β is one of the cytokines that affect downstream signaling. An important EGFR ligand – AREG- was shown to be decreased in presence of the combination. We observed a downregulation of COX-2 in the presence of the AREG neutralizing antibody. We believe that AREG is one of the drivers of the crosstalk between macrophages and tumor cells in the tumor microenvironment. In order to substantiate our claims that these two ligands are important downstream targets, we combined both neutralizing antibodies to see if it mimics the action observed when the cells were incubated with the combination. We were able to validate the inhibition of COX-2 in both the adenocarcinoma cell lines. This shows that the combination of neutralizing antibodies showed a synergistic effect and mimicked the result obtained by the combination. We also found that VEGF might be another driver for M2 macrophages in the tumor microenvironment. IL-1β is an inducer of cell migration in breast cancer. We tested whether the combination has an effect on the migration of tumor cells incubated with macrophage conditioned media. We saw a negligible amount of cells migrating to form a closure of the scratch. This validates the idea that the combination might have an effect on migratory proteins like fibroblast activating protein (FAP). Further experiments should be carried out to investigate the role of migratory proteins in the crosstalk between macrophages and tumor cells.

FUTURE DIRECTIONS:

Apart from measuring cytokines at a protein level, we can thereby verify our results on mRNA level for various M1 (CXCL9, CXCL10, CXCL11, NOS2) and M2 (Mrc1, tgm2, Fizz1, Ym1/2, Arg1) markers. Using RNA analysis to check for inhibiting COX-2 expression is an alternative. We also suspect that VEGF may be one of the prominent factors responsible for induction of M2 macrophages. It is worth observing if recombinant VEGF can activate COX-2 and stimulate tumor cell proliferation and promote a protumorigenic environment. In order to understand the sensitivity of the combination on VEGF it will be interesting if we use a VEGF neutralizing antibody and downregulate COX-2 to inhibit proliferation. A double conditioned co-culture experiment could help us understand how the pathways are modulated in presence of the combination. These same set of experiments could also be repeated in bone marrow derived macrophages or tumor associated macrophages (TAMs) for verifying the regulation of COX-2/PGE2 pathway. In order to establish a hypothesis describing the modulation of the ER and PPAR pathways it is important to repeat these experiments in a superior model like Transwell migration assay. The transwell migration assay will be suitable to characterize additional ligands that might only be observed via co-culture when cells are in close contact. Based on the above results, an addition of a specific COX-2 inhibitor or a drug that targets AREG and IL-1β secretion could be worth investigating. Apart from macrophages, it would be interesting to study the

effects of the combination on PDL-1, HGF and cAMP signaling in M2 macrophages and other immune cells. Lastly an i*n vivo* tumor model to test the combination of Fulvestrant and Pioglitazone**.**

CONCLUSION

In summary, we have established that Amphiregulin, and Interleukin-1b are important in the expression of COX-2 and its product PGE2. Their expression can be maximally reduced by combination treatment with pioglitazone and fulvestraent **(Fig 53).** This has been further bolstered by the fact that we were able to show decreased cell proliferation and functional reduction in M2 macrophage ligands by combining an ER degrader with a PPAR activator. This interaction was observed in both the adenocarcinoma cell lines (201T and A549). There was also a dual effect of this drug combination on the communication between macrophages and tumor cells, in which M1 cytokines were induced and M2 cytokines were inhibited. As this drug combination also substantially decreased the levels of estrogen in the tumor microenvironment, we believe that this therapy can be useful for both males and females. The drug combination also blocked the compensatory increase in E2 and AREG found with fulvestrant alone.

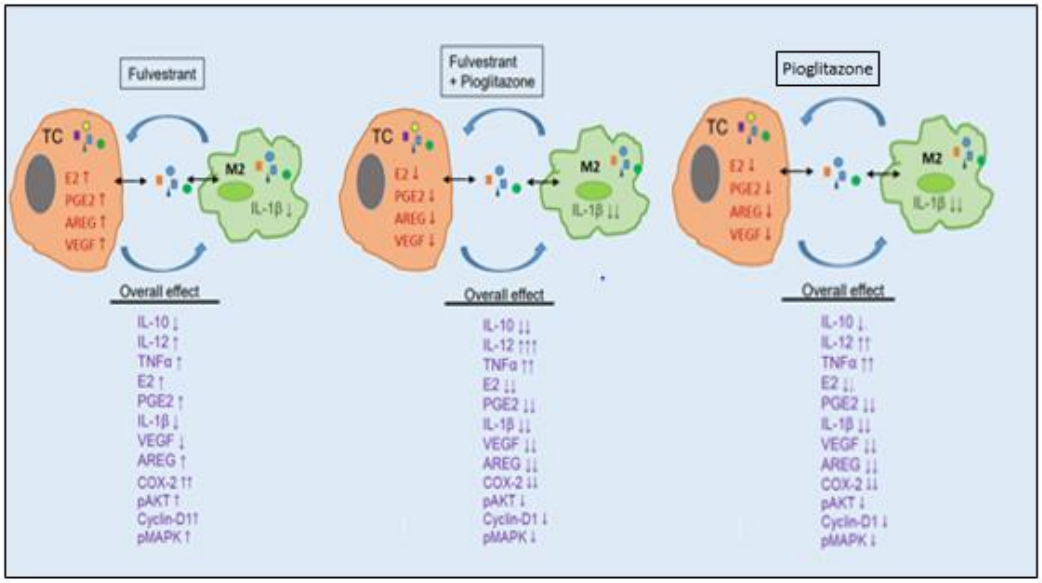


FIG 53: The combination targets the crosstalk between tumor cells and macrophages in the tumor microenvironment. There is downregulation of M2 biomarkers like IL-10, E2, PGE2, VEGF, AREG, COX-2 and IL-1β. Indirectly targeting various common tumor proliferative pathways, the combination promotes cell apoptosis and arrests cell migration. The combination ultimately shifts the macrophage paradigm towards the anti-tumorigenic stage thus, promoting its therapeutic activity.

MATERIALS AND METHODS:

Reagents and cell culture

NSCLC cell lines 201T (was established in our laboratory from primary tissue) and A549 (obtained from American Type Culture Collection, Manassas, VA, USA) , were maintained in 37ºC and 5% CO2. 201T and A549 were grown in basal medium eagle (BME) with 10% fetal bovine serum, 2mM of L-Glutamine and 50X Penicillin Streptomycin all purchased from GIBCO by Life technologies. THP-1 cells were obtained from Kaylee Schwertfeger laboratory and were grown in Roswell Park Memorial Institute Medium (RPMI) with 10% fetal bovine serum, and 50X Penicillin Streptomycin. Rabbit monoclonal COX-2 (12282S ;1:1000), p44/p42 MAPK (9102S ;1:1000), pAkt (4060S ;1:1000), cyclin d1 (2922S ;1:1000) and GAPDH (5174S;1:1000) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Human AREG neutralizing antibody (AF269), Normal goat IgG antibody (AB-108-C) and IL-1β neutralizing antibody (AF269) were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human AREG antibody (2170950010) was purchased from Tonbo (CA, USA). Recombinant IL-1β antibody was purchased from Shenandoah (PA, USA). The antiestrogen (fulvestrant; S1191) and thiazolidone (pioglitazone; CDS021593) were purchased from Selleckchem (TX, USA) and Sigma (M0, USA) respectively. Phorbol 12 Myristate 13-Acetate (PMA) (P1585), LPS (L2630), IL-4 (H7291), IL-13 (SRP3274) was purchased from Sigma (MO, USA). Human Amphiregulin ELISA kit (DY262), Human IL-10 ELISA kit (DY217B), Human il-1b ELISA kit (DY201), Human TNF-a ELISA kit (DY210), Human VEGF ELISA kit

(DY293B), Human IL-12 ELISA kit (DY1270) and Estradiol ELISA assay (KGE014) were purchased from R&D Systems (Minneapolis, MN, USA). PGE2 ELISA kit (500141) was purchased from Cayman (MI, USA). Cell Titre Aqueous One Solution Cell Proliferation Assay MTS was obtained from Promega (Madison, WI, USA). SuperSignal West Pico Chemiluminescent Substrate (1:1) was purchased from Thermo Scientific (Waltham, MA, USA) and Luminata Forte Western HRP Substrate was obtained from Millipore Corporation (Billerica, MA, USA). Immobilon-P transfer membrane was obtained from Millipore Corporation (Billerica, MA, USA). Image analysis was done by LAS core v4.7 from Leica (2015).

Experimental procedures

For measuring the basal expression of RTKs, 450,00cells of 201T and A549 cells were grown in a 6 well plate separately with BME medium, both containing 10% fetal bovine serum. 1,200,000 THP-1 cells along with PMA (1ul/ml media from a stock which is 5ug/ml) were plated in a 6 well plate in RPMI medium containing 10% FBS for 24 hours. The cells were then starved for 24 hours with serum free RPMI media. Based on the necessary conditions the adherent THP-1 cells were then differentiated based on the inducers added in the media – M0 macrophages (no inducer), M1 macrophages (LPS-concentration: 10ng/ml), M2 macrophages (IL-4 – concentration: 20ng/ml and IL-13 – concentration: 20ng/ml) for 24 hours. Cells were washed with phosphate-buffered saline and lysed with RIPA whole lysis buffer. The insoluble fraction was cleared by centrifugation at 12,000 x g for 30 minutes at 4°C. Protein concentrations were measured by Bradford assay (BIO-RAD). Samples were prepared containing 40 μg of proteins, 4X

NuPAGE LDS Sample buffer with BME and RIPA buffer. Prepared samples were then subjected to denaturation by heating at 100°C for 5 minutes. 50ul of the samples were loaded in each well of the gel. Gels were prepared using NEXT gel 7.5% Acrylamide solution, 10% APS and TMED. Gels were run at 100 volts for two hours. Proteins were then transferred to a PVDF membrane (Immobilon-P) over a period of one and a half hours. Membranes were then blocked with 5% milk for one hour followed by overnight incubation with the primary antibodies at 4°C. The membranes were then subjected to three washes of fifteen minutes each in TBST. Following this they were incubated with the secondary antibody at room temperature for one hour. Membranes were then washed four times with TBST for ten minutes each. Developing solution used was SuperSignal West Pico Chemiluminescent Substrate (1:1). Developed films were obtained from the X-ray film developer.

In order to determine the level of M1 and M2 cytokines in the media- 450,000 cells of each of the two adenocarcinoma cell lines were plated per well in a 6 well plate in BME medium containing 10% FBS. The cells were then starved for 24 hours with serum free DMEM medium containing 5% glutamine and 5% penicillin- streptomycin. The cells were then treated with fulvestrant or/and pioglitazone for 6 hours from the date of plating. The media was then stripped off and replaced with serum free DMEM medium for 24 hours. Simultaneously, 1,200,000 THP-1 cells along with PMA (1ul/ml media from a stock which is 5ug/ml) were plated in a 6 well plate in RPMI medium containing 10% FBS for 24 hours. The cells were then starved for 24 hours with serum free RPMI media. Based on the necessary conditions the adherent THP-1 cells were then differentiated based on the

inducers added in the media – M0 macrophages (no inducer), M1 macrophages (LPS- concentration: 10ng/ml), M2 macrophages (IL-4 – concentration: 20ng/ml and IL-13 – concentration: 20ng/ml) for 24 hours. The macrophages were the subjected to the treated tumor conditioned media incubation for 24 hours. The media samples were collected for ELISA analysis of various M1 and M2 markers. The conditioned media then were collected and centrifuged at 12,000 g for 10 minutes at 4ºC to remove any insoluble fractions. Following the manufacture's protocol, IL-12, TNF-a, AREG, VEGF and IL-10 were measured in triplicates.

To determine the effect of the concentration on tumor cell proliferation in the presence of macrophage conditioned media. 1,200,000 THP-1 cells along with PMA (1ul/ml media from a stock which is 5ug/ml) were plated in a 6 well plate in RPMI medium containing 10% FBS for 24 hours. The cells were then starved for 24 hours with serum free RPMI media. Based on the necessary conditions the adherent THP-1 cells were then differentiated based on the inducers added in the media – M0 macrophages (no inducer), M1 macrophages (LPS- concentration: 10ng/ml), M2 macrophages (IL-4 – concentration: 20ng/ml and IL-13 – concentration: 20ng/ml) for 24 hours. The macrophages were then subjected to treatments with fulvestrant and/or pioglitazone for 6 hours. The media was then replaced by fresh serum free media and incubated for 24 hours. Simultaneously, 8000 cells of each of the two adenocarcinoma cell lines were plated per well in a 96 well plate in BME medium containing 10% FBS. The cells were then starved for 24 hours with serum free DMEM medium containing 5% glutamine and 5% penicillin- streptomycin. The

adenocarcinoma cell lines were then subjected to 24 hour incubated with the macrophage conditioned media (with/ without treatments).

To determine Absorbance was measured using the Cell Titre Aqueous One Solution Cell Proliferation Assay MTS, 48 hours from the time of treatment at 490 nm.

Effect of combination in presence of E2 in the co-culture experiment, was measured by plating 450,000 A549/ 201T cells per well in BME medium containing 10% FBS in a 6 well plate. The cells were then allowed to grow to the desired confluence and starved for 24 hours with serum free BMEM media. Cells were treated with fulvestrant and/or pioglitazone for 6 hours. The media was later replaced with fresh serum free media for 24 hours. Simultaneously, 1,200,000 THP-1 cells along with PMA (1ul/ml media from a stock which is 5ug/ml) were plated in a 6 well plate in RPMI medium containing 10% FBS for 24 hours. The cells were then starved for 24 hours with serum free RPMI media. Based on the necessary conditions the adherent THP-1 cells were then differentiated based on the inducers added in the media – M0 macrophages (no inducer), M1 macrophages (LPS-concentration: 10ng/ml), M2 macrophages (IL-4 – concentration: 20ng/ml and IL-13 – concentration: 20ng/ml) for 24 hours. The precursor Androstenedione (concentration-300uM) was added in the media along with the macrophages for 24 hours. The macrophages were incubated with the treated tumor conditioned media along with an additional amount of Androstenedione for 24 hours. The media samples then were collected and centrifuged at 12,000 g for 10 minutes at 4ºC to remove any insoluble fractions. Following the manufacture's protocol, E2 was measured in triplicates.

To show that combination could downregulate COX-2/PGE2 and other common proliferative pathways. 450,000 A549/ 201T cells per well in BME medium containing 10% FBS in a 6 well plate. The cells were then allowed to grow to the desired confluence and starved for 24 hours with serum free BMEM media. Simultaneously, 1,200,000 THP-1 cells along with PMA (1ul/ml media from a stock which is 5ug/ml) were plated in a 6 well plate in RPMI medium containing 10% FBS for 24 hours. The cells were then starved for 24 hours with serum free RPMI media. Based on the necessary conditions the adherent THP-1 cells were then differentiated based on the inducers added in the media – M0 macrophages (no inducer), M1 macrophages (LPS- concentration: 10ng/ml), M2 macrophages (IL-4 – concentration: 20ng/ml and IL-13 – concentration: 20ng/ml) for 24 hours. Cells were treated with fulvestrant and/or pioglitazone for 6 hours. The media was later replaced with fresh serum free media for 24 hours. Lysates and media samples were collected. Immunoblotting was conducted as mentioned above and the blots were probed for COX-2, pMAPK, pAKT, cyclin d1and GAPDH. The media samples then were collected and centrifuged at 12,000 g for 10 minutes at 4ºC to remove any insoluble fractions. Following the manufacture's protocol, PGE2 was measured in triplicates.

To validate the effects of combination on COX-2 by blocking AREG alone. We plated 450,000 cells of 201T and/or A549 per well were plated in BME medium containing 10% FBS in a 6 well plate. The cells were allowed to grow to the desired confluence and starved for 24 hours in BMEM serum free media. Following the starvation, the cells were then subjected to pre-incubation without/ with the neutralizing AREG antibody (0.8ug/ul) for 2 hours. The above conditions were incubated with the macrophage treated with IL-4 and

IL-13 conditioned media for 24 hours. Lysates were collected after 24 hours and were subjected to immunoblotting as described previously. The membranes were then probed for COX-2 and GAPDH.

In order to determine the level of IL-1B in the media- 450,000 cells of each of the two adenocarcinoma cell lines were plated per well in a 6 well plate in BME medium containing 10% FBS. The cells were then starved for 24 hours with serum free DMEM medium containing 5% glutamine and 5% penicillin- streptomycin. Simultaneously, 1,200,000 THP-1 cells along with PMA (1ul/ml media from a stock which is 5ug/ml) were plated in a 6 well plate in RPMI medium containing 10% FBS for 24 hours. The cells were then starved for 24 hours with serum free RPMI media. Based on the necessary conditions the adherent THP-1 cells were then differentiated into M2 macrophages (IL-4 – concentration: 20ng/ml and IL-13 – concentration: 20ng/ml) for 24 hours. The cells were then treated with fulvestrant or/and pioglitazone for 6 hours from the date of plating. The media was then stripped off and replaced with serum free medium for 24 hours. The adenocarcinoma cells were then subjected to the treated macrophage conditioned media incubation for 24 hours. The media samples were collected for ELISA. The conditioned media then were collected and centrifuged at 12,000 g for 10 minutes at 4ºC to remove any insoluble fractions. Following the manufacture's protocol, IL-1b were measured in triplicates.

To validate the effects of combination on COX-2 by blocking IL-1b alone. We plated 450,000 cells of 201T and/or A549 per well were plated in BME medium containing 10% FBS in a 6 well plate. The cells were allowed to grow to the desired confluence and starved

for 24 hours in BMEM serum free media. Following the starvation, the cells were then subjected to pre-incubation without/ with the neutralizing IL-1b antibody (1.5ug/ul) for 2 hours. The above conditions were incubated with the macrophage treated with IL-4 and IL-13 conditioned media for 24 hours. Lysates were collected after 24 hours and were subjected to immunoblotting as described previously. The membranes were then probed for COX-2 and GAPDH.

In order to reaffirm that combination targets IL-1b and AREG. 450,000 cells of 201T and/or A549 each were plated per well in BME medium containing 10% FBS, in a 6 well plate, followed by starvation in medium containing 1% FBS for 24 hours. We pre-incubated the starved tumor cells with rAREG (10ug/ul) and/or rIL-1b (0.1ug/ul)/ rAREG, neutralizing AREG antibody (0.8ug/ul) and/or neutralizing IL-1b antibody (1.5ug/ul) for 2 hours. The above conditions were incubated with the macrophage treated with IL-4 and IL-13 conditioned media for 24 hours. Lysates were collected after 24 hours and were subjected to immunoblotting as described previously. The membranes were then probed for COX-2 and GAPDH.

In order to validate that the combination might affect cell migration. We plated 450,000 cells of A549 in BME medium containing 10% FBS in a 6 well plate. The cells were then allowed to grow to the desired confluence following which they were starved in DMEM medium containing 1% FBS for 24 hours. Simultaneously, 1,200,000 THP-1 cells along

with PMA (1ul/ml media from a stock which is 5ug/ml) were plated in a 6 well plate in RPMI medium containing 10% FBS for 24 hours. The cells were then starved for 24 hours with serum free RPMI media. Based on the necessary conditions the adherent THP-1 cells were then differentiated based on the inducers added in the media – M0 macrophages (no inducer), M1 macrophages (LPS- concentration: 10ng/ml), M2 macrophages (IL-4 – concentration: 20ng/ml and IL-13 – concentration: 20ng/ml) for 24 hours. Cells were treated with fulvestrant and/or pioglitazone for 6 hours. The media was later replaced with fresh serum free media for 24 hours. The scratch was made using a 200ul pipette on starved tumor cells. The images were taken before and after the 72 hour incubation period. The images were then analyzed and the extent of the closure of the gap was calculated.

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