Bone Morphogenetic Protein-7 (bmp-7) Polarizes Monocytes Into M2 Macrophages

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Crystal Rocher
University of Central Florida

BONE MORPHOGENETIC PROTEIN-7 (BMP-7) POLARIZES MONOCYTES INTO M2 MACROPHAGES

by

CRYSTAL ROCHER
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ABSTRACT

Atherosclerosis is an inflammatory disease in which an accumulation of fatty acids and cholesterol occurs to form a plaque in small and large arteries. Monocyte polarization to classic M1 macrophages or alternative M2 macrophages is an important area of research that can determine the severity of disease progression. BMP-7 is a key growth factor responsible for directing differentiation of mesenchymal stem cells into brown fat cells, suggesting a role of BMP-7 in cellular plasticity; however, its role in monocyte polarization is yet to be revealed. In the current study, we hypothesize that monocyte treatment with BMP-7 will significantly result in increased polarization of monocytes into M2 macrophages and increased expression of anti-inflammatory cytokines. To that effect, we have established a stress induced cell culture system with monocytes (THP-1 cells) and apoptotic conditioned medium (ACM), simulating injury, to understand the effects of BMP-7 on M2 macrophage polarization from monocytes. Our data demonstrates that the BMP type 2 receptor (BMPR2) is found on monocytes and its activation is significantly ($p<0.05$) increased in both monocytes and M2 macrophages following treatment with BMP-7. Furthermore, a significant ($p<0.05$) increase of M2 macrophages in the BMP-7 treated group was shown following immunostaining with CD206 and arginase-1, two M2 macrophage markers, whereas a significant ($p<0.05$) decrease of iNOS expression, an M1 macrophage marker, was shown. Moreover, treatment with BMP-7 resulted in significantly ($p<0.05$) increased expression of IL-10 and IL-1ra, two anti-inflammatory cytokines, but significantly ($p<0.05$) decreased levels of the pro-inflammatory cytokines, MCP-1, IL-6 and TNF-α. We also hypothesize that polarization of monocytes to M2 macrophages occurs through activation of SMAD1/5/8

and PI3K-Akt-mTOR pathways. Upon BMP-7 binding to its receptor, BMPR2, activation of SMAD1/5/8 occurs which then activates the p85 subunit of PI3K resulting in downstream activation of Akt and mTOR. Our data shows that following treatment with BMP-7, expression of p-SMAD1/5/8, p-PI3K, p-Akt and p-mTOR is significantly ($p<0.05$) increased compared to controls whereas p-PTEN, an inhibitor of the PI3K pathway, is significantly ($p<0.05$) decreased in the BMP-7 treated group compared to controls. In conclusion, our data reveals that BMP-7 polarizes monocytes into M2 macrophages and it achieves this through activation of the PI3K-Akt-mTOR pathway, which will have significant applications for atherosclerosis treatment.

"The lord is my shepherd, I shall not want. He makes me lie down in green pastures, He leads me beside quiet waters, He restores my soul. He guides me in paths of righteousness for His name's sake. Even though I walk through the valley of the shadow of death, I will fear no evil, for You are with me. You prepare a table before me in the presence of my enemies. You anoint my head with oil; my cup overflows. Surely goodness and love will follow me all the days of my life, and I will dwell in the house of the Lord forever."

– Psalm 23: 1-6

First and foremost, I dedicate this to my Lord and Savior, Jesus Christ. I also dedicate this to my wonderful family, whom continually provided love and support throughout this time. Without my parents, I would not be the woman I am today. They have allowed me to have a wonderful life and allowed me to go after my dreams. For that I am eternally grateful. To my beautiful sister, thank you for having patience with me and understanding during this time. You are my best friend.

"*There is no friendship, no love, like that of the parent for the child"*

– Henry Ward Beecher

ACKNOWLEDGMENTS

"*Every great dream begins with a dreamer. Always remember, you have within you the strength, the patience, and the passion to reach for the stars to change the world.*"

–Harriet Tubman

I am sincerely grateful and thankful for my mentor, Dr. Dinender Singla, for his guidance and support during my time in the lab. He taught me to be hard working and instilled in me a desire to always strive for the best. It is because of him that I have had the success I have had as a Master's student and the success I will have in all my future endeavors. Furthermore, I thank my committee members, Dr. Shadab Siddiqi and Dr. Kimunobu Sugaya, for their encouragement and advice.

"The most beautiful discovery true friends make is that they can grow separately without growing apart."

— Elisabeth Foley

I am also very thankful for Reetu Singla for her support and encouragement as well as guidance and knowledge. Lastly, I would like to thank my fellow labmates and friends, Latifa Abdelli, Princess Urbina and Hilda Merino Chavez. They picked me up when I was down and pushed me to be my best and work my hardest. I am so proud to have worked with such amazing, smart and wonderful women.

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LIST OF ABBREVIATIONS

ACM	Apoptotic Conditioned Media
Akt	Adenosine triphosphate-dependent tyrosine kinase
BMP-7	Bone Morphogenetic Protein-7
DAPI	4',6-diamidino-2-phenylindole
ELISA	Enzyme-linked immunosorbent assay
Foll	Follistatin
IL-1ra	Interleukin 1 receptor antagonist
IL-6	Interleukin 6
IL-10	Interleukin 10
MCP-1	Monocyte Chemoattractant Protein-1
mTOR	Mammalian Target of Rapamycin
p-Akt	Phosphorylated Akt
PI3K	Phosphoinositide-3 Kinase
p-PI3K	Phosphorylated PI3K
p-mTOR	Phosphorylated mTOR
p-PTEN	Phosphorylated phosphatase and tensin homolog
PTEN	Phosphatase and tensin homolog
RIPA	Radioimmunoprecipitation assay buffer
TNF-α	Tumor Necrosis Factor-Alpha

CHAPTER 1: INTRODUCTION

Atherosclerosis and Inflammation

Atherosclerosis is a disease which results in the accumulation of fat and cholesterol in large and small arteries (Seimon and Tabas, 2009;Moore and Tabas, 2011;Shimizu et al., 2012). This plaque formation that occurs causes hardening and narrowing of the arteries which can lead to further complications such as a myocardial infarction or an ischemic stroke (Libby et al., 2009;Rocher et al., 2012). Recently studies have focused on the inflammatory response that occurs with atherosclerosis and refer to this condition as an inflammatory disease (Libby et al., 2009). In atherosclerosis, monocytes migrate to the area of injury and polarize based on the environment and the factors being secreted (Seimon and Tabas, 2009). In early stages of atherosclerosis, polarization of M2 macrophages is greater and allows for secretion of anti-inflammatory cytokines and efferocytosis to occur but in later stages of atherosclerosis, the injury becomes too great and cellular debris becomes necrotic causing a pro-inflammatory environment and M1 polarization from monocytes to occur (Thorp and Tabas, 2009;Libby et al., 2009;Seimon and Tabas, 2009;Moore and Tabas, 2011).

Monocyte Polarization

Monocyte polarization involving classical M1 macrophages and alternative M2 macrophages is an emerging area of research that plays a significant role in various conditions and diseases (Xu et al., 2006;Weis et al., 2009;Rocher et al., 2012). Classical M1 macrophages are known to be pro-inflammatory and secrete such

cytokines as iNOS, IL-6, TNF-α, and MCP-1, while alternative M2 macrophages are anti-inflammatory and secrete such cytokines as IL-10 and arginase-1 (Sinha et al., 2005;Xu et al., 2006;Weis et al., 2009;Rocher et al., 2012). Therefore, having a greater ratio of M2 macrophages is essential for injury repair and wound healing. Monocyte polarization is also influenced by the stimuli received from the external environment based on their ability to exhibit high plasticity (Woollard and Geissmann, 2010;Rocher et al., 2012).

Bone Morphogenetic Protein-7 and SMAD1/5/8 Pathway

Bone morphogenetic protein-7 (BMP-7), which is part of the TGFβ family, is essential for the embryonic development of the eyes, kidneys and skeleton, is shown to be involved in cell polarization and demonstrated to reduce expression of such pro-inflammatory cytokines as IL-6 and MCP-1 in the kidney (Wang et al., 2003;Boon et al., 2011;Rocher et al., 2012). Furthermore, we recently published the effect of BMP-7 treatment on THP-1 cells as well as the effects of its inhibitor, follistatin (Rocher et al., 2012). We showed that BMP-7 treatment was able to successfully increase anti-inflammatory cytokine expression such as IL-10 and induce increased polarization of monocytes to M2 macrophages (Rocher et al., 2012). BMP-7 works through the SMAD1/5/8 pathway following binding to the BMP type 2 receptor (BMPR2) on the surface of the cell (Boon et al., 2011). Follistatin works to inhibit BMP-7 by preventing the binding of BMP-7 with BMPR2 and thus preventing the activation of the SMAD1/5/8 pathway (Amthor et al., 2002;Rocher et al., 2012). Binding of BMP-7 with its receptor,

however, has also been shown to activate other non-SMAD pathways such as the PI3K-Akt-mTor pathway (Shimizu et al., 2012).

PI3K-Akt-mTOR Pathway

The PI3K pathway has been shown to be essential to immune regulation and induces a more anti-inflammatory environment (Weichhart and Saemann, 2008). Studies have reported that this pathway inhibits the release of pro-inflammatory cytokines and its activation is required for M2 macrophage polarization (Rauh et al., 2005;Weichhart and Saemann, 2008;Weichhart and Saemann, 2009). This pathway, works through the activation and phosphorylation of the p85 subunit of PI3K which results in downstream phosphorylation of PIP2 to PIP3 (Weichhart and Saemann, 2008). This results in phosphorylation of Akt and downstream activation of mTOR (Weichhart and Saemann, 2008). PTEN, an inhibitor of this pathway, works by dephosphorylating PIP3 and thus preventing activation of Akt (Weichhart and Saemann, 2008). In this study we will show that following treatment of BMP-7, THP-1 cells will polarize into M2 macrophages. Furthermore, we will show that activation of the SMAD1/5/8 pathway will activate the p85 subunit of PI3K resulting in activation of the PI3K-Akt-mTOR pathway.

Hypothesis

In the present study, we hypothesize that:

I. Monocyte treatment with BMP-7 under stressed conditions will greatly increase polarization of monocytes into M2 macrophages and increase expression of anti-inflammatory cytokines.

II. Polarization by BMP-7 is mediated through both the SMAD1/5/8 and PI3K-Akt-mTOR pathways.

Aims

Aim 1. Determine whether under stressed conditions, BMP-7 can induce M2 macrophage polarization from THP-1 cells and primary CD14 monocytes after 48 hours of treatment.

Aim 2. Determine the mechanism involved in the observed M2 macrophage polarization.

CHAPTER 2: MATERIALS AND METHODS

THP-1 Cell Culture

THP-1 cells are a cell line for human monocytes that were used in this study and purchased from ATCC. The monocytes were cultured with RPMI 1640 (ATCC) which contained 10% fetal bovine serum and 0.05mM β-mercaptoethanol. THP-1 cells were grown in suspension in 25cm^2 flasks and media was added every other day as per manufacturer's instructions (Rocher et al., 2012).

Apoptotic Conditioned Media

The H9C2 cell line was used to generate apoptotic condition media which is an established method and has been published by us (Singla and McDonald, 2007;Rocher et al., 2012). A 60mm tissue culture plate was plated with 5x10^5 H9C2 cells for 24 hours. Following 24 hours, 400uM of H2O2 was added to the plate for 2 hours. After 2 hours, the media was removed from the plate and filtered.

BMP-7 and Follistatin Treatment

A total of 40,000 THP-1 cells were plated per well in a 24 well plate for 24 hours. The cells were split up into the following groups: Control, ACM, ACM+BMP-7 and ACM+BMP-7+Foll. Following 24 hours, cells were treated according to the designated groups for 48 hours. Cells treated with BMP-7 were given a concentration of 660ng/ml and cells treated with follistatin were given a concentration of 500ng/ml (Rocher et al., 2012).

Double Label Immunostaining of BMPR2 with CD14 and CD206

As previously reported, after 48 hours of treatment, cells were removed from the wells and centrifuged for 5 minutes at 300g (Rocher et al., 2012). Following centrifugation, cell smears were made using ColorFrost Plus glass slides and allowed to dry. When smears were ready blocking was done with 10% normal goat serum (Vector Labs) for one hour at room temperature. Primary antibodies for anti-CD14 or anti-CD206 were added and incubated for 24 hours. Smears were then left in secondary antibody goat anti-rabbit Alexa 568 for one hour. Next smears were blocked with 10% donkey serum and then primary antibody anti-BMPR2 was added for 24 hours. Secondary antibody donkey anti-goat Alexa 488 was then added. Smears were then counter stained to visualize all blue nuclei with DAPI (4', 6-diamidino-2-phenylindole) (Vector Labs) and the slides were cover-slipped. Four images per smear were taken and analyzed.

Immunohistochemistry of Macrophage Markers Arginase-1 and iNOS

First, smears were blocked with 10% normal goat serum (Vector Labs). Next, anti-iNOS was added and kept for 24 hours. Goat anti-mouse Alexa 568, the secondary, was then added. Next, smears were blocked with 10% donkey serum and then anti-arginase-1 was added also for 24 hours. Smears were next given secondary antibody donkey anti-goat Alexa 488. Smears were then counter stained to visualize all blue nuclei with DAPI (4', 6-diamidino-2-phenylindole) (Vector Labs) and the slides were cover-slipped. Four images per smear were taken and analyzed.

Pro and Anti-Inflammatory Cytokine ELISAs

Supernatant from each group was collected after 48 hours of treatment and centrifuged at 13,000 rpm for 5 minutes as previously reported (Rocher et al., 2012). In brief, pro (IL-6, TNF-α, and MCP-1) and anti-inflammatory (IL-10) ELISA kits were purchased from Raybiotech and followed under manufacturer's instructions.

Western Blot Analysis

Cells were divided and plated in 25cm2 flasks per group. Following 48 hours of treatment, cells were collected, centrifuged and lysed with RIPA buffer containing protease inhibitor cocktail, PMSF, sodium orthovanadate and sodium fluoride for 30 minutes. The cell lysates were then used to measure the protein concentration and following, protein concentration determination, samples were made from the cell lysates and were run on either an 8% or 10% sodium-dodecyl sulfate polyacrylamide gel at 100V for 5 minutes and then 150V for 55 minutes. Next the gels were transferred onto a PVDF membrane (Bio-Rad). After transferring the gels onto the membrane, the membranes were blocked with 5% milk and primary antibodies p-Smad 1/5/8, p-PI3K, Total PI3K, p-Akt, p-PTEN, p-mTOR or Total mTOR were added followed by secondary antibody. Finally the membranes were detected with a Visualizer.

Statistical Analysis

Statistical analysis was done using one-way analysis of variance (ANOVA) then Tukey Test. Data are given as a mean ± SEM with p-value <0.05 to show statistical significance.

CHAPTER 3: RESULTS

BMPR2 Expression is Increased Following BMP-7 Treatment

To confirm the presence of BMPR2 on THP-1 cells, a double immunostaining was performed. Figure 1A shows representative photomicrographs of THP-1 cells stained with CD14, a monocyte marker, and BMPR2. Furthermore, quantitative analysis shows that expression of BMPR2 is significantly increased in the ACM+BMP-7 group following treatment with BMP-7 compared to control (Figure 1C). A double immunostaining with CD206, an M2 macrophage marker, and BMPR2 further demonstrates that the receptor is located on M2 macrophages as well and expression of the receptor is significantly increased in the ACM+BMP-7 group following treatment with BMP-7 compared to the control (Figure 1B and D).

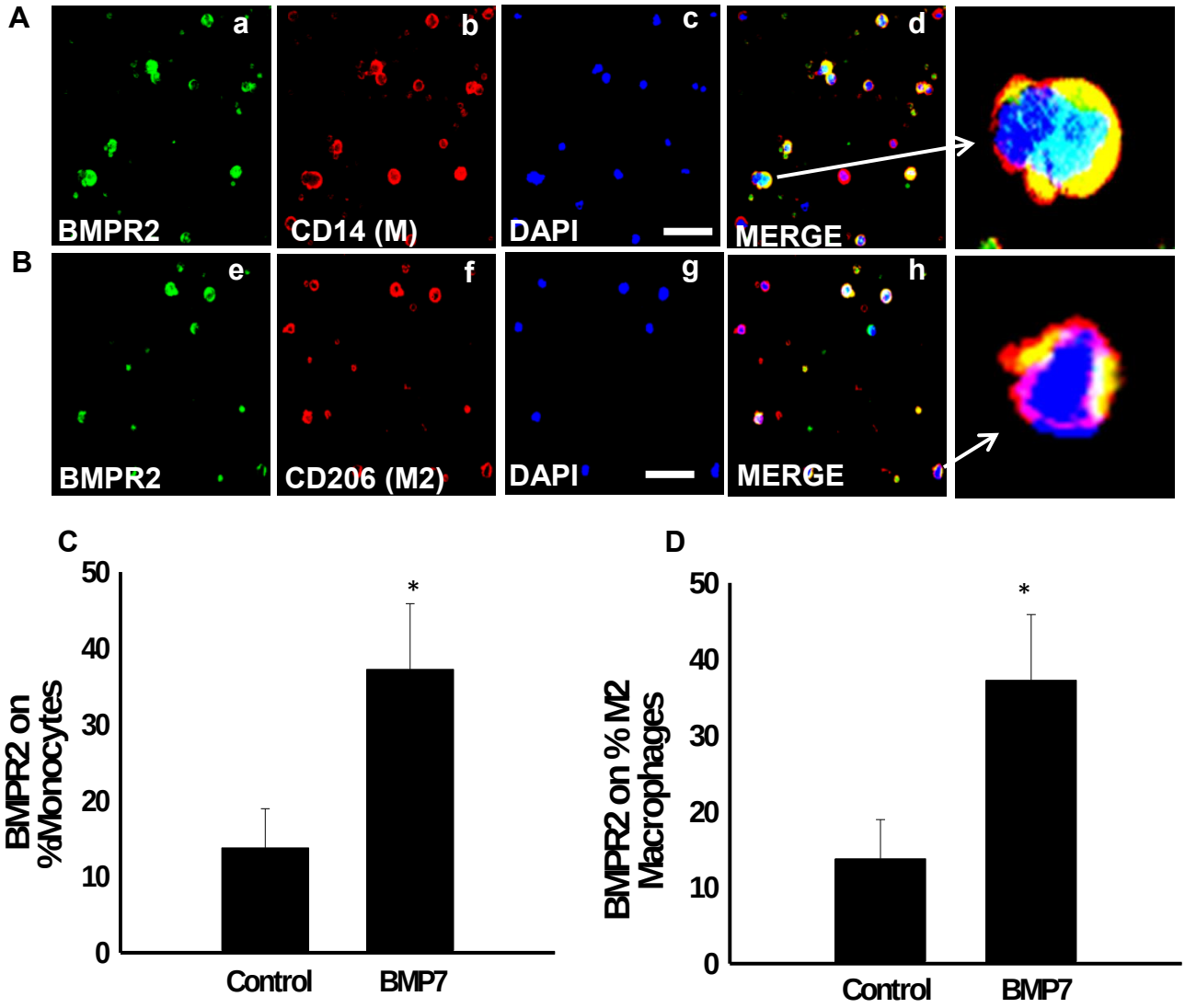

Figure 1: Expression of BMPR2 is increased following treatment with BMP-7. Top panel **(A)** shows representative photomicrographs of cell smears stained with anti-BMPR2 in green (a), anti-CD14, a monocyte marker, in red (b), DAPI in blue (c) and the merged image (d). Bottom panel **(B)** shows representative photomicrographs of anti-BMPR2 in green (e), anti-CD206, an M2 macrophage marker, in red (f), DAPI in blue (g) and the merged image (h). Bottom left histogram **(C)** shows quantitative analysis of percent BMPR2 positive CD14 cells. Right histogram **(D)** shows quantitative analysis of percent BMPR2 positive CD206 cells. *p<0.05 vs Control.

BMP-7 Treatment Increases M2 Macrophage Polarization

Polarization of THP-1 cells following treatment with BMP-7 was next determined by immunohistochemistry. Figure 2A shows quantitative analysis of CD14 immunostaining showing a significant decrease of CD14 positive cells in the ACM, ACM+BMP-7 and ACM+BMP-7+Foll groups. This suggests that the cells are polarizing into another cell type following treatment. To determine what the cells are polarizing into, THP-1 cells were stained with CD206 and arginase-1, two M2 macrophage marker, and iNOS, an M1 macrophage marker (Sinha et al., 2005;Xu et al., 2006;Weichhart and Saemann, 2008). Figure 2B shows quantitative analysis of CD206 positive cells with a significant increase in the ACM+BMP-7 group and a significant decrease in the ACM+BMP-7+Follistatin group suggesting that BMP-7 treatment successfully polarizes THP-1 cells into M2 macrophages and follistatin, an inhibitor of BMP-7, prevents the polarization from occurring (Boon et al., 2011). Next, Figure 2C shows quantitative analysis of arginase-1 positive cells with a significant increase in the ACM+BMP-7 group which further suggests that BMP-7 is successfully polarizing the THP-1 cells into M2 macrophages. Lastly, Figure 2D shows quantitative analysis of iNOS positive cells with a significant increase in the ACM and ACM+BMP-7+Foll groups and a significant decrease in the ACM+BMP-7 group. This suggests that without treatment of BMP-7 cells polarize into an M1 macrophage-expressing cell.

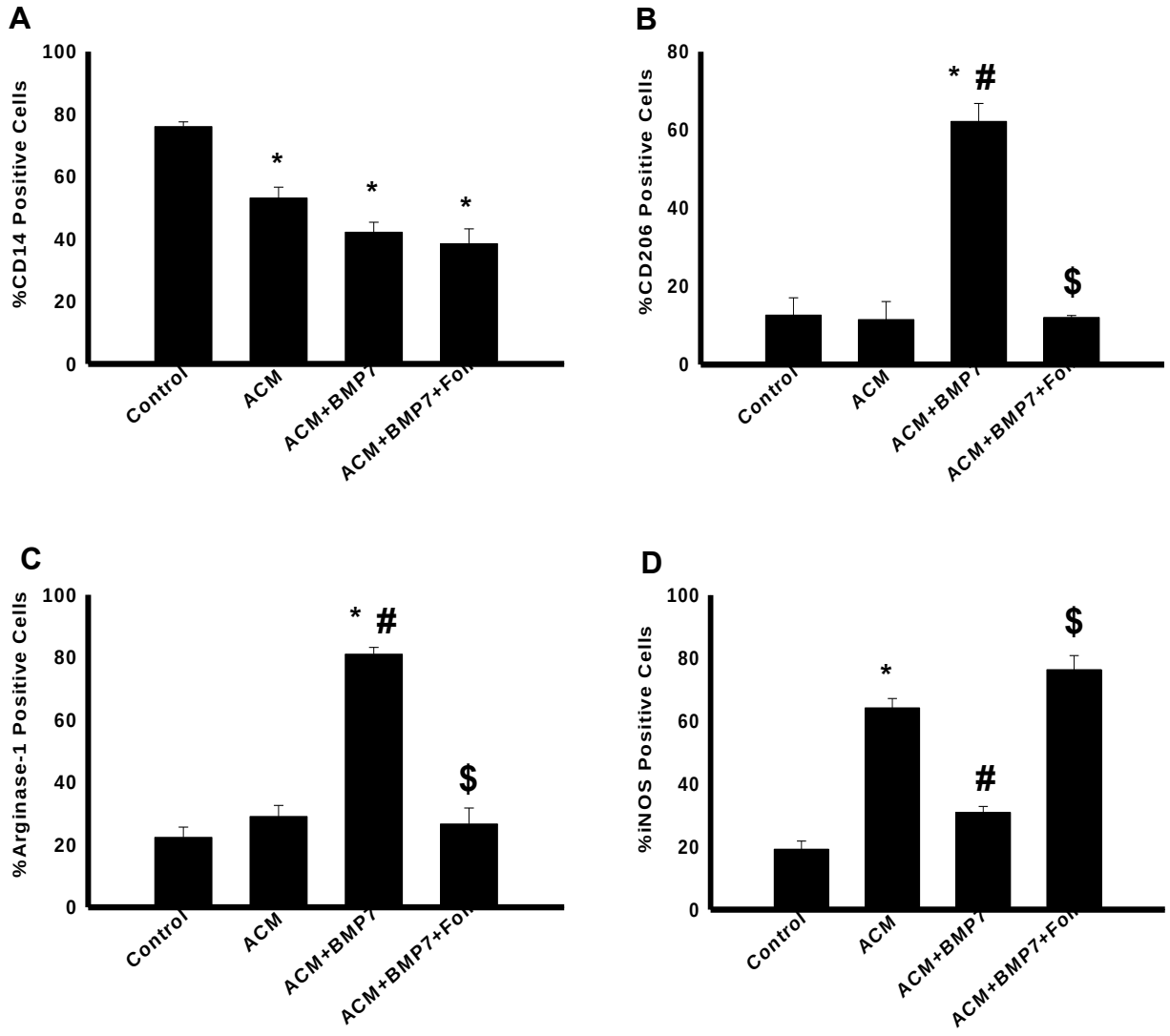

Figure 2: Treatment with BMP-7 increases M2 macrophage markers while decreasing M1 markers. Top left histogram **(A)** shows quantitative analysis of percent CD14 positive cells. Top right histogram **(B)** shows quantitative analysis of percent CD206 positive cells. Bottom left histogram **(C)** shows quantitative analysis of percent arginase-1 positive cells. Bottom right histogram **(D)** shows quantitative analysis of percent iNOS positive cells. *p<0.05 vs Control, #p<0.05 vs ACM, $p<0.05 vs ACM+BMP-7.

Expression of Inflammatory Cytokines Following Treatment with BMP-7

Another method of determining M1 and M2 macrophage polarization is by expression of pro and anti-inflammatory cytokines (El et al., 2012;Rocher et al., 2012). ELISA kits were used to measure the levels of cytokines per group. IL-10, an anti-inflammatory cytokine and marker of M2 macrophages, was measured and demonstrated a significant increase in the ACM+BMP-7 group and a significant decrease in the ACM and ACM+BMP-7+Foll group (Figure 3A). Figure 3B shows expression of IL-1ra, another anti-inflammatory cytokine, with also a significant increase in the ACM+BMP-7 group. IL-6, TNF- α and MCP-1 are pro-inflammatory cytokines that are known to be expressed by M1 macrophages and contribute to the progression of inflammatory diseases, were also measured (Weis et al., 2009;Rocher et al., 2012). Figures 3C-D show quantitative analysis of expression of the pro-inflammatory cytokines with a significant decrease in the ACM+BMP-7 group suggesting that treatment with BMP-7 inhibits expression of these cytokines.

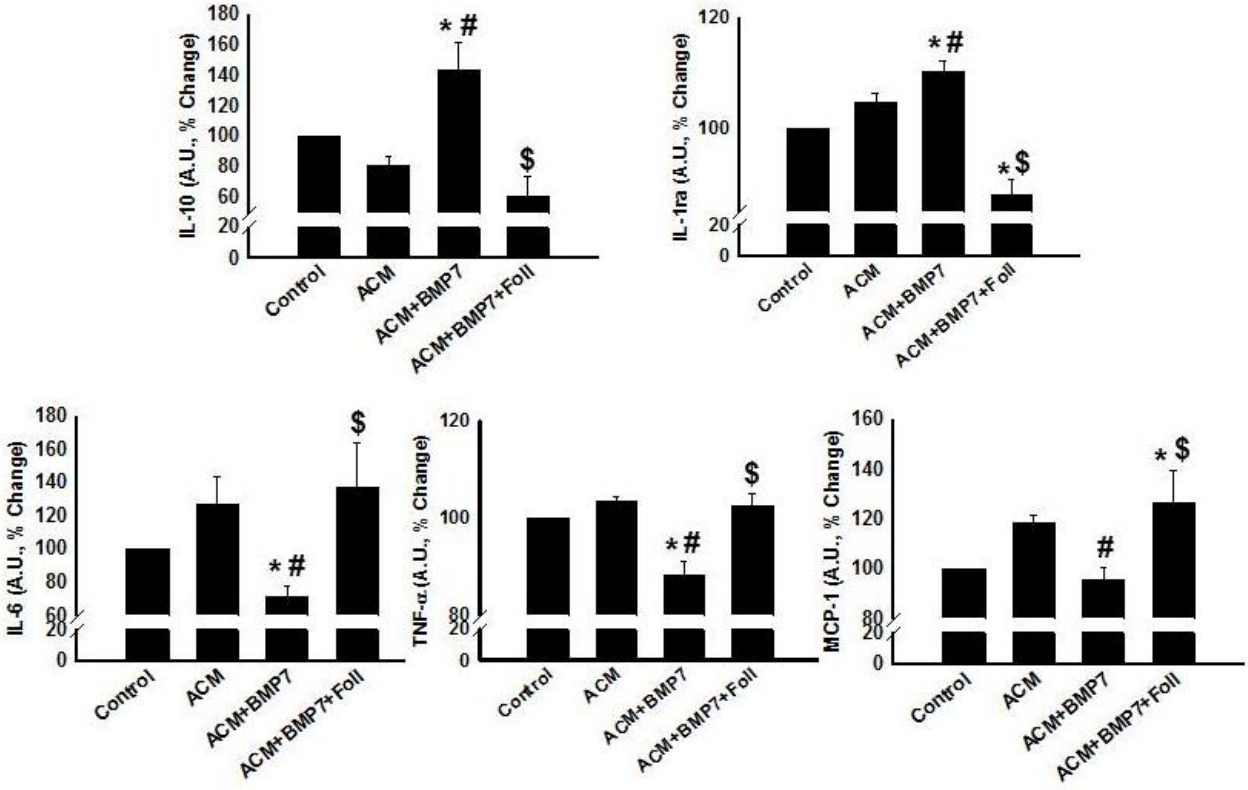

Figure 3: BMP-7 increases anti-inflammatory cytokine expression and decreases pro-inflammatory cytokine expression. Histogram **(A)** shows quantitative analysis of IL-10. Histogram **(B)** shows quantitative analysis of IL-1ra. Histogram **(C)** shows quantitative analysis of IL-6**.** Histogram **(D)** shows quantitative analysis of TNF- α. Histogram **(E)** shows quantitative analysis of MCP-1. *p<0.05 vs Control, #p<0.05 vs ACM, $p<0.05 vs ACM+BMP-7.

BMP-7 Treatment Activates the SMAD1/5/8 Pathway

When BMP-7 binds to BMPR2 located on the THP-1 cells, the SMAD1/5/8 pathway gets activated which results in gene expression (Perron and Dodd, 2009;Boon et al., 2011). Western blot analysis was utilized to ascertain the expression of SMAD1/5/8 per group. Figure 4A shows representative western blot bands for SMAD1/5/8 and β-actin. Densitometry analysis of the western blot bands showed that expression of SMAD1/5/8 was significantly higher in the ACM+BMP-7 group suggesting that BMP-7 is successfully binding the receptor and further confirming that the receptor is active on THP-1 cells. (Figure 4B).

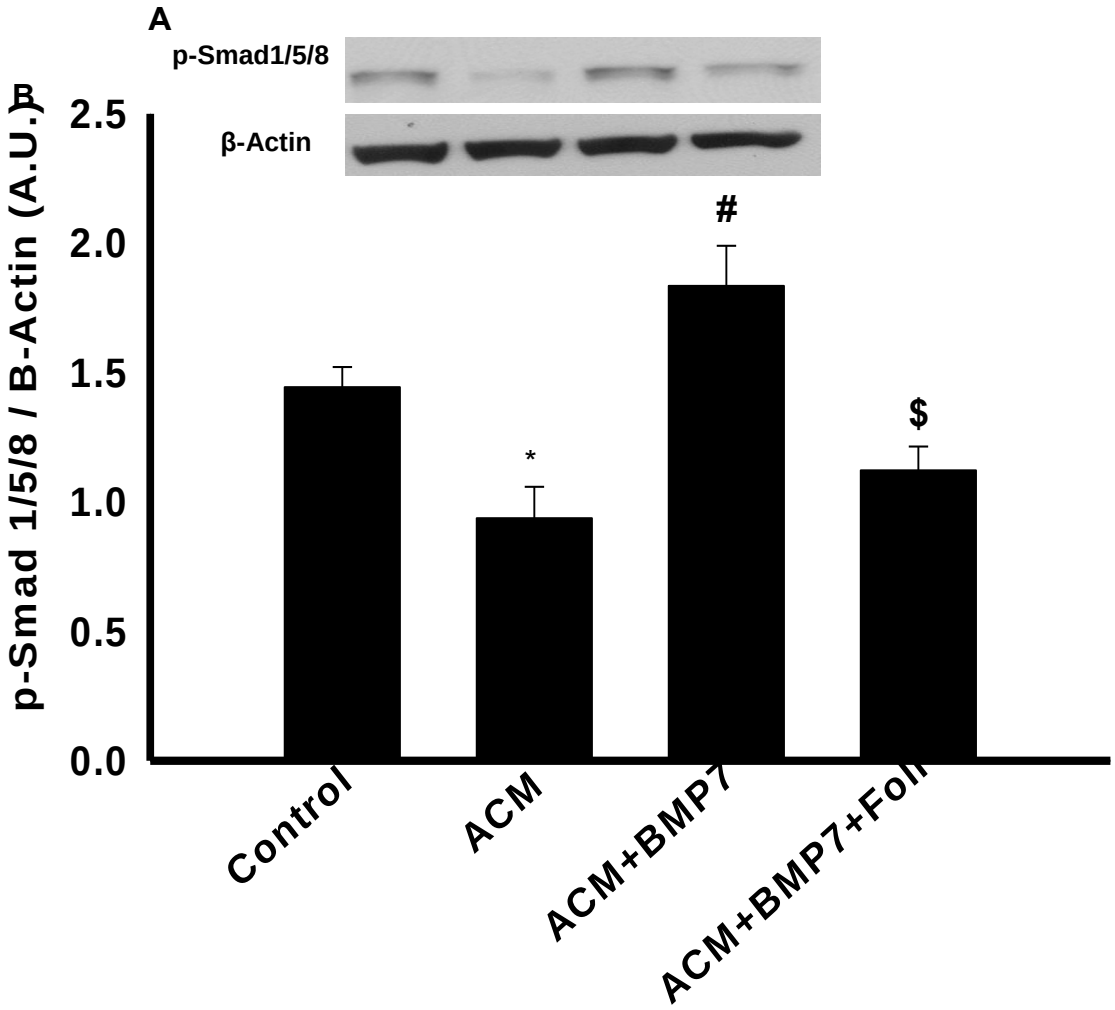

Figure 4: Treatment with BMP-7 increases activation of SMAD1/5/8 pathway. Representative western blot bands **(A)** are shown of p-SMAD1/5/8 and β-actin. Histogram **(B)** shows analysis of p-SMAD1/5/8. *p<0.05 vs Control, #p<0.05 vs ACM, $p<0.05 vs ACM+BMP-7.

Binding of BMPR2 Activates Non-SMAD Pathway PI3K-Akt-mTOR

Studies have shown that activation of the BMPR2 can also result in activation of non-SMAD related pathways such as the PI3K pathway (Shimizu et al., 2012). When binding occurs, the p85 subunit of PI3K gets activated which allows for downstream activation of Akt and mTOR (Weichhart and Saemann, 2008;Weichhart and Saemann, 2009;Shimizu et al., 2012). Western blot analysis was used to measure the expression levels of each protein. Figure 5A shows representative western blot bands for p-PI3K and total PI3K. Densitometric analysis of the western blot bands showed significantly increased expression of p-PI3K in the control and ACM+BMP-7 groups (Figure 5B).

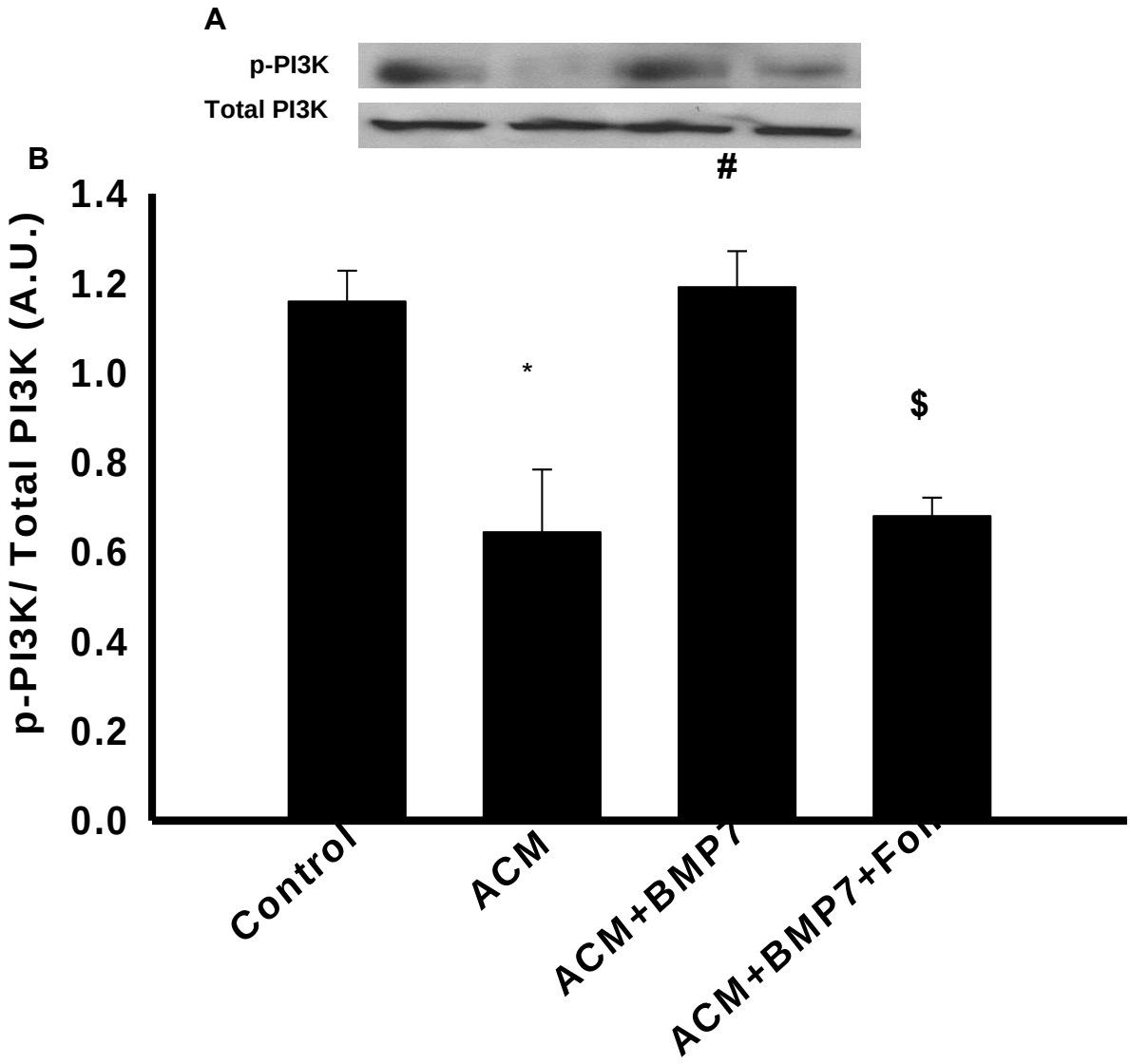

Figure 5: Activation of SMAD1/5/8 pathway results in activation of the p85 subunit of PI3K. Representative western blot bands **(A)** are shown of p-PI3k and total PI3K. Histogram **(B)** shows analysis of p-PI3K. *p<0.05 vs Control, #p<0.05 vs ACM, $p<0.05 vs ACM+BMP-7.

Furthermore, Figure 6A and 6C show representative western blot bands for p-Akt and p-PTEN with β-actin. Densitometric analysis of the p-Akt western blot bands show a significant increase in expression of the ACM+BMP-7 group (Figure 6B). Figure 6D, however, shows densitometric analysis of p-PTEN, an inhibitor of Akt, with a significant decrease in the ACM+BMP-7 group and a significant increase in the ACM and ACM+BMP-7+Foll groups. This suggests that PTEN is significantly inhibiting the PI3K pathway. Figure 7A shows representative western blot bands of p-mTOR and total mTOR. Densitometric analysis of the western blot bands show a significant increase in the ACM+BMP-7 group (Figure 7B).

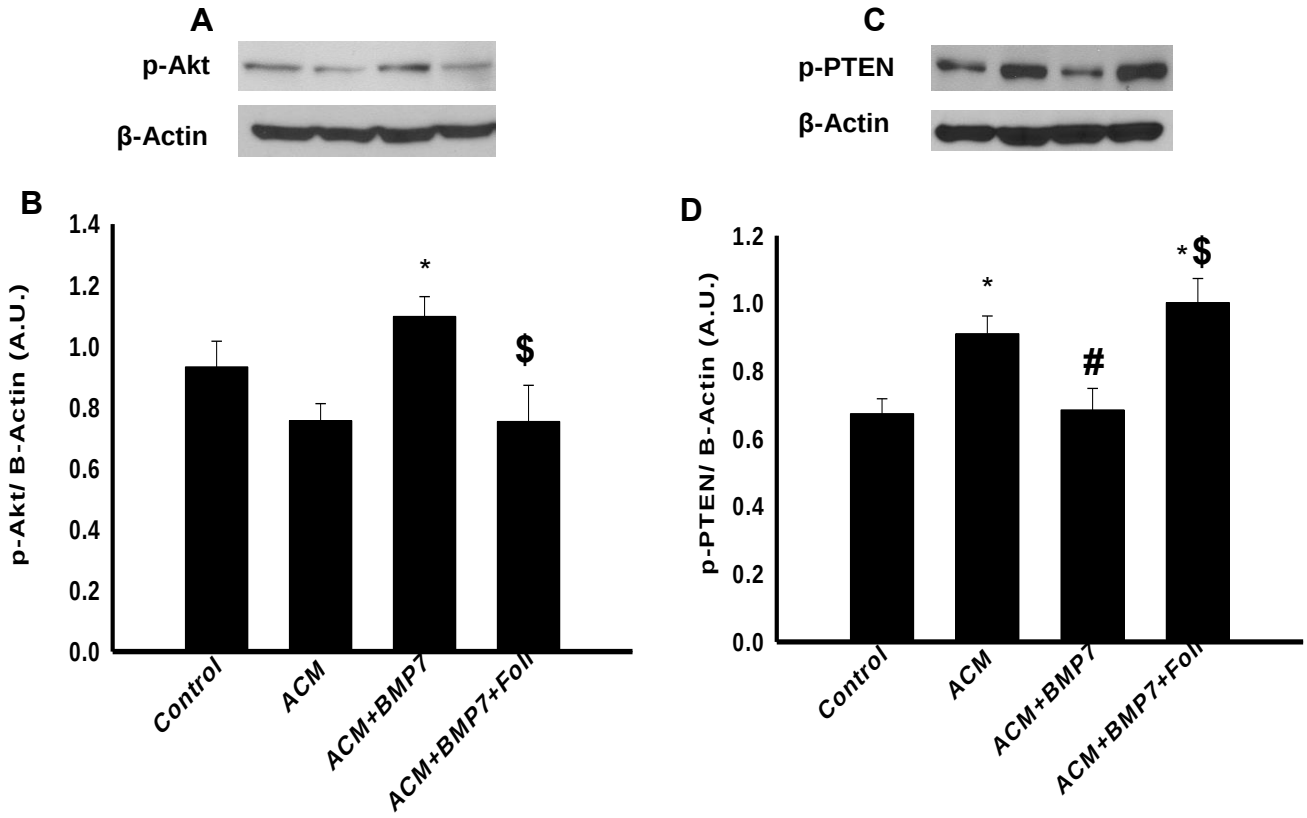

Figure 6: Activation of PI3K results in downstream phosphorylation of Akt. Representative western blot bands **(A)** are shown of p-Akt and β-actin. Histogram **(B)** shows analysis of p-Akt. Representative western blot bands **(C)** are shown of p-PTEN and β-actin. Histogram **(D)** shows analysis of p-PTEN. *p<0.05 vs Control, #p<0.05 vs ACM, $p<0.05 vs ACM+BMP-7.

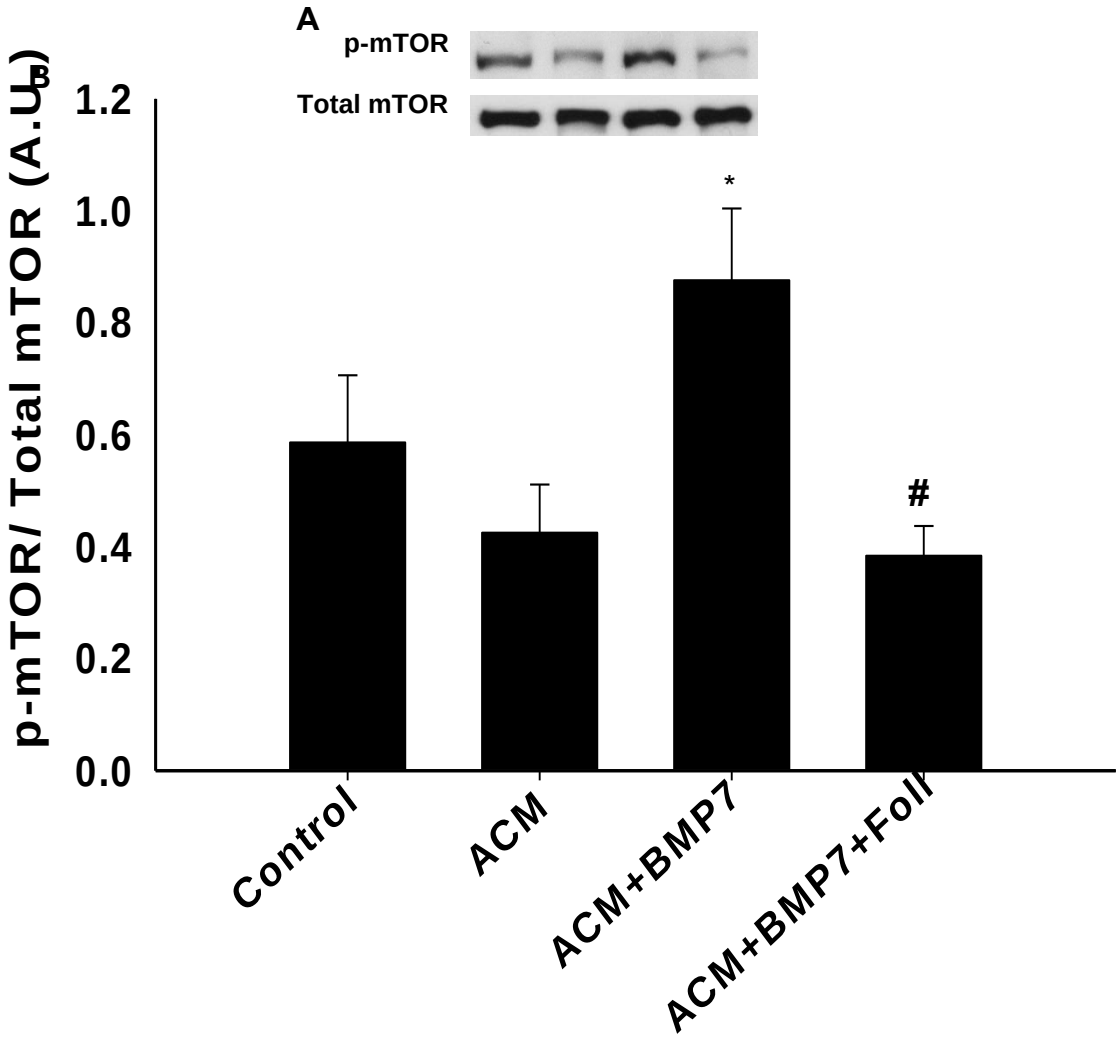

Figure 7: Downstream activation of mTOR occurs following phosphorylation of Akt. Representative western blot bands **(A)** are shown of p-mTOR and total mTOR. Histogram **(B)** shows densitometric analysis of the western blot bands for p-mTOR. *p<0.05 vs Control, #p<0.05 vs ACM+BMP7.

CHAPTER 4: DISCUSSION

Monocyte polarization is an important area of research that plays a significant role in disease progression. During the onset of atherosclerosis, monocytes move to the artery and polarize based on the stimuli they receive from the environment (Boon et al., 2011;Moore and Tabas, 2011;Rocher et al., 2012). In the early stages of atherosclerosis, monocytes polarize to M2 macrophages, which secrete anti-inflammatory cytokines to decrease the inflammatory response and prevent further injury and also phagocytize the apoptotic cellular debris (Libby et al., 2009;Galkina and Ley, 2009;Moore and Tabas, 2011). Unfortunately, as the atherosclerosis progresses and the injury worsens, apoptosis increases and the cellular debris becomes necrotic resulting in secretion of pro-inflammatory cytokines, which then induce the infiltrating monocytes to polarize to an M1 macrophage cell type (Thorp and Tabas, 2009;Seimon and Tabas, 2009). Being able to control the ratio of M1 to M2 macrophages could inhibit the severity of the inflammatory response and allow for injury repair.

Studies have shown that monocytes are triggered to polarize based on the stimuli they receive such as apoptotic cell death or increased cytokine levels (Voll et al., 1997;Weigert et al., 2006;Rocher et al., 2012). In this study, we wanted to mimic the inflammatory environment by adding apoptotic conditioned media (ACM) which has been published by others and us (Xu et al., 2006;Weis et al., 2009;Rocher et al., 2012). Adding this stressed condition induced the THP-1 cells to polarize from monocytes to macrophages. To increase the polarization of monocytes to M2 macrophages BMP-7 was added to the THP-1. In this study when the THP-1 cells are treated with BMP-7 under stressed conditions for 48 hours, we see an increase in M2 macrophage markers

suggesting that BMP-7 is successfully polarizing the THP-1 cells. We further confirmed that BMPR2 is located on THP-1 cells through double label immunostaining with CD14, a monocyte marker (Urbich et al., 2003;Rocher et al., 2012). In addition, we showed that expression of BMPR2 is significantly increased following treatment with BMP-7 compared to control. We also showed that BMPR2 is found on M2 macrophages, which was demonstrated through the double immunostaining performed on the THP-1 cells following treatment, and was seen with the CD14 positive cells, expression was significantly increased in the ACM+BMP-7 group compared to control.

Cell polarization was demonstrated through multiple methods, the first being immunohistochemistry of the various monocyte and macrophage markers. Following 48-hour treatment of the THP-1 cells with BMP-7 under stressed conditions; the cell smears were stained with anti-CD14, anti-CD206, anti-arginase-1 and anti-iNOS. The CD14 marker confirmed that the THP-1 cells are monocytes and that following treatment the percentage of CD14 positive cells decreased. Seeing that the percentage decreased suggested that the cells are polarizing to another cell type.

CD206 and arginase-1, two markers of M2 macrophages, stainings were next performed to see if indeed BMP-7 was successfully polarizing the cells to an M2 macrophage cell type (Sinha et al., 2005;El et al., 2012). As shown in the results section, the percentage of cells expressing CD206 and arginase-1 was significantly increased in the ACM+BMP-7 group and significantly decreased in the ACM and ACM+BMP-7+Foll groups. This suggests that THP-1 cells are polarizing into M2 macrophages do to treatment with BMP-7. Furthermore, the decrease of expression of CD206 and arginase-1 in the follistatin group demonstrates that inhibition of BMP-7 is

occurring and that since expression of M2 macrophage markers decreases when BMP-7 is inhibited then BMP-7 must play an important role in the M2 macrophage polarization. This is further shown by the iNOS immunostaining performed on the THP-1 cells. A significant increase of iNOS expression was shown in the ACM and the ACM+BMP-7+Foll groups but a significant decrease in the ACM+BMP-7 group showing that BMP-7 treatment induces M2 macrophage polarization and not M1.

Cell polarization was further demonstrated through ELISA analysis of pro and anti-inflammatory cytokine expression for each group. Levels of pro-inflammatory cytokines IL-6, TNF- α and MCP-1 and anti-inflammatory cytokines IL-10 and IL-1ra were performed (Libby et al., 2009; Wolfs et al., 2011; El et al., 2012; Rocher et al., 2012). The BMP-7 treated group was able to significantly lower levels of these pro-inflammatory cytokines compared to the ACM group but significantly increase levels of IL-10 and IL-1ra. We show that following treatment with BMP-7, THP-1 cells polarize into M2 macrophages. This data confirms studies done by others and us that BMP-7 can reduce levels of pro-inflammatory cytokines and be used as a treatment for inflammatory diseases such as atherosclerosis (Rocher et al., 2012).

Understanding the mechanism for how BMP-7 polarizes THP-1 cells was previously unknown. In this study, we show that BMP-7 works through two pathways. The first pathway is SMAD1/5/8, a known mechanism for BMP-7 when binding occurs through BMPR2 (Boon et al., 2011;Rocher et al., 2012). Western blot analysis confirms that activation of the SMAD1/5/8 pathway occurs in the ACM+BMP-7 group. Furthermore, it not only demonstrates that successful binding of BMP-7 occurs but also

correlates with the data from Figure 1A regarding the location of the receptor on the cell surface, since activation of the SMAD1/5/8 pathway occurs following binding of BMPR2.

In this study we also show that following binding of BMPR2 with BMP-7, the PI3K-Akt-mTOR pathway is also activated. Studies have shown that this pathway is very important to M2 macrophage polarization and its activation is seen as almost a requirement for M2 macrophage polarization to occur (Martin et al., 2003;Rauh et al., 2005;Weichhart and Saemann, 2008). Inhibition of either PI3K or mTOR, by their respective inhibitors results in polarization toward an M1 macrophage type with increased pro-inflammatory cytokine expression (Martin et al., 2003; Rauh et al., 2005; Weichhart and Saemann, 2009; Araki et al., 2011). Although studies have shown that activation of this pathway can occur through BMPR2, no one has yet to show the correlation of this activation with the polarization of M2 macrophages. In this study, through western blot analysis, we show that expression of p-PI3K, p-Akt and p-mTOR is significantly increased in the ACM+BMP-7 group. Conversely, western blot analysis of p-PTEN shows that in the ACM and ACM+BMP-7+Foll groups, inhibition of the PI3K pathway occurs which correlates with our previous data regarding cell polarization. The groups that had increased expression of the PI3K pathway also had increased expression of M2 macrophage markers whereas groups that had decreased expression of PI3K had increased expression of M1 macrophage markers. This data suggests that activation of the PI3K pathway is necessary for M2 macrophage polarization.

In conclusion, this study demonstrates that treatment with BMP-7 polarizes THP-1 cells into M2 macrophages. We showed that THP-1 cells treated with BMP-7 had increased expression of M2 macrophage markers as well as increased levels of anti-

inflammatory cytokines. Furthermore, our data suggests that polarization occurs through activation of the PI3K-Akt-mTOR pathway following binding of BMPR2.

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