BMP-7 Inhibits p38 and JNK Pathways and Increases M2 Macrophage Differentiation to Reduce Atherosclerosis in Apolipoprotein E-/- Mice

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BMP-7 INHIBITS P38 AND JNK PATHWAYS AND INCREASES M2 MACROPHAGE DIFFERENTIATION TO REDUCE ATHEROSCLEROSIS IN APOLIPOPROTEIN $E^{-/-}$ MICE

by

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ABSTRACT

We have previously shown that treating atherosclerosis with bone morphogenetic protein-7 (BMP-7) affects the presence of macrophage subtypes *in vitro*, however it remains unknown whether BMP-7 treatment affects development and progression of atherosclerosis *in vivo* at an early and mid-stage of the disease. We therefore performed a Day 5 (D5) and Day 28 (D28) study to examine BMP-7's potential to affect monocyte differentiation. Atherosclerotic plaque formation was developed using our standard method and $ApoE^{-/-}$ mice were sacrificed at D5 and D28 post-surgery. Treatment animals received intravenous injections of BMP-7 at 200µg/kg of bodyweight. Hematoxylin and Eosin morphological stain shows that BMP-7 is capable of significantly reducing plaque accumulation at D28 post-surgery vs. PLCA group, $p<0.05$. At D5, plaque formation was reduced but not significant. Immunohistochemistry staining was performed to determine BMP-7's effect on monocytes (CD14), inflammatory M1 (iNOS) and anti-inflammatory M2 (CD206, Arginase-1) macrophages. Immunohistochemistry results show BMP-7 administration reduced pro-inflammatory monocytes and M1 macrophages at D5 and D28 compared to PLCA animals; however, monocytes were not statistically lower at D28. The anti-inflammatory M2 macrophage population was significantly less in PLCA animals compared to SHAM animals at D5 and D28. There was no significant difference in M2 macrophages between PLCA and PLCA + BMP7 animals at D5, however, by D28, PLCA + BMP7 animals showed a significant increase in M2 macrophages compared to PLCA animals. Western blot analysis confirms a significant increase in pro-survival kinase ERK and a significant reduction in pro-inflammatory kinases p38 and JNK in BMP-7 treated mice (D5 and D28, $p<0.05$). ELISA

showed a significant reduction in pro-inflammatory cytokines IL-6, MCP-1, and TNF-α (D5 and D28, $p<0.05$) and a significant increase in anti-inflammatory cytokine IL-10 in BMP-7 treated mice (D5 and D28, $p<0.05$). In summary, our data indicate BMP-7 treatment induces monocyte to M2 macrophage differentiation, increases anti-inflammatory cytokine levels (IL-1ra and IL-10), and improves blow flow velocity (D5 and D28, $p<0.05$) compared to untreated animals. The mechanisms of monocyte to M2 macrophage differentiation appear to be mediated by the p38, JNK, and ERK pathways. This study suggests BMP-7 is capable of reducing inflammation and slowing progression of atherosclerosis at both an early and mid-stage of the disease.

ACKNOWLEDGMENTS

I would like to acknowledge Singla Lab members past and present for their help and support through this process, especially my PI Dr. Singla for his continual guidance and encouragement.

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CHAPTER ONE: INTRODUCTION

Atherosclerosis is an inflammatory disease initiated by injury to the endothelium generated by one or a combination of the following risk factors: dyslipidemia, hypertension, smoking, or familial hypercholesteremia [1]. Inflammation of the endothelium causes expression of adhesion molecules such as VCAM or ICAM, which capture circulating immune cells [2;3]. The immune cells to arrive first and in greatest number are monocytes. Upon capture, monocytes signaled via chemokines released by endothelial and smooth muscle cells will enter the subendothelial space and differentiate into macrophages of varying phenotypes [4-6]. The characteristics of the different macrophage phenotypes play a major role in the pathogenesis of atherosclerosis, as M1 inflammatory macrophages exacerbate the immune response and increase inflammation, whereas M2 macrophages produce anti-inflammatory cytokines and are linked to plaque regression [2]. As atherosclerosis progresses, there is continued immune cell recruitment and monocyte differentiation, increased macrophage cell death due to lipid-overload, smooth muscle cell proliferation into the plaque from the tunica media, and changes to the extracellular matrix [7]. Continued release of inflammatory cytokines by monocytes and M1 macrophages causes destabilization of the plaque, which may lead to a thrombotic event [7-9]. Alternatively, M2 macrophages promote efficient clearing of excess lipids, known as efferocytosis, and release anti-inflammatory cytokines which dampen the chronic inflammation promoting atherosclerosis. The beneficial characteristics of M2 macrophages have prompted scientists to search for novel mechanisms to increase monocyte differentiation toward the M2 phenotype. Previously, *in vitro* studies conducted by our lab

showed that human monocytes treated with bone morphogenetic protein-7 (BMP-7) exhibit increased monocyte to M2 macrophage differentiation demonstrated by a significant increase in M2 macrophage markers on differentiated cells and increase anti-inflammatory cytokines in culture media [10;11].

BMP-7 is an important mediator of cellular differentiation and growth especially during embryonic development. In fact, BMP-7 knockout mice die soon after birth because of failed kidney function and exhibit other signs of developmental malfunction, including poorly formed eyes and skeletal tissue [12]. Other studies tracking BMP-7 expression in normal animals shows that protein expression continues into adulthood and is under tissue-specific transcriptional regulation [13]. This indicates BMP-7's important role not only in differentiation during development but also in maintaining tissue homeostasis in adulthood. The purpose of this study was to determine how administration of BMP-7 to Apolipoprotein E Knockout mice affects ATH development, specifically regarding monocytes and macrophages within the plaque, at an early (D5) as well as a mid-stage of the disease (D28).

Apolipoprotein E (ApoE) knockout mice lack the important cholesterol transport protein apolipoprotein E, resulting in severe hypercholesteremia. Blood serum analysis shows these mice can have as much as six times the number of circulating lipids compared to non-knockouts, and exhibit early signs of atherosclerotic development at three months of age when fed a Western diet [14;15]. Although diet-induced obesity is clearly linked to ATH, many people develop the disease while maintaining a healthy weight often because of familial hypercholesteremia. To study atherosclerosis independently of diet, this lab developed a surgical

technique known as partial ligation of the carotid artery (PLCA). This method ligates three of the four branches of the carotid artery leading to activation of the endothelium by lowering the fluid shear stress through the carotid artery in ApoE KO mice [16].

Here we present evidence BMP-7 beneficially directs monocytes to anti-inflammatory M2 macrophages, reduces activation of inflammatory kinases p38 and JNK, and reduces atherosclerosis. ERK activation of treated animals suggest utilization of this pathway to direct monocyte differentiation.

CHAPTER TWO: METHODOLOGY

Animals

All protocols used were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Central Florida. Apolipoprotein E knockout mice were originally purchased from The Jackson Laboratory, B6.129P2-*Apoe*^{tm1Unc}/J. Male and female mice between 10 and 12 weeks of age fed a normal chow diet were used for both the D5 and D28 time point.

Partial Ligation of the Carotid Artery

Animals were sedated using 3.5% inhalatory isoflurane and 2.5% oxygen. Upon sedation, mice were weighed and placed on a heated surface where they continued to receive isoflurane administered via nose cone. They received an intramuscular injection of pain medication (buprenephrine) prior to undergoing surgery. Immediately preceding surgery, animals were placed in one of three experimental groups: SHAM, PLCA, or PLCA + BMP7. Both PLCA and PLCA + BMP7 groups underwent surgery as follows: the left carotid artery was exposed and three of the four branches were ligated using a medical grade 7/0 silk suture. The external and internal carotid, as well as the occipital artery were sutured while the branch leading to the thyroid was left undisturbed. SHAM animals, serving as the control group, underwent all aforementioned procedure but the suture was only looped around the artery, not tied, to generate similar levels of tissue damage to the surrounding area. PLCA + BMP7 animals also received a

tail vein injection of BMP-7 immediately following surgery at a dosage of 200µg/kg of bodyweight. Dosage was determined from previous publications by us and others [11;17]. PLCA + BMP7 animals also received two additional doses of BMP-7, one the day after surgery and a final injection two days after surgery. The animals were allowed to recover for either 5 or 28 days post-surgery.

Preparation of Carotid Artery Tissue for Analysis

Upon removal of the carotid artery from animals, the tissue was washed in 1X PBS before dividing into either 4% paraformaldehyde (PFA) or RNA Later (RNAL). Samples were in solution for at least 24 hours. Following incubation, 4% PFA samples were washed 3 times in 1X PBS for five minutes each before being placed in 70% ethanol until further processing. RNAL artery tissues were blotted dry and placed in -80^{o}C until homogenization for Western blot.

Tissues originally preserved in 4% PFA were processed using a tissue-processing machine before being embedded in paraffin wax, with the trachea placed vertically, for sectioning (Leica 15053). Tissue sections of 5µm made from paraffin blocks were heat-fixed to charged glass slides at 60^{o}C (Fisher Scientific, 12-550-17). Prior to any staining procedure, all slides were deparaffinized and rehydrated by incubation in xylene followed by a series of decreasing alcohol dilutions starting with 100% alcohol and ending with 30% alcohol before a final wash in double distilled water.

Hematoxylin and Eosin Staining

Arterial sections from each animal were stained using hematoxylin and eosin (H&E) as previously described to identify areas of plaque formation [16]. Plaques were quantified using Image J software. In brief, %Plaque Formation was calculated as follows: Plaque Area/Total Arterial Area * 100 [16].

Immunohistochemistry

Once regions of plaque accumulation were identified, arteries were stained for antibodies against monocytes (CD14, Abbiotech, #251561), M1 macrophages (iNOS, Abcam, ab15323), and M2 macrophages (CD206, Abcam, ab64693, and Arginase-1, Santa Cruz, sc18351). Immediately after the deparaffinization and rehydration process described above, arterial sections were blocked for an hour in either 10% goat or donkey serum prepared in 1X PBS. After an hour, the blocking agent was decanted and excess fluid removed before addition of the primary antibody was added at a concentration of 1:50 prepared in 10% goat or donkey serum and left overnight between 4 and 7^{o}C. After overnight incubation, slides were washed twice for three minutes each in 1X PBS before addition of the secondary antibody at a concentration of 1:50 prepared in 1X PBS (Alexa Flour, A11011, A11055, and A11008). The secondary antibody incubated for at least one hour at room temperature or longer as needed before being washed twice for three minutes in 1X PBS. Finally, slides were cover slipped using DAPI mounting medium. Images for analysis were taken using the 20X objective on an Olympic IX-70 microscope and quantified using Image J. Using monocytes as an example, the percentage of monocytes was determined using the formula $CD14^{+ve}$ Cells/Total $DAPI^{+ve}$ Cells *100.

ELISA

ELISA of blood serum was performed to measure various cytokines associated with atherosclerosis. Inflammatory cytokines MCP-1(RayBiotech, ELM-MCP-1-001), IL-6 (RayBiotech, ELM-IL6), and TNF-α (RayBiotech, ELM-TNF-α-001) were assayed according to manufacturer's directions and as previously described [17]. Additionally, anti-inflammatory cytokines IL-10 (RayBiotech, ELM-IL10-001) and IL-1RA (ELM-IL1RA-001) were measured per package directions. Finally, ELISA was also used to determine circulating BMP-7 at both D5 and D28 (TSZ Scientific, M7485).

Western Blot Analysis

Arterial tissue collected the day of sacrifice, originally preserved in RNAL, and stored at -80^{o}C was homogenized in a solution of radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail, phenylmethane sulfonyl fluoride, sodium orthovandate, and sodium fluoride before being centrifuged at13,000 G for 10 minutes at 4^{o}C. A Bradford assay was performed using the protein obtained from the supernatant. Using results from the Bradford Assay, 80µg of protein was loaded into a 10% SDS-PAGE gel. After loading, gels were run at 150V for 45 minutes to 1 hour as needed, and then transferred to a PVDF membrane using a Bio Rad Semi-Dry Transfer Machine at 15V for 45 minutes. Following transfer, membranes were blocked for one hour at room temperature using 5% milk prepared in 1X TBS-T. After an hour, membranes received 5mLs of primary anti-body at a concentration of 1:1000 in 5% milk and left in 4^{o}C overnight on a shaker. Primary antibodies used were p-p38 (Cell Signaling, 4631S), p-JNK (Cell

Signaling, 4668S), p-ERK (Santa Cruz, sc7383), and β-actin (Cell Signaling, 4967L). Following overnight incubation, the membranes were washed three times for 5 minutes each using 1X TBS-T before addition of the secondary antibody. The secondary antibody used for all proteins except p-ERK was HRP conjugated anti-rabbit IgG from Cell Signaling (7074S), the secondary for p-ERK was HRP conjugated anti-mouse IgG from Santa Cruz (sc2964). The secondary antibody incubated on membranes for 1 hour at room temperature and then membranes were washed again three times for 5 minutes each with 1X TBS-T. At this point, 2 mLs of the chemiluminescent substrate (Thermo Scientific, 32106) were added to the membranes and incubated for 2 to 3 minutes before the excess was decanted and membranes placed in a cassette for exposure. Film was exposed to the membrane between 5 minutes and overnight as deemed necessary before development (MidSci, EBA45). Western blot densitometry analysis performed using Image J software as previously reported [10].

Functional Analysis of Blood Flow Through the Left Carotid Artery

Upon conclusion of the time point, animals were assessed for arterial function via transesophageal echocardiography. Animals were sedated using 2% inhalatory isoflurane and 2.5% oxygen before being placed in the supine position under nosecone ventilation. Transesophageal echocardiography was performed using a Phillips Sonos 5500 Ultrasound system and snapshot images taken in B mode, at least three images taken per animal for statistical analysis of peak systolic velocity. Height during peak systolic velocity was measured using the ultrasound machine and the highest peak used for comparison between groups. After imaging, animals were exposed to 5% inhalatory isoflurane for 5 minutes prior to cervical

dislocation. Following cervical dislocation, the chest cavity was opened and blood collected following aortic puncture.

CHAPTER THREE: FINDINGS

BMP-7 Significantly Reduces Plaque Development by D28 vs. PLCA Animals

To determine the extent of plaque formation after partial ligation of the carotid artery, hematoxylin and eosin (H&E) stains of arterial sections for both time points was completed. At D5, there was no significant difference in % Plaque Area between groups. This is likely because of the short duration between ligation and sacrifice. The trend at D5, however, became significant by D28 and BMP-7 significantly prevented the development of plaque compared to PLCA animals. PLCA animals had significantly more plaque development compared to SHAM animals as well at D28. There was no significant difference at D28 between SHAM and PLCA + BMP7 animals.

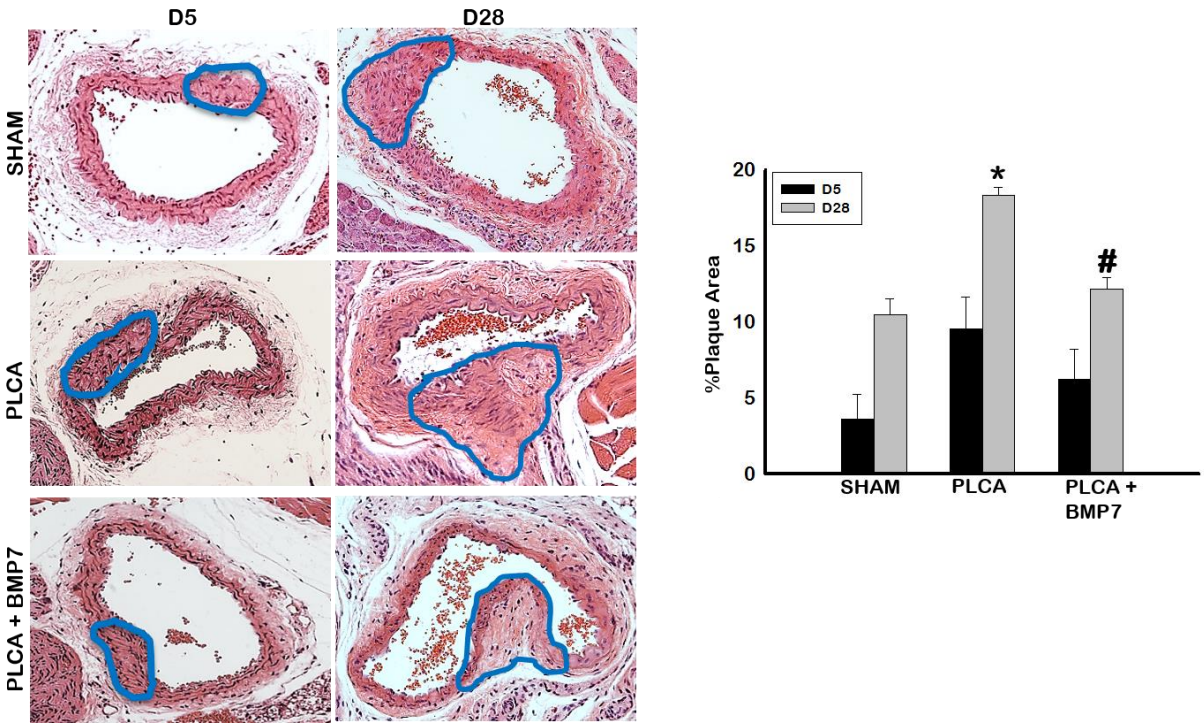


Figure 1: Effect of BMP-7 on Atherosclerotic Plaque Accumulation at both D5 and D28. Hematoxylin and Eosin staining of arterial sections show regions of plaque development in the left carotid artery. **A** shows representative arteries from SHAM, PLCA, and PLCA + BMP7 groups, there was no significant difference between groups at D5 (left column). In contrast, images in the right column show by D28 PLCA animals had significantly more plaque accumulation compared to SHAM and PLCA + BMP7 animals. Bar graph (**B**) shows quantitative analysis done using Image J software. Blue circle indicates region of plaque formation. *:$p<0.05$ vs. SHAM, #:$p < 0.05$ vs PLCA, n = 6 animals for both D5 and D28.

Hematoxylin and Eosin results are useful in identifying the area of plaque formation and size of the plaque but yield no indication regarding the plaque cellular profile, which plays an important role in the progression of ATH [18;19]. To identify specific cell types within the plaque, immunohistochemistry (IHC) of arterial sections was performed.

Monocyte Infiltration into the Inflamed Arterial Region

As described earlier, many of the first cells attracted to the activated endothelium are monocytes. Monocytes in circulation will attach to adhesion molecules expressed on injured endothelial cells and undergo diapedesis as signaled by chemokines released from arterial cells [5;20]. Monocytes were identified using antibody against CD14, a well characterized monocyte marker, with the results shown in Figure 2 [21]. At D5, there is a large influx of monocytes to the area of injury for all three groups compared to D28. This influx of cells follows the traditional inflammatory response pattern, with dramatic inflammation occurring immediately post-injury and subsequently waning as the wounded area begins to heal [22]. Interestingly, animals in the PLCA + BMP7 group had significantly less monocytes present within the artery at D5 compared to the PLCA. This may indicate that BMP-7 mediates monocyte recruitment to the area of injury immediately following trauma. This initial benefit at D5 is lost, however, by D28, and there is no significant reduction in monocyte presence within the artery of PLCA + BMP7 animals compared to PLCA animals.

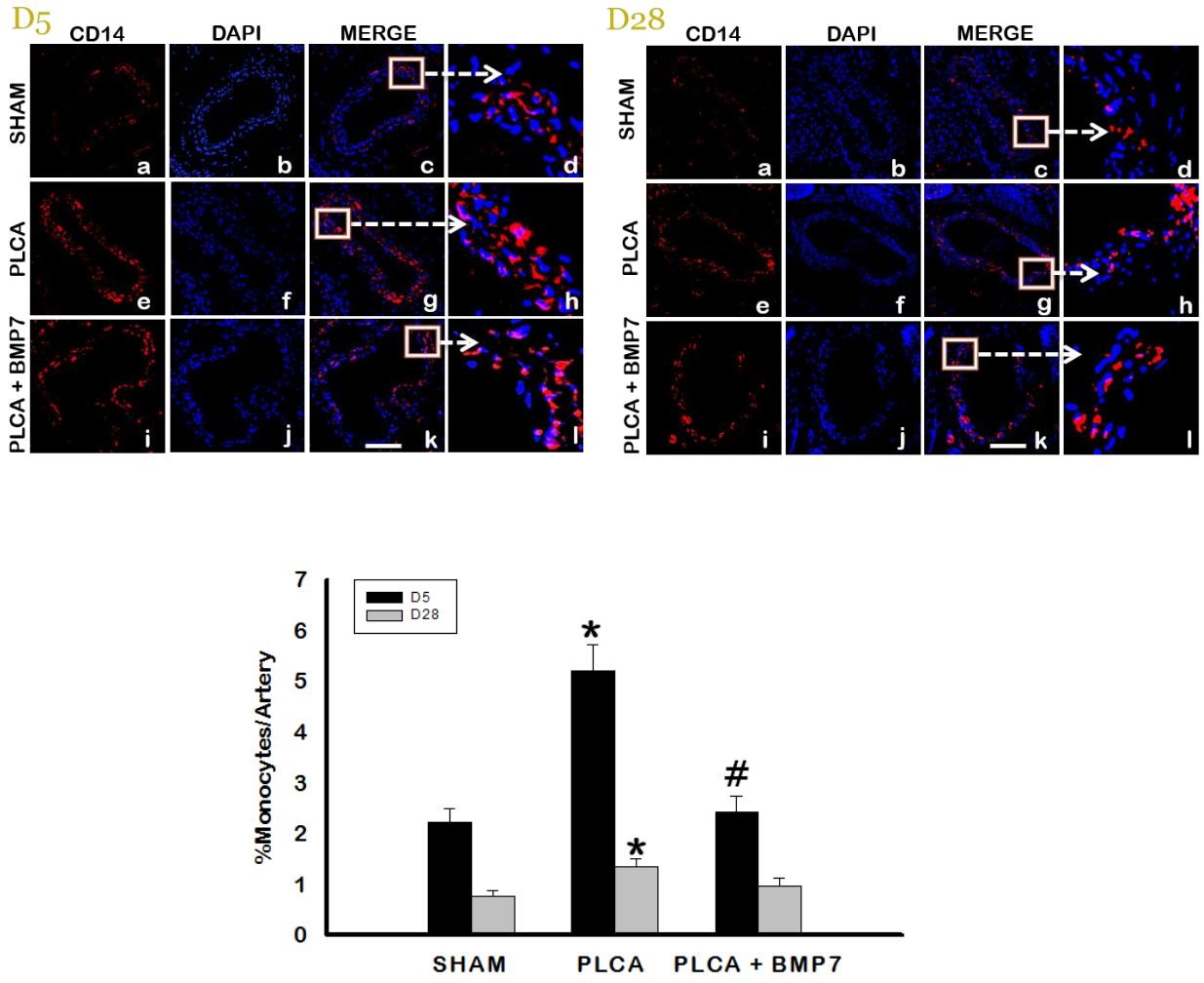


Figure 2: Monocyte Recruitment to the Area of Injury. Photomicrographs of immunohistochemistry performed using antibody against the characteristic monocyte marker CD14 at D5 (**left**) and D28 (**right**). $CD14^{+ve}$ cells shown in red (a, e, i), DAPI stained nuclei in blue (b, f, j), merged images (c, g, k), and selected enlarged areas of merged images (g, h, i). Bar graph depicts quantitative analysis indicating that animals treated with BMP-7 showed significantly less initial recruitment of monocytes to the area of inflammation (D5) but that significance was lost by D28 compared to PLCA animals. At both D5 and D28 PLCA animals had significantly more monocytes present compared to SHAM animals. *: $p<0.05$ vs. SHAM, #$p<0.05$ vs. PLCA, n = 6-7 for both D5 and D28. Scale bar = 100µm.

BMP-7 Decreases Inflammatory Macrophage Presence at D5 and D28

Following monocyte infiltration into the subendothelial space, they will undergo differentiation into various macrophage subtypes based on signals from the microenvironment [21]. Inflammatory cytokines such as TNF-α and IFN-γ released from T_H1 or resident macrophages direct monocyte differentiation toward the inflammatory macrophage phenotype known as M1, characterized by their subsequent release of additional inflammatory cytokines and high production of reactive oxygen and nitrogen species [6;23]. Identification of M1 macrophages within the plaque was done using antibody against inducible nitric oxide synthase (iNOS), a highly expressed enzyme by M1 macrophages. Results shown in Figure 3 indicate that BMP-7 administration significantly reduces the presence of M1 macrophages at both D5 and D28.

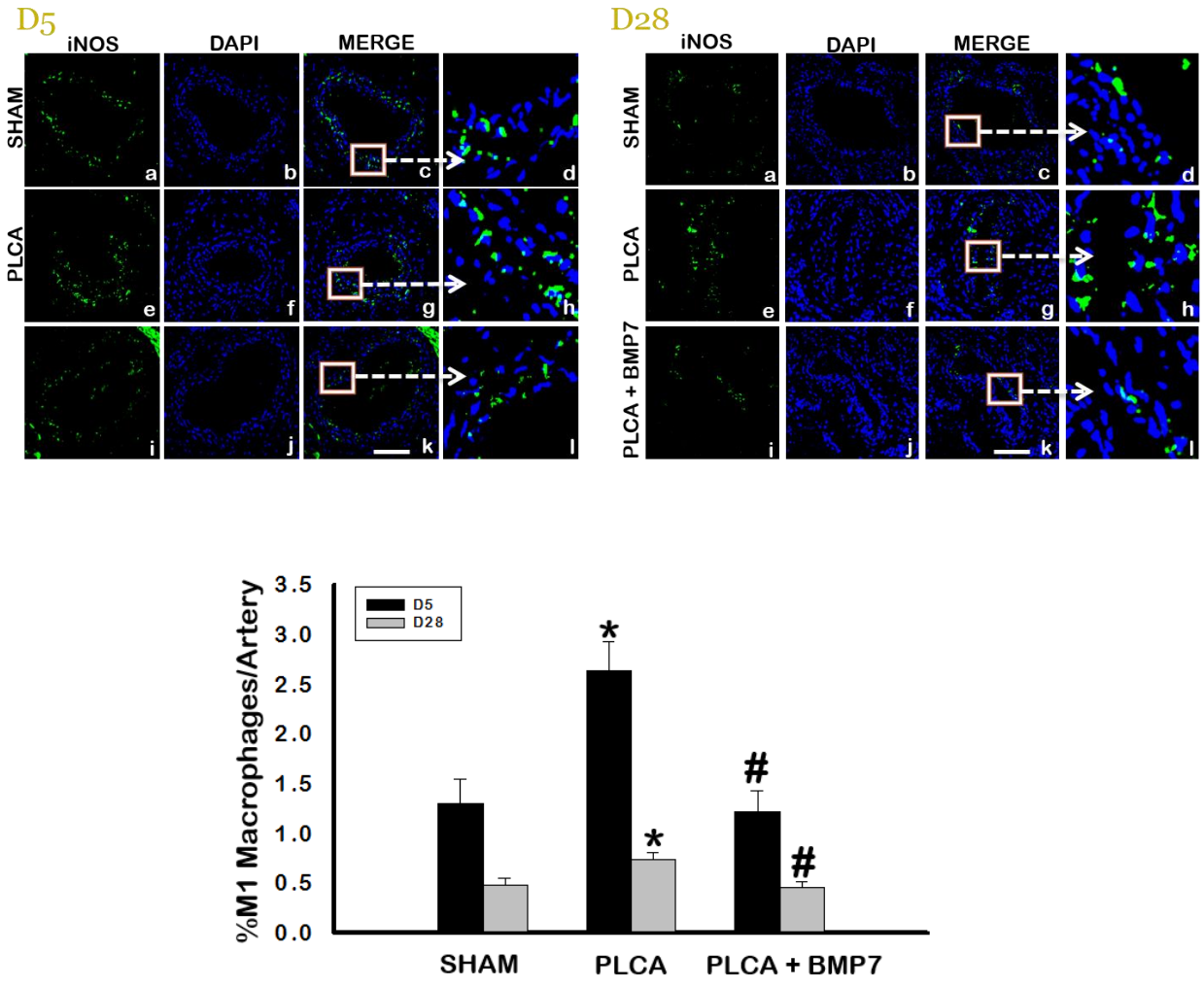


Figure 3: Reduced Monocyte Differentiation into the Inflammatory M1 Macrophage. Monocytes differentiated into the inflammatory M1 phenotype were identified using antibody against iNOS. iNOS^{+ve} cells are shown in in green (a, e, i), DAPI stained nuclei in blue (b, f, j), merged images (c, g, k), and selected enlarged areas (d, h, l) for both D5 (**left**) and D28 (**right**). Quantitative analysis indicates that animals treated with BMP-7 showed a significant reduction of M1 macrophages present at both D5 and D28 compared to PLCA animals (**bar graph**). PLCA animals had significantly more iNOS^{+ve} cells vs. SHAM at D5 and D28. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, n = 6 (D5) and n = 6-7 (D28). Scale bar = 100µm.

IHC results of the iNOS stain show that BMP-7 reduces inflammatory macrophage presence at both D5 and D28 post-PLCA surgery. As previously mentioned, monocyte differentiation can result in a distinct macrophage phenotypes, another being the anti-inflammatory M2 macrophage.

BMP-7 Directs Monocyte Differentiation Toward the Anti-Inflammatory M2 Phenotype

Immunohistochemical identification of M2 macrophages was done using antibody against characteristic M2 markers CD206 and Arginase-1 [4;23]. Prior to this study, previous *in vitro* results indicate BMP-7's ability to differentiate human monocytes in culture toward the anti-inflammatory M2 phenotype [10]. We therefore hypothesized BMP-7 administration in this study would promote monocyte to M2 differentiation *in vivo*. Results indicate that by D5, there was not a significant increase in M2 macrophage presence in BMP-7 treated animals compared to the PLCA artery (Figure 4). However, by D28, the trend seen at D5 became significant and BMP-7 treated animals had significantly more M2 macrophages present compared to PLCA.

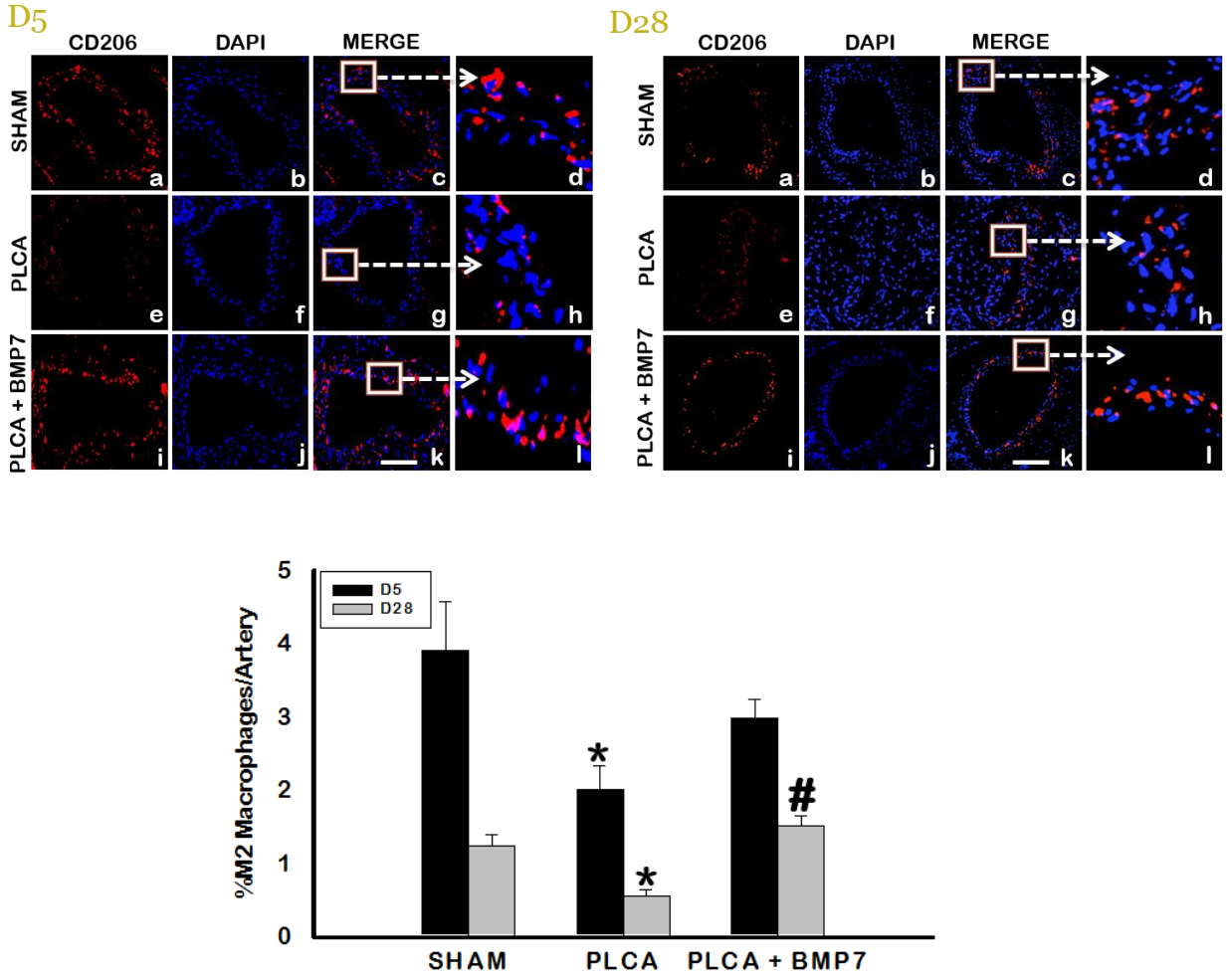


Figure 4: BMP-7 Directs Monocyte Differentiation Toward the Anti-Inflammatory Phenotype by D28. CD206 is a characteristic marker of M2 macrophages. At D5 (**left**), there was not a significant increase in $CD206^{+ve}$ macrophages of PLCA + BMP7 animals compared to PLCA animals, although the PLCA group was significantly lower than SHAM animals. By D28 (**right**), there were significantly more M2 macrophages in PLCA + BMP7 animals compared to PLCA animals. PLCA animals also had significantly less M2 macrophages compared to SHAM animals at D28. Representative images show CD206 positive cells in red (a, e, i), DAPI stained nuclei in blue (b, f, j), merged images (c, g, k) and selected enlarged areas of merged images (d, h, l) (D5 and D28). Bar graph shows results of quantitative analysis using Image J software. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, n = 6-7 animals (D5 and D28). Scale bar = 100µm.

Additional staining for M2 macrophages was performed using the marker Arginase-1. Results show a similar trend to the CD206 stain.

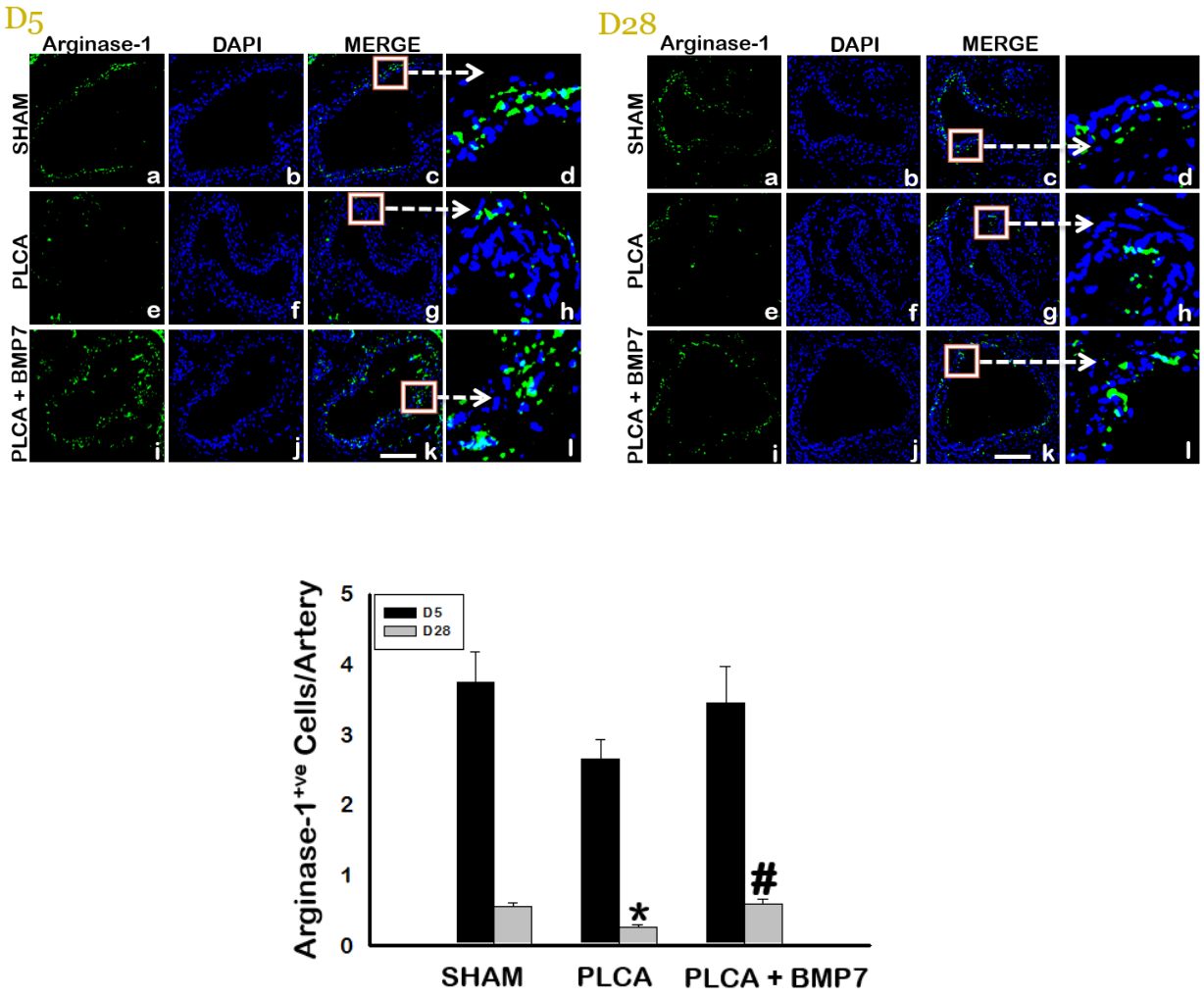
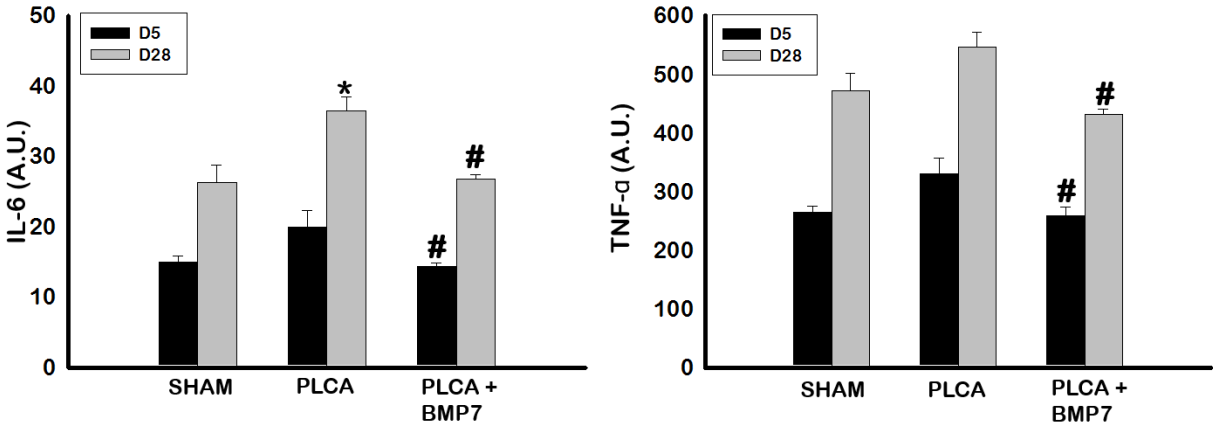


Figure 5: Arginase-1^{+ve} M2 Macrophages Significantly Increased in PLCA + BMP7 Animals at D28. M2 macrophages were also quantified using the additional M2 macrophage marker Arginase-1. Representative photos of Arginase-1^{+ve} cells are shown in green (a, e, i), DAPI stained nuclei in blue (b, f, j), merged images (c,g,k), and enlarged selected areas of merged images (d, h, l) (D5 and D28). Quantitative analysis (**bar graph**) shows at D5 there is no significant difference between groups. By D28 there is a significant increase of Arginase-1^{+ve} macrophages in the PLCA + BMP7 group compared to the PLCA. PLCA group was significantly less than the SHAM animals as well (D28). *: $p<0.05$ vs. SHAM, #$p<0.05$ vs. PLCA, n = 6 (D5) and n = 6-7 (D28). Scale bar = 100µm.

Results of the CD206 and Arginase-1 stain suggest that like our *in vitro* study, BMP-7 is capable of differentiating monocytes toward the anti-inflammatory M2 macrophage. To corroborate these findings and assess inflammation on a larger scale occurring in these animals at D5 and D28 post-surgery, ELISA of blood serum was performed.

BMP-7 Reduces Blood Serum Inflammatory Cytokine Levels

M1 macrophages release inflammatory cytokines such as IL-6, TNF-α, and MCP-1. These cytokines influence the progression of atherosclerosis by causing increased lipid deposition and retention in the artery, induction of apoptosis, and recruitment of monocytes to the affected area [24]. Results show that treated animals have significantly lower blood levels of IL-6, TNF-α, and MCP-1 at D5 and D28 compared to PLCA animals.



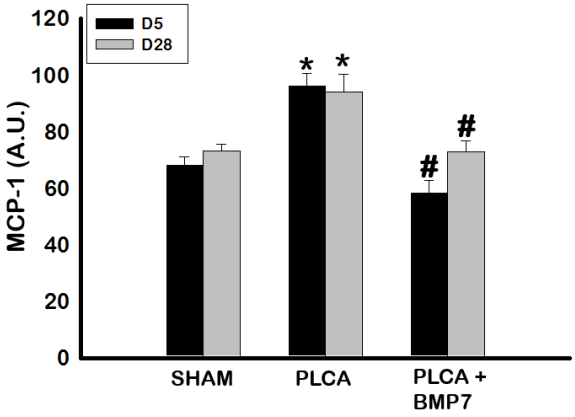


Figure 6: BMP-7 Reduces Inflammatory M1 Cytokines at D5 and D28. ELISA of blood serum collected immediately post-sacrifice of cytokines IL-6, TNF-α, and MCP-1. Results show that animals treated with BMP-7 had significantly less inflammatory cytokines present at both D5 and D28 compared to PLCA animals. PLCA animals also had significantly more IL-6 (D28 only) and MCP-1 (D5 and D28) compared to SHAM animals. *: $p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, n = 5-7 (D5 and D28).

BMP-7 Increases Circulating Levels of Anti-Inflammatory Cytokines IL-1 and IL-1RA

Like M1 macrophages, M2 macrophages have a unique cytokine profile reflective of their anti-inflammatory nature. Anti-inflammatory cytokines produced by M2 macrophages include IL-10 and IL-1RA. IL-10 plays an important role in inducing monocyte to M2 differentiation, phenoswitching of M1 macrophages towards the M2 phenotype, and deactivation of other immune cells such as T lymphocytes [25-27]. IL-1RA is a naturally produced antagonist of the receptor for IL-1, an inflammatory cytokine linked to expression of adhesion molecules by endothelial cells to continue capture of circulating monocytes [28]. ELISA shows that at D5, PLCA animal's level of IL-1RA was significantly lower than SHAM and PLCA +

BMP7 animals. The significance was lost by D28. Further, by D28, atherosclerosis progression was enough so that PLCA + BMP7 show significantly less IL-1RA compared to SHAM animals. IL-10 shows a slightly different story, with levels of IL-10 being significantly lower in the PLCA group vs. SHAM only at D5, but vs. PLCA + BMP7 at both D5 and D28.

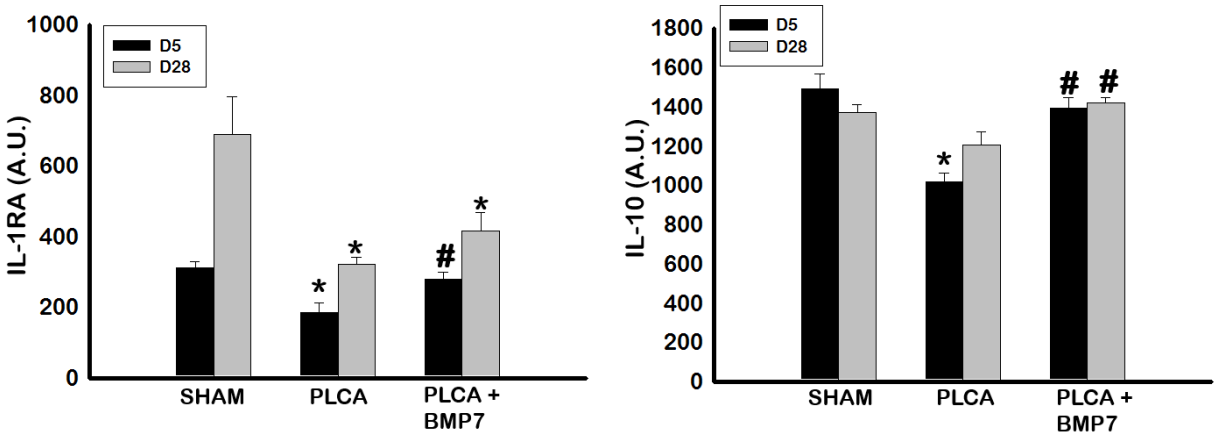


Figure 7: BMP-7 Increases Levels of Anti-Inflammatory Cytokines. ELISA of IL-1RA and IL-10. At D5, PLCA animals had significantly less IL-1RA compared to both SHAM and PLCA + BMP7 animals. By D28, treatment animals had significantly less IL-1RA compared to SHAM and there was no significant difference between PLCA + BMP7 and PLCA animals. ELISA also showed that at D5, the PLCA group had significantly less circulating IL-10 compared to both SHAM and PLCA + BMP7 animals. PLCA + BMP7 animals had significantly more IL-10 compared to PLCA at D28 as well. *:$p<0.05$ vs. SHAM, #: $p< 0.05$ vs. PLCA, n = 5 (D5), n = 4-5 (D28).

The increased levels of anti-inflammatory cytokines in the PLCA + BMP7 animals compared to PLCA animals reflects the higher presence of M2 macrophages seen in affected arterial regions by IHC. At this point, it is becoming clear that BMP-7 administration reduces inflammation by causing plaque cellular profile changes. The increased presence of M2

macrophages at D5 and D28 leads to an overall reduction of circulating inflammatory cytokines and corresponding increase of anti-inflammatory cytokines.

BMP-7 Decreases Activation of p38 and JNK

JNK and p38 proteins are activated in response to stress stimuli such as oxidized low-density lipoprotein (oxLDL) and TNF-α. Both kinases were assessed using Western blot to see if BMP-7 affected activation of either [29;30]. PLCA + BMP7 animals showed a significant reduction in the activated form of p38 at D5 compared to SHAM and PLCA animals. At D28, p38 pathway activation was still significantly less than PLCA, but no longer significantly less than SHAM.

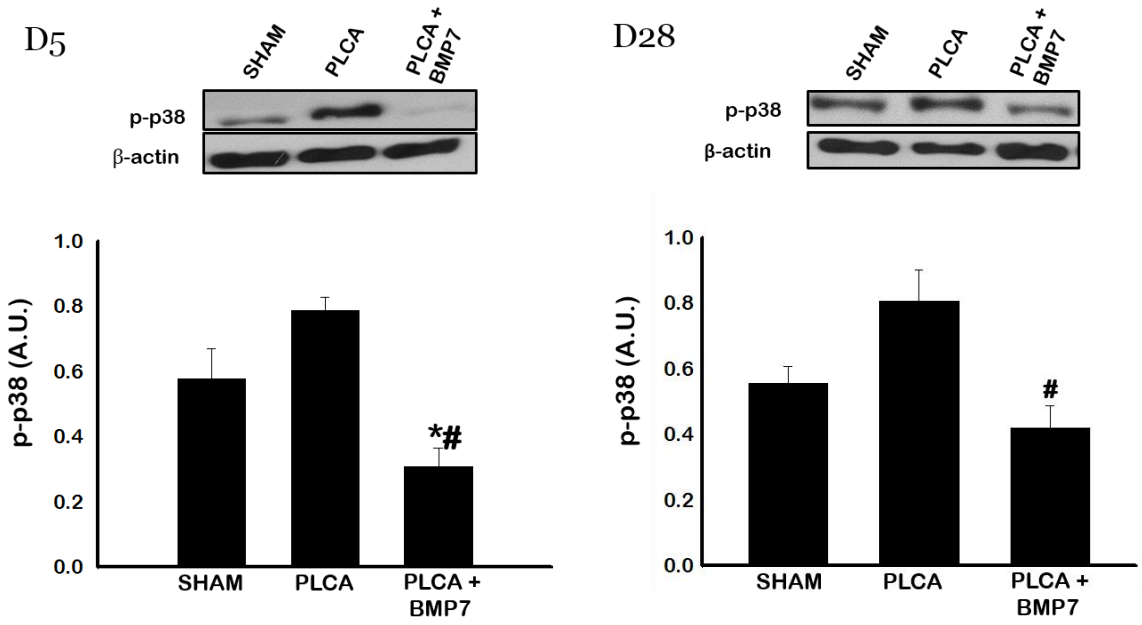


Figure 8: BMP-7 Decreases Activation of p38 Pathway. Western blot analysis of carotid artery tissue demonstrates a significant reduction in the activated form of p38 protein, p-p38, in BMP-7 treated animals compared to PLCA at both D5 and D28. PLCA + BMP-7 was also

significantly lower compared to SHAM animals at D5. *: p <0.05 vs. SHAM, #: p<0.05 vs. PLCA, n = 4 (D5) and n = 4-5 (D28).

Analysis of activated JNK protein showed p-JNK was significantly lower in PLCA + BMP7 mice compared to PLCA mice at both D5 and D28. PLCA animals had significantly more activation compared to SHAM animals at both D5 and D28 as well.

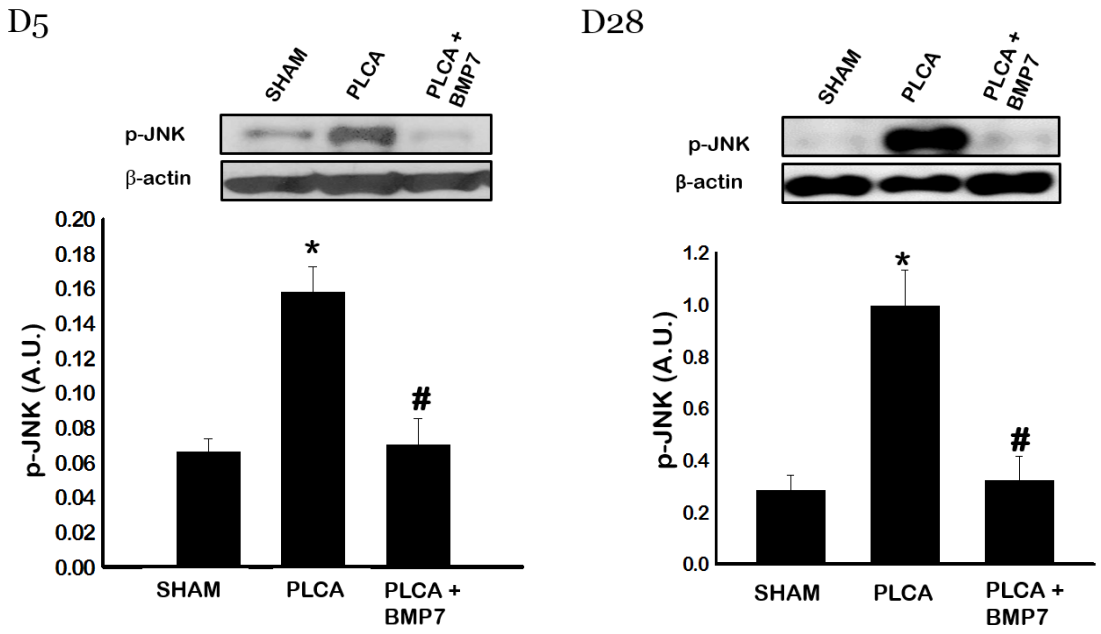


Figure 9: BMP-7 Decreases Activation of JNK. Animals who received BMP-7 showed significantly less activation of inflammatory kinase JNK vs. PLCA animals at both D5 and D28. PLCA animals showed significantly more JNK activation compared to SHAM (D5 and D28). *:p<0.05 vs. SHAM, #: p<0.05 vs. PLCA, n = 3-4 (D5) and 4-5 (D28).

Western blot analysis of p38 and JNK protein indicate that BMP-7 plays a role in mitigating inflammation by decreasing activation of these two proteins linked to increased

production of inflammatory cytokines, increased apoptosis, and increased plaque formation [31;32].

BMP-7 Activates Cellular Differentiation and Growth Related Protein ERK

Unlike p38 and JNK, ERK is a kinase associated with cellular differentiation and anti-inflammatory cytokine production [29;33]. ERK activation was assessed using Western blot technique and at both D5 and D28, PLCA animals had significantly less of the activated form of the ERK protein, p-ERK, compared to SHAM animals. Further, BMP-7 treated animals showed a significant increase in p-ERK compared to PLCA animals at D5 and D28.

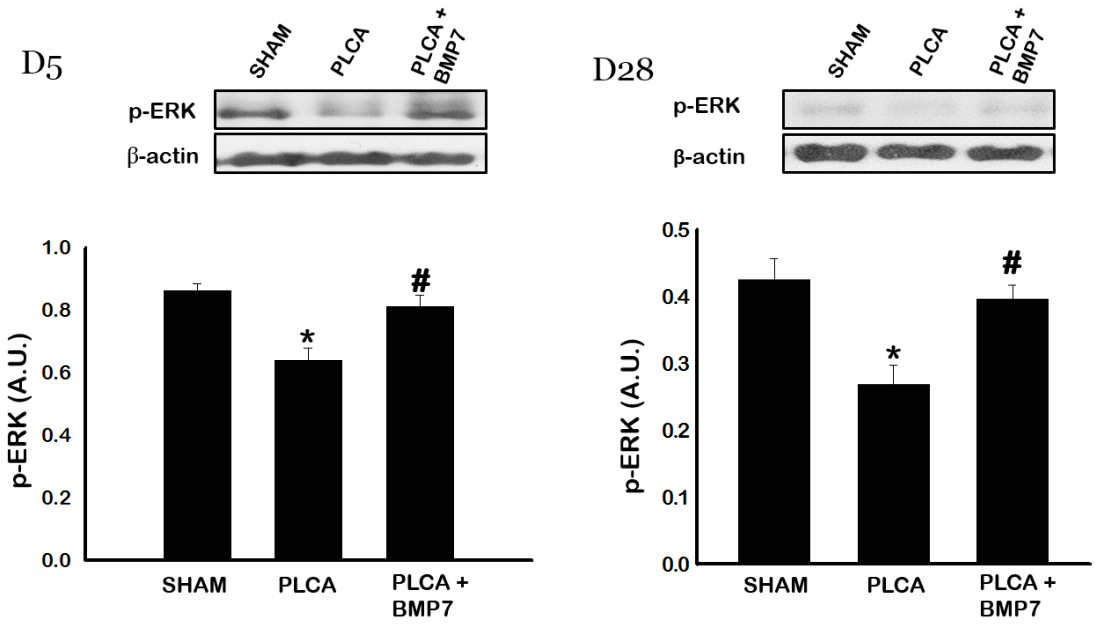


Figure 10: BMP-7 Increases Activation of ERK at D5 and D28. Results of Western blot analysis of carotid artery tissue show a significant increase in ERK activation at both D5 and D28

compared to PLCA animals. PLCA animals also had significantly less p-ERK vs. SHAM (D5 and D28). *:p<0.05 vs. SHAM, #:p<0.05 vs. PLCA, n = 4 (D5) and n = 4-5 (D28).

Since ERK has been linked to cellular differentiation, it is possible BMP-7 is utilizing this pathway to direct monocyte differentiation to the anti-inflammatory M2 macrophage phenotype.

Progression of Atherosclerosis Affects Normal Production of BMP-7

Results from previous studies indicate that inflammation can affect endogenous levels of BMP-7 in adult animals. To determine if the inflammation associated with atherosclerosis may affect production of BMP-7 at D5 and D28, ELISA of blood serum was performed. The results show that at D5 and D28, PLCA animals exhibited significantly less circulating BMP-7 compared to PLCA + BMP7 animals (Figure 11).

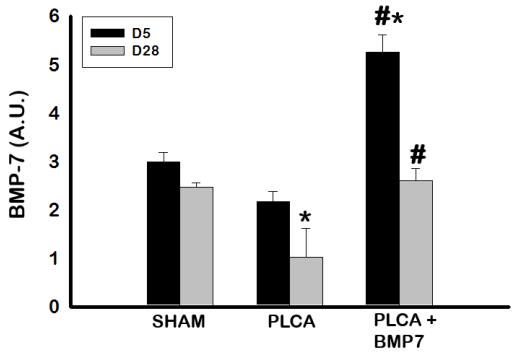


Figure 11: BMP-7 Production Affected by Atherosclerosis. ELISA of blood serum collected immediately post-sacrifice suggests that increased atherosclerosis can affect normal production of BMP-7. At D5, PLCA + BMP-7 animals had significantly more BMP-7 in circulation compared to both SHAM and PLCA animals. At D28, PLCA + BMP7 animals had significantly more BMP-7 in circulation compared to PLCA but not compared to SHAM. PLCA animals had

significantly less BMP-7 present compared to SHAM as well at D28. *: $p<0.05$ vs. SHAM, #$p<0.05$ vs. PLCA, n = 7-8 (D5) and n = 5-6 (D28).

BMP-7 Improves Blood Flow Through the Carotid Artery Post-Ligation

Finally, analysis of blood flow through the artery was assessed to study arterial function as a measure of systolic velocity. Data was acquired using transesophageal echocardiography in B mode. At least three images per animal were analyzed and the tallest peak of each image used to calculate the average systolic velocity per animal. Results show that PLCA animal's systolic velocity was significantly lower than SHAM animals at D5 and D28. PLCA + BMP7 animals showed a significant improvement compared to PLCA animals at both D5 and D28, however by D28 PLCA + BMP7's systolic velocity was significantly lower than SHAM. There should be some loss of arterial function in the PLCA + BMP7 group compared to SHAM animals since the PLCA + BMP7 group received a partial ligation of the carotid artery while no branches of the carotid artery were ligated in SHAM animals.

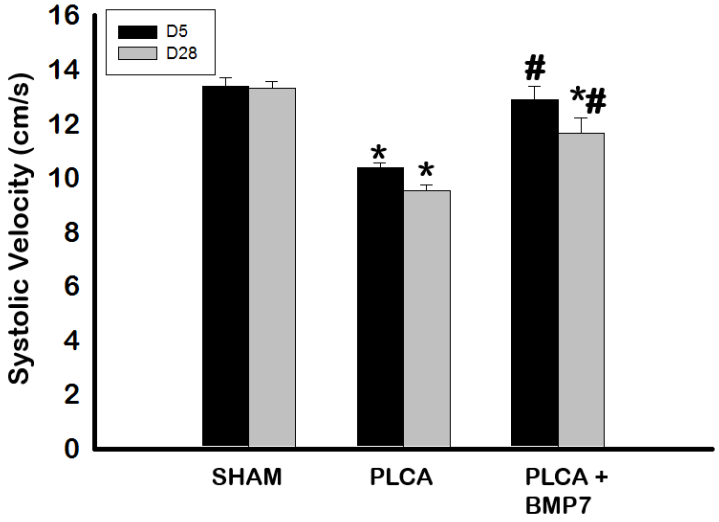


Figure 12: BMP-7 Improves Blood Flow Velocity Through the Carotid Artery at D5 and D28. Transesophageal echocardiography of the left carotid artery in B Mode. Analysis of systolic velocity as a measure of arterial function shows that at both D5 and D28, PLCA animal's systolic velocity was significantly lower compared to SHAM animals. PLCA + BMP7 animals showed significant improvement in systolic velocity compared to PLCA animals at D5 and D28. PLCA + BMP7 animals did show a significant decrease in systolic velocity compared to SHAM animals at D28. *:$p<0.05$ vs. SHAM, #: $p<0.05$ vs. PLCA, n = 8-9 (D5) and n = 9 (D28).

CHAPTER FOUR: CONCLUSION

Atherosclerosis (ATH) is quickly becoming a worldwide epidemic, with risk factors including smoking, hypertension, dyslipidemia, diabetes, and a high-fat diet [34]. Interestingly, atherosclerosis tends to develop in areas of low fluid shear stress, an observation made in both humans and animals [35;36]. Previous work done by this lab generated a surgical method to develop atherosclerosis in Apolipoprotein E knockout mice exploiting the knowledge that low shear stress causes endothelial damage initiating atherosclerosis [16]. The technique is termed partial ligation of the carotid artery, in which three of the four branches of the carotid artery are sutured to trigger ATH. The use of this procedure is unique in that it does not require the animals consume a Western diet to develop atherosclerosis, akin to patients who develop atherosclerosis regardless of a healthy diet and lifestyle. This study utilized our current knowledge regarding surgically induced atherosclerosis to study how treatment using bone morphogenetic protein-7 (BMP-7) affects ATH development and progression at both an early (D5) and mid-stage (D28) of the disease.

Carotid artery sections with plaque accumulation were identified using the Hematoxylin and Eosin (H & E) stain. The results of the H & E stain indicate that at D5 there was no significant difference in plaque accumulation between the three groups, however there was a trend of increased plaque in the PLCA group. This trend became significant at D28, with significantly more plaque present in PLCA animals compared to both SHAM and PLCA + BMP7 animals (Figure 1). This suggests BMP-7 treatment reduces plaque accumulation within the carotid artery up to D28 post-surgery. Established plaques are characterized by infiltrated

immune cells, extracellular matrix components, and cholesterol crystals [14]. We therefore approached plaque analysis via examination of specific cell markers [37].

Immunohistochemistry of carotid artery sections using antibody against the characteristic monocyte marker CD14 showed a surge in monocyte recruitment to the area at D5 compared to D28. This should be attributed to the immediate immune response occurring following any trauma, and this pattern follows for all other IHC stains as well (iNOS, CD206, Arginase-1). Regarding monocyte presence at observed time points, by D5 post-surgery there was a significant increase in the PLCA animals compared to both SHAM and the PLCA + BMP7 (Figure 2). This suggests that BMP-7 reduces the initial inflammatory response occurring because of trauma to the area. Other studies have shown a similar anti-inflammatory result of BMP-7 administration. One study found that intravenous administration of BMP-7 significantly reduced inflammation by controlling cytokine production in a rat model of renal ischemia [38]. Another study presented that BMP-7 given to rats with induced colitis exhibited reduced levels of inflammatory cytokines and less overall damage to gut tissue when compared to non-treated animals [39]. This initial decrease in the burst of inflammation occurring in the area at D5 was lost by D28, and there was no longer a significant reduction in monocytes in PLCA + BMP7 animals vs. PLCA (Figure 2). It is likely the loss of significance is a result of progression of atherosclerosis. Results of the H & E stain indicate that BMP-7 animals exhibited increased plaque development at D28 vs. D5 meaning BMP-7 is not capable of completely preventing monocyte recruitment to the area of inflammation at a mid-stage of the disease.

Monocytes recruited to the area of inflammation will differentiate into M1 or M2 macrophages once they enter the subendothelial space and receive various environmental stimuli. Anti-inflammatory stimuli generates M2 macrophages, while inflammatory stimuli causes monocyte differentiation to M1 macrophages [18]. Of the potential differentiation outcomes, the most beneficial would be M2 macrophages, which promote lipid clearance from the area and release anti-inflammatory cytokines. To determine if BMP-7 had any effect on monocyte differentiation, immunohistochemistry (IHC) was utilized to detect the presence of M1 and M2 macrophages within the arterial area. At both D5 and D28, there was a significant increase in inflammatory M1 macrophages in PLCA animals vs. SHAM animals. Further, BMP-7 treated animals exhibited significantly less M1 macrophages compared to PLCA animals. BMP-7 may therefore be able to reduce monocyte differentiation into M1 macrophages.

To determine the extent of BMP-7's monocyte differentiation potential, we stained arterial sections for characteristic M2 markers CD206 and Arginase-1. The results of the CD206 stain show that at D5, PLCA animals had significantly less M2 macrophages present compared to SHAM. There was an increase in $CD206^{+ve}$ cells in animals treated with BMP-7 compared to PLCA animals, though it was not significant at D5. In comparison, by D28, disease-related inflammation progression in PLCA mice resulted in a significant reduction of CD206 positive cells compared to SHAM animals and PLCA + BMP7 had significantly more $CD206^{+ve}$ cells vs PLCA. To confirm this finding, arterial sections were assessed for Arginase-1, an additional M2 macrophage marker. There was no significant difference of Arginase-1 positive cells between groups at D5, likely because of the influx of immune cells immediately post-injury, though the trend seen in the CD206 stain was again present. There was a significant increase at D28 in

BMP-7 treated animals compared to PLCA animals, which agrees with the CD206 findings. PLCA animals showed a significant reduction in Arginase-1 positive cells compared to SHAM animals as well at D28. Altogether, the IHC results indicate that BMP-7 results in increased monocyte differentiation to the M2 phenotype, thereby increasing the M2 to M1 ratio. As M2 macrophages are beneficial in promoting plaque regression, this suggests BMP-7 as a possible therapeutic for preventing plaque development in ATH [27;40].

Arterial section staining is useful to identify what is happening at the area of injury, but using ELISA to detect ATH-related cytokines present in blood serum gives a good indication of the overall inflammation occurring in the organism. Both M1 and M2 macrophages have characteristic cytokine profiles. M1 macrophages express inflammatory cytokines IL-6, TNF-α, and MCP-1. These cytokines cause increased apoptosis, immune cell activation promoting chronic inflammation, and continued recruitment of monocytes to the area [41]. ELISA of blood serum showed that at D5 and D28, the PLCA + BMP7 group had significantly less IL-6, TNF-α, and MCP-1 in their blood compared to PLCA animals. The significant reduction in inflammatory cytokine presence in treated animals compared to untreated animals corroborates our IHC that BMP-7 prevents monocyte polarization toward the inflammatory M1 macrophage [35;42].

M2 macrophages characteristic anti-inflammatory profile includes IL-10 and IL-1RA. At D5, IL-1RA was significantly lower in PLCA animals compared to both SHAM and PLCA + BMP7 agreeing with immunohistochemistry results. By D28, both PLCA and PLCA + BMP7 animals had significantly less IL-1RA compared to SHAM, confirming BMP-7 cannot

completely halt ATH. There is, however, still the trend of increased IL-1RA in treated animals compared to untreated PLCA animals at D28. IL-10 showed that in PLCA animals there was a significant reduction at both D5 and D28 compared to SHAM, and a significant increase in PLCA + BMP7 animals compared to PLCA. Taken together, the immunohistochemical results and ELISA suggest BMP-7 is capable of slowing development of plaque within the injured artery, increasing the M2 to M1 ratio, and improving the blood cytokine profile by decreasing inflammatory cytokines and increasing anti-inflammatory cytokines.

Western blot analysis of arterial tissue homogenate interrogated how BMP-7 affects various pathways related to atherosclerosis *in vivo*. Assessment of pro-inflammatory kinases JNK and p38 indicated BMP-7 successfully prevented activation of both pathways at D5 and D28. Activation of these kinases occurs in response to inflammatory cytokines such as TNF-α and IL-1 and can generate enough inflammation to promote apoptosis [43;44]. As apoptosis alone is an inflammatory process, this means reduced pathway activation is doubly beneficial. Further, as atherosclerosis advances, inefficient clearance of apoptotic macrophages within the plaque lead to the development of a necrotic core. Atherosclerosis becomes dangerous when an advanced plaque containing a necrotic core becomes destabilized and leads to a thrombotic event resulting in a possible heart attack or stroke [7].

IHC and ELISA indicate BMP-7 increases the M2 macrophage population. ERK is a signaling kinase linked to cellular growth and differentiation and shown to play a role in monocyte differentiation to M2 macrophages [29;33]. Western blot analysis of carotid artery tissue showed BMP-7 increased ERK activation at both D5 and D28 compared to PLCA

animals. Compiling these results with the IHC results, it appears BMP-7 triggers an intracellular signaling cascade involving ERK to differentiate monocytes into M2 macrophages [10].

BMP-7 expression occurs throughout an organism's lifetime, indicating its role not only during development but also in maintaining adult tissues. The majority of the research related to BMP-7's role in adult animals is regarding kidney homeostasis. Interestingly, following renal injury, one study found BMP-7 expression decreases in adult rats suggesting increased inflammation down regulates BMP-7 expression [45]. To see if inflammation associated with atherosclerosis has a similar effect, ELISA of blood serum measured circulating levels of BMP-7 at both D5 and D28. At each timepoint, PLCA animals revealed significantly less BMP-7 in their blood serum compared to PLCA + BMP7 animals. These results support the hypothesis that inflammation negatively affects normal production of BMP-7.

Finally, in order to determine how surgery affected blood flow through the carotid artery, transesophageal echocardiography images taken in B mode were assessed. At both D5 and D28, PLCA + BMP7 animals showed significant improvement in arterial function as a measure of systolic velocity compared to PLCA animals. At D28, however, PLCA + BMP7 animals systolic velocity was significantly lower than SHAM. The decrease in arterial function by D28 is likely a result of increased plaque accumulation present at D28 in PLCA + BMP7 group compared to SHAM.

In conclusion, BMP-7 is capable of reducing inflammation associated with atherosclerosis at both D5 and D28 post surgically induced endothelial injury. BMP-7 can direct monocyte differentiation into the anti-inflammatory M2 phenotype, possibly through utilization

of the ERK pathway. Increased M2 macrophages means an overall decrease in inflammatory cytokines associated with increased atherosclerotic development. BMP-7 also lowers activation of inflammatory kinases, improves blood flow through the artery post ligation, and ultimately slows progression of the disease.

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