Abstract

Role of Macrophage Polarization and Apolipoprotein E in Granuloma Resolution

by

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Sarcoidosis is a chronic disease characterized by the development and accumulation of granulomas. Through multiwall carbon nanotube (MWCNT) instillation of C57 Bl/6 (wild-type) mice, we have established a murine model that induces granulomatous formations, histologically resembling the granulomas in human sarcoidosis. This model has proven to have a large number of commonly upregulated genes and pathways with human sarcoidosis. Matrix metalloproteinase-12 (MMP12), a protein elastase implicated in several chronic inflammatory diseases, was found to be the most upregulated gene expressed by alveolar macrophages of both human sarcoid patients and MWCNT-instilled wild-type mice. Previous studies have found that MMP12 levels are correlated to disease severity in human sarcoid patients.

These findings led to the development of a MMP12 KO model of granulomatous formation via MWCNT-instillation. As in the wild-type model, granulomatous formations present at ten days post-instillation. Granulomas in wild-type MWCNT-instilled mice persist past 60D post-instillation, while granulomas in MMP12 KO mice are resolving at 60D.

We observed a change in macrophage morphology between the MMP12 KO and wild-type bronchoalveolar lavage (BAL) cells. This led us to investigate the macrophage phenotypes

M1 and M2. We stained for the presence of M2 macrophages via their unique surface protein mannose receptor 1 (CD206, MRC1). At 60D post-instillation, we found that CD206 was highly prevalent in the MMP12 KO strain and very low in the wild-type strain. In the MMP12 KO model we utilized dectin-1 and the receptor for advanced glycation end-products (RAGE) to stain for M2a and M2c macrophages, respectively. In sham-instilled MMP12 KO the predominant macrophage subtype is M2c, while in MWCNT-instilled MMP12 KO the predominant macrophage subtype is M2a. At 10D, both sham and MWCNT-instilled MMP12 KO had a high prevalence of M2c cells. The shift in the phenotype of the macrophage population from M2c to M2a and the subsequent release of IL-13 by M2a macrophages corresponds with the resolution of granulomas in the MMP12 KO model. Thus, M2a macrophages may be critical to granuloma resolution.

The knock out of apolipoprotein E (ApoE) has been utilized to induce granulomatous formations in a murine model of sarcoidosis. Thus, we hypothesized that high levels of ApoE would be associated with granuloma resolution. At 10D in the MMP12 KO model, when granulomatous formations are present, ApoE intracellular protein levels were found to be low. However, ApoE gene and protein expression were increased at 60D in MWCNT-instilled MMP12 KO mice, compared to low levels in wild-type MWCNT-instilled mice. Thus, we observed that increased ApoE levels are associated with granuloma resolution. These observations suggest that ApoE may be playing a role in the resolution of granulomatous formations, potentially through enhanced phagocytosis of degraded collagen fragments.

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LIST OF ABBREVIATIONS

°C: degree Celsius

µL: microliter

2°: secondary

10D: ten-days post-instillation

60D: sixty-days post-instillation

9/11: September 11th

AM: alveolar macrophages

ANOVA: analysis of variance

ApoE: apolipoprotein E

AUP: Animal Use Protocol

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

CCL2: chemokine (C-C) motif ligand 2

CD206: mannose receptor 1

Ct: cosine transform

DAPI: 4′,6-diamidino-2-phenylindole

dCt (ΔCt): discrete cosine transform

ELISA: enzyme linked immunosorbent assay

GAPDH: glyceraldehyde 3 phosphate dehydrogenase

HLA: human leukocyte antigen

IFNγ: interferon gamma

IL-1R: interleukin-1 receptor

IL-4: interleukin-4

IL-10: interleukin-10

IL-13: interleukin-13

LPS: Lipopolysaccharides

LRP1: low density lipoprotein receptor-related protein 1

LXR: Liver X receptors

LYM: lymphocytes

KO: knock out

mL: milliliter

MMP: matrix metalloproteinase

MMP12: matrix metalloproteinase 12

MMP12 KO: matrix metalloproteinase 12 knock out

MRC1: mannose receptor 1

MWCNT: multiwall carbon nanotubes

N: number (sample size)

PBS: phosphate buffered saline

PCR: polymerase chain reaction

Pg: picogram

PMN: polymorphonuclear cells

PPARγ: peroxisome proliferator-activated receptor gamma

qPCR: quantitative real-time PCR

RAGE: receptor for glycation end-products

rpm: rotations per minute

RXR: retinoid X receptor

SEM: standard error of the mean

TBE: tribromoethanol

TNF-α: tumor necrosis factor alpha

CHAPTER 1:
Introduction

1.1 Sarcoidosis etiology and epidemiology

Sarcoidosis is a disease characterized by the development and accumulation of granulomatous formations (1). There are approximately 185,000 cases of sarcoidosis in the United States, and approximately 1.2 million worldwide (2, 3). Prevalence of sarcoidosis varies immensely geographically, making the etiology difficult to determine (4). While a universal cause of sarcoidosis has not been established, associations have been drawn to a number of airborne agents including inorganic particles, tree pollen, and insecticides (1). An increase in sarcoidosis incidence has also been attributed to geographical region, as more cases are observed in northern latitudes potentially due to decreased sunlight (5). Associations have also been made between sarcoidosis and a number of occupations including those in the U.S. Navy, metalworking, firefighters, and specifically with first responders to the September 11th, 2001 attacks on the World Trade Center (5-8). The airborne nature of these environmental triggers is explanatory of the common presentation of sarcoid granulomas in the eyes, ears, and lungs (1).

While the presence of these environmental triggers appears essential to the development of sarcoid granulomas, various genetic and demographic factors increase an individual's susceptibility to development of the disease. A 2018 study found that the heritability of sarcoidosis is 39% (9). Siblings of individuals with sarcoidosis are at a greater risk of developing the disease, thus suggesting a genetic component (10). The primary genes associated with sarcoidosis are class I and class II HLA antigens, while no other genes have been consistently been correlated with disease presentation (1). Several demographics have been linked to increased incidence of sarcoidosis, including Black Americans, individuals of Northern

European descent, and individuals aged between 20 and 39 (11, 12). Black Americans have also been found to have worse clinical outcomes from the disease, with Black women having the highest frequency and severity of sarcoidosis out of any demographic (13, 14).

1.2 Sarcoidosis pathology and current treatments

Granulomas are aggregates of giant, multinucleated macrophages and leukocytes that can occur in various tissues (15). As previously mentioned, sarcoid granulomas typically present in the eyes, skin, and lungs (1). The multinucleated giant cells are of the macrophage lineage, and typically form in response to constitutive expression of cytokines including interferon gamma (IFNγ) and different interleukins (16). In more developed granulomas, a combination of collagen, fibroblasts, mast cells, and proteoglycans forms in the periphery as a protective shell surrounding the granuloma (17). These granulomatous formations have previously been considered a host-defense mechanism to prevent the spread of foreign pathogens (18).

Approximately 90% of sarcoidosis patients have pulmonary granulomas (19). While 67% of sarcoidosis cases will spontaneously resolve, pulmonary granulomatous formations are associated with worse outcomes, primarily due to the pulmonary fibrosis that develops in 20-25% of sarcoidosis patients (1). Accordingly, sarcoidosis cases are staged based off lung and lymph node presentation. Stage 1, the most benign, is characterized by granuloma presence solely in the lymph nodes. In patients with Stage 1 sarcoidosis, spontaneous remission will occur in 60-90% of cases. Sarcoidosis is considered Stage 2 when granulomas are present in both the lymph nodes and the lungs, and spontaneous remission will occur in 40-70% of these cases. Stage 3 of the disease is characterized by granulomas present in the lung and absent from the lymph nodes, with spontaneous remission only occurring in 10-20% of cases. The most severe

form of this disease, Stage 4, is due to the development of pulmonary fibrosis. Once sarcoidosis reaches this stage, it cannot spontaneously reach remission (20).

Much of the mechanism regarding the pathology and progression of sarcoidosis is not currently understood. There is no cure for sarcoidosis today, despite many different drugs being tested for efficacy. TNF-α blockers were found to have a significant, yet slight, impact on pulmonary sarcoidosis in a limited study (21). Tetracyclines, namely minocycline, were found to have positive effects on pulmonary sarcoidosis in two out of twelve patients in another limited study (22). These treatments, along with a few others, have only been found to be effective in small trials, and thus adequate data has not been provided for them to become commonplace treatment options. Today, the primary treatment therapy involves corticosteroids for at least 3 months. However, this treatment is only used when organ function is threatened due to the significant adverse effects of the corticosteroids (1). Recently, methotrexate has been added to this treatment regimen due to its action in potentiating the effects of the corticosteroids, despite having no effect on the disease as a monotherapy (23). Due to this lack of sufficient pharmaceuticals, we must continue to expand our understanding of sarcoidosis pathogenesis in order to one day develop a better treatment for this disease.

1.3 Murine model of pulmonary sarcoidosis

Sarcoidosis is associated with several different occupations, but has only been linked to one event: the 9/11 World Trade Center attacks. First responders to this event have had a significantly increased incidence of sarcoidosis relative to unexposed, demographically similar individuals (24). Correspondingly, carbon nanoparticles have been observed during postmortem examination of the lungs of individuals present at these attacks or in the immediate aftermath,

suggesting a potential link between carbon nanoparticles and sarcoidosis (25). Multi-wall carbon nanotubes (MWCNT), which are concentric structures of carbon, are found in high strength materials, electronics, paint, and are even emitted by the combustion of some materials (25). Carbon nanotubes have since been shown to cause sarcoidosis-like effects in exposed animals, inducing pulmonary inflammation and granuloma like lesions (26)

One of the primary limiting factors in the study of sarcoidosis pathogenesis is the lack of a widely accepted and implemented animal model of the disease (27). However, our laboratory has successfully developed a chronic murine model of granulomatous inflammation through the use of MWCNT (28). When C57Bl/6 (wild-type) mice are instilled with MWCNT, granuloma-like lesions are observed at 10, 60, and 90 days post-instillation, and these lesions have been found to have a similar histological presentation to human granulomas (28). In C57Bl/6 mice instilled with phosphate buffered saline (PBS/surfactant) serving as the sham treatment group, no physiological change is observed (28).

1.4 Alveolar macrophage activity in sarcoidosis

Alveolar macrophages are the predominant immune cell in the airways of healthy individuals. These macrophages serve as the front line of defense against pathogens that survive the mechanical defenses of the immune system. This function is achieved through a variety of mechanisms including phagocytosis and the secretion of proteases. When the alveolar macrophages are unable to uptake the foreign stimuli, they surround the agent and release cytokines to signal for the infiltration of other immune cells (29).

Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcriptional factor that negatively regulates pro-inflammatory macrophage activation (30). In normal, healthy individuals, PPARγ is constitutively expressed by alveolar macrophages (31). However, in individuals with sarcoidosis, alveolar macrophage expression of PPARγ is decreased (30). The same effect is seen in our murine model, as alveolar macrophages from sham-instilled wild-type mice constitutively express PPARγ, while its expression is decreased in wild-type MWCNT-instilled mice (32). Thus, the stimuli that leads to the formation of granulomas decreases PPARγ activity resulting in pro-inflammatory macrophage activation in both sarcoid patients and our murine model.

A transcriptional survey comparing our MWCNT model and human sarcoidosis yielded twelve commonly upregulated pathways and a large number of commonly differentially expressed genes (33).This survey strengthens this use of MWCNT as a representative model of the pathology of human sarcoidosis.

1.5 Matrix metalloproteinase-12

Matrix metalloproteinases (MMPs) are a class of proteases, or enzymes that degrade proteins. These enzymes are involved a number of biological processes, primarily involved in degrading the extracellular matrix and in the immune and inflammatory responses, but are also involved in tissue repair, tissue remodeling, and cell proliferation. The activity of MMPs is low in healthy individuals, with several different modulators. However, environmental triggers can cause the release and activation of MMPs, leading to inflammation and tissue remodeling in the form of fibrosis. Accordingly, the MMPs have previously been indicated in several pulmonary diseases

including idiopathic pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease (34).

Matrix metalloproteinase-12 (MMP12) is a macrophage elastase that has been indicated in a number of chronic inflammatory disease, both pulmonary and systemic (34). This enzyme is produced by alveolar macrophages, and it is released in response to pathogens (35). However, MMP12 is regulated by a multi-faceted system, with different factors influencing gene expression, compartmentalization, zymogen activation, and proteolysis (36). Through these processes, MMP12 is only produced, released, activated, and preserved under conditions allowing all of these factors to function synergistically, such as in the immune response (36). MMP12's role in chronic disease has previously been attributed to its functions in macrophage recruitment and regulation of inflammation through TNF-α (35, 37).

In the gene expression profile previously mentioned, MMP12 was determined to be the most upregulated gene in both sarcoidosis and the MWCNT model (33). PCR data confirmed this result, with MMP12 significantly elevated in sarcoid patients relative to control individuals and in our wild-type MWCNT model relative to sham-instilled mice (33, 38). Additionally, MMP12 levels have been found to be correlated with disease severity in sarcoid patients, with elevated expression observed in areas of active granulomatous inflammation (39).

1.6 A MMP12 KO model of pulmonary sarcoidosis

In response to these findings from both the wild-type MWCNT model and human sarcoidosis, our lab decided to utilize a MMP12 KO mouse strain to help delineate the role of MMP12 in sarcoidosis. Through the administration of MWCNT, we were able to induce

pulmonary granulomas at 10D, similar to those observed in the wild-type MWCNT. However, histological differences were seen at 60D (chronic stage) between the wild-type and MMP12 KO. In the chronic phase of MMP12 KO mice instilled with MWCNT granulomas are resolving, suggesting that MMP12 is critical to the persistence of granulomatous formations (38).

Previously, our lab has shown that at 10D post-instillation, gene expression of proinflammatory cytokines including chemokine ligand 2 (CCL2) and IFNγ are increased in MWCNT-instilled mice of both strains (unpublished data). However, differences in several proinflammatory genes between the two strains at 60D was observed (38). PPARγ, a negative modulator of proinflammatory responses, is decreased in wild-type MWCNT and increased in MMP12 KO MWCNT (38). Thus, proinflammatory responses are negatively modulated to a greater extent in MMP12 KO mice than in wild-type mice at 60D. Correspondingly, IFNγ gene expression is elevated in wild-type MWCNT relative to sham, while no difference in expression between MWCNT- and sham-instilled MMP12 KO mice is observed (38). Additionally, an increase in the alveolar macrophage population of the MMP12 KO MWCNT was observed (38).

These results led to our first proposed mechanism for the role of MMP12 in granuloma persistence. Following MWCNT instillation, the alveolar macrophage expression of PPARγ is decreased. This, in turn, leads to the increased expression of the proinflammatory cytokine IFNγ, resulting in the formation of granulomas. This response causes elevated MMP12 expression, which plays a role in maintaining the low expression level of PPARγ and the high level of IFNγ expression, enabling granulomas to persist. However, in the absence of MMP12, PPARγ expression increases and IFNγ expression decreases, leading to granuloma resolution (38).

1.7 Macrophage polarization

Alveolar macrophages are the predominant immune cell in the lower respiratory airways, and thus are the primary immune response to foreign antigens or pathogens in this region (29). There are two primary classes of macrophages: M1 and M2. M1 macrophages, also known as classically activated macrophages, are activated by IFNγ and LPS (40). M1 macrophages are pro-inflammatory with their primary function being to kill or clear foreign antigens or pathogens via phagocytosis or microbicidal activity (41). Meanwhile, M2 macrophages are also known as alternatively macrophages, and can be activated by a wider variety of substances and cytokines, including IL-4 and IL-13 (40). The primary function of M2 macrophages is to heal, with anti-inflammatory activity in tissue repair and the maintenance of tissue integrity (41, 42). Phenotypically, M2 macrophages express several unique surface proteins including Mannose Receptor 1 (CD206, MRC1), Arginase-1 (Arg1), and Hemoglobin-Haptoglobin Scavenger Receptor (CD163), which have previously been used to identify the population (43). Thus, macrophage phenotype is very important to the immune response.

M2 macrophages can be categorized into three different subpopulations: M2a, M2b, and M2c (40). M2a polarization is driven by IL-4 and IL-13, and phenotypically characterized by surface expression of Dectin-1 and IL-1R (44). These macrophages primarily function to stimulate and potentiate the M2 immune response through the release of M2-related cytokines (45). M2b macrophages are induced by toll-like receptor (TLR) agonists, and do not have a uniquely expressed surface protein (44). M2b macrophages serve as regulatory macrophages, acting essentially as the biological counterpart to M1 macrophages (40, 44). M2c macrophages are activated by IL-10 and are characterized by the surface expression of the receptor for advanced glycation end-products (RAGE) and Mer receptor tyrosine kinase (MerTK) (44, 46). M2c

macrophages have strong anti-inflammatory action and are involved in tissue repair and remodeling through the release of interleukin cytokines and high levels of phagocytosis (44). Despite M2 macrophages sharing a common, anti-inflammatory function, the different subtypes achieve this function in different ways.

1.8 Apolipoprotein E

Apolipoprotein E (ApoE) is a glycoprotein that is involved with various physiological systems and processes (47). ApoE primarily functions in lipid metabolism and clearance, as it will initiate the formation of high-density lipoproteins which enter the circulation (47). In circulation, ApoE associates with remnants of enzymatic lipolysis, transporting them back to the liver via a process called reverse cholesterol transport (48). ApoE also functions in the nervous system by forming lipoproteins that distribute smaller lipoproteins to cells in the system (47). When variants, or isoforms, of ApoE are made and enter circulation, they can cause issues in both the heart and nervous system, with a main linkage to Alzheimer's disease (47, 49). ApoE carries out other functions as well, including cytoskeletal assembly and stability (50).

ApoE has been linked to pulmonary diseases as well. In 2010, an ApoE knock out murine strain on a cholate-containing high-fat diet developed granulomas with similar pathological presentation to pulmonary granulomas, suggesting a potential relationship between low ApoE levels and granuloma development (51). ApoE has also recently been linked to resolution of pulmonary fibrosis, a condition that develops in severe human sarcoidosis cases (52). One of the main driving forces of pulmonary fibrosis is the degradation of collagen by metalloproteinases at a faster rate than the resulting fragments can be cleared (52). In this process, ApoE functions by binding collagen fragments, making them more accessible for phagocytosis by the LRP1

receptors on macrophages (52). As ApoE has been found to stimulate resolution of the most severe pathological development in sarcoidosis, high levels may be potentially be involved in granuloma resolution. As collagen is a component of the surrounding environment in mature granulomas, ApoE may potentially expose the granuloma to the immune responses necessary for resolution (52).

ApoE has also previously been linked to macrophage polarization. Endotoxins and bacterial infections in ApoE KO murine models have led to increased levels of M1-related cytokines such as TNF-α and INFγ (53, 54). This effect can be mitigated via exogenous ApoE administration (53). Subsequent studies have yielded a direct effect of ApoE on macrophage differentiation towards the M2 macrophage phenotype (55). Exposure to ApoE leads to the surface expression of M2 macrophage markers and decreased M1 related macrophage activity (55). M2 macrophages have also been observed to have increased production of ApoE due to their increased LXR and PPARγ activity (56). Thus, ApoE expression is involved in the conversion of M1 macrophages to M2, anti-inflammatory macrophages. Additionally, as we see increased PPARγ at 60D in MMP12 KO MWCNT-instilled mice, we would anticipate a corresponding increase in ApoE.

1.9 Hypotheses

i) We observed a change in macrophage morphology with an increased number of smaller cells in MMP12 KO MWCNT mice compared to wild-type MWCNT and MMP12 KO sham. We hypothesize that the changes in macrophage morphology in the MMP12 KO MWCNT mice is attributed to a shift in macrophage phenotype, resulting in granuloma resolution.

ii) The absence of apolipoprotein E results in granulomatous formations in mice. We hypothesize that increased ApoE may be associated with granuloma resolution in MMP12 KO mice.

CHAPTER 2:

Materials and Methods

2.1 Mice

All studies were conducted in conformity with Public Health Service (PHS) Policy on human care and use of laboratory animals. Experimental approval was granted by the institutional animal care committee (AUP #J199c) (AUP #J207a). Two strains were used in this study, C57Bl/6 and MMP12 KO. Both strains were obtained from Jackson Laboratories (Bar Harbor, ME). The gene knock out in the MMP12 KO strain is complete. Mice were set up for breeding at seven weeks, and breeders were replaced every six months. All experimentation was conducted using sex-matched littermates and/or multiple age-matched litters.

2.2 Instillation with Multi-Wall Carbon Nanotubes

Following sedation by aerosolized isoflurane, mice were instilled oropharyngeally with 100 µg of freshly prepared MWCNT (catalogue number 900-1501, lot GS1802, SES Research, Houston, TX) in a 35% surfactant in PBS solution. Correspondingly, sham groups were administered a 35% surfactant in PBS solution via oropharyngeal administration. All animals received a single dose based on their treatment group. At either ten- or sixty-days post-instillation, all mice were euthanized with tribromoethanol (TBE) and samples were collected.

2.3 Bronchoalveolar Lavage

At either ten- or sixty-days post-instillation, mice were euthanized via an intraperitoneal injection of TBE. Post-mortem, mice were cannulated with plastic tubing. 5mL of a 0.2% lidocaine in PBS solution was used to lavage the lungs. The lavage was centrifuged at 4°C and 1800 rpm for 20 minutes. This centrifugation led to the separation of cells, in the form of a pellet, and BAL fluid (BALF). The BALF from each sample was collected in 1mL increments and the cell pellets were subsequently resuspended in 4mL of PBS. 120µL of this cell suspension were spun in a cytocentrifuge at room temperature and 500 rpm for 5 minutes to yield a cytospin. The cytospins were stained with Diff Quick solution (Thermo Fisher Scientific, Waltham, MA) for differential counts to determine the composition, by percentage, of the following immune cell populations: macrophages, lymphocytes, and polymorphonuclear cells. The remaining cell suspension was centrifuged again. The fluid was aspirated, and the cell pellet was resuspended in 4 mL of PBS. This cell suspension was used to count the total cell number of immune cells in the pellet via a hemocytometer. Once again, the cell suspension was centrifuged under the same conditions. The fluid was aspirated, and the pellet was stored at -70°C.

2.4 Immunocytochemistry

Cytospins containing approximately 50,000 freshly isolated BAL cells were fixed with 4% paraformaldehyde for 20 minutes. Cells were permeabilized with a 0.3% Triton X-100 in PBS solution, followed by blocking with 10% goat serum in PBS. A primary antibody for each desired protein was diluted in 10% goat serum solution and added to the corresponding samples, which were incubated at 4°C overnight. A goat-anti-rabbit 2° antibody (Life Technologies,

Carlsbad, CA) at a 1:1000 dilution in 10% goat serum was added for an incubation at room temperature. Slides were counterstained with DAPI to identify the nucleus and sealed with nail polish. Following completion of the protocol, slides were stored at -20°C. Images were taken on the Zeiss LSM 700 Confocal (Zeiss, Oberkochen, Germany).

The primary antibodies used in this study and their corresponding concentrations in 10% goat serum are: unconjugated CD206 (1:100, ab64693 abcam, Cambridge, MA), ApoE (1:500, ab183596 abcam, Cambridge, MA), Dectin-1 (1:300, ab140039 abcam, Cambridge, MA), RAGE (1:100, ab27647 abcam, Cambridge, MA), and an Alexa 488 conjugated CD206 (1:200, MCA2235A488T Bio-Rad Laboratories, Hercules, CA). Please note that the aforementioned procedure is the base staining protocol; some protocols included slight variations from this, which will be explicitly mentioned in the corresponding figure legends.

2.5 RNA Purification and Analysis

Using miRNeasy kits (Qiagen, Germantown, MD), total RNA was isolated from the cell pellets collected by bronchoalveolar lavage (BAL). This total RNA was subsequently converted to cDNA via a RT2 First Strand Kit (Qiagen, Germantown, MD). The cDNA was used to determine relative gene expression levels using the StepOne Plus PCR system (Thermo Fisher Scientific, Waltham, MA), Qiagen qPCR primer (Germantown, MD), and SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). Each sample was run in duplicate with the desired gene and compared to glyceraldehyde 3 phosphate dehydrogenase (GAPDH), our control gene. The difference between the Ct of the control gene and the Ct of the tested gene yielded the ΔCt, which will in turn be used to calculate fold change via the $2^{-\Delta\Delta Ct}$ method. Samples were excluded

due to decreased levels of GAPDH, which signals the deterioration of a sample, or inconsistent duplicates.

2.6 Protein Analysis

BALF was stored at -80°C until used in protein enzyme-linked immunosorbent assays (ELISA). ApoE (AB215086 abcam, Cambridge, MA) and IL-13 (M1300CB R&D Systems Inc., Minneapolis, MN) following the respective manufacturer's protocol.

2.7 Statistical Analysis

Using Prism 7 software (Graphpad, Inc., San Diego, CA), the data was analyzed via Student's t-test and/or two-way ANOVA with Tukey's multiple comparison test.

CHAPTER 3:

Macrophage Polarization in MMP12 KO and Wild-Type Murine Models of Granulomatous Disease at 60D.

RESULTS

3.1 M2 Surface marker, CD206, is predominantly expressed in MMP12 KO

Previous research from our laboratory has shown that bronchoalveolar lavage (BAL) has increased cell numbers in the MMP12 KO strain compared to the wild-type. Furthermore, MWCNT instillation results in increased BAL cells relative to sham-instilled mice in the MMP12 KO strain. In the process of performing differential counts, a population of smaller alveolar macrophages was observed in MMP12 KO mice and are indicated by red arrows (Figure 1A). Recent research has begun to classify macrophages based on their physical characteristics rather than the tissue they reside in (41). Macrophage populations can be subdivided into M1 and M2 phenotypes. Human M2 macrophages are smaller in size than M1 macrophages; however, limited size data is available regarding murine macrophages (57).

This led us to test for the presence of M2 macrophages. To accomplish this, we stained BAL cells with an antibody to the mannose receptor-1 (CD206, MRC1), a C-type lectin that is specifically expressed on the surface of M2 macrophages (43). Few cells stained positive for CD206 within the wild-type strain, with a slightly increased number of positive cells in the MWCNT-instilled relative to the sham group (Figure 1B). However, in the MMP12 KO strain, nearly the entire population of cells stained positive for CD206 in both the sham and MWCNT-instilled groups (Figure 1B). Thus, the MMP12 KO macrophage population is predominantly M2, while the wild-type macrophage population is predominantly M1. We hypothesized that M2 macrophages may be involved in the resolution of granulomas observed in the MWCNT-instilled MMP12 KO mice.

3.2 Instillation with MWCNT leads to conversion of macrophage phenotype from M2c to M2a in MMP12 KO.

As M2 macrophages were highly prevalent in the MMP12 KO model, we aimed to further define this population of macrophages. In doing so, we hoped to determine the macrophage subpopulation present in MMP12 KO MWCNT, along with their respective function that may be responsible for the resolution of granulomas.

There are three primary M2 macrophage subpopulations: M2a, M2b, and M2c. Each of these macrophage subtypes has unique functions in the immune response. M2a and M2c macrophages can be characterized by surface expression of proteins unique to their respective macrophage subpopulation. One of the surface proteins that is unique to M2a macrophages is Dectin-1 (40). The surface expression of the receptor for advanced glycation end-products, or RAGE, is unique to M2c macrophages (46). There are currently no identified unique surface proteins on M2b macrophages, as the surface proteins expressed are also expressed by M1, M2a, and/or M2c macrophages (44). Thus, we decided to co-stain CD206 and Dectin-1 to identify M2a macrophages in our immune cell population, and to co-stain CD206 and RAGE to identify M2c macrophages, a previously published mechanism for determining phenotype (44). While M2b macrophages cannot specifically be stained for, we theorized that if there is a population of macrophages which stains positively for CD206 but not for Dectin-1 or RAGE, we could assume M2b macrophages are present.

Our focus was on the MMP12 KO mice, as the wild-type had little expression of M2 macrophages. Co-staining for CD206 and RAGE revealed that the majority of M2 macrophages from MMP12 KO PBS-instilled mice stain positive for RAGE, while very few M2 macrophages from MMP12 KO MWCNT-instilled mice stained positive (Figure 2A). Co-staining CD206 and Dectin-1 revealed that Dectin-1 was predominantly expressed by the macrophages in MMP12

KO instilled with MWCNT and expressed on very few cells from MMP12 KO sham-instilled (Figure 2B). This led us to conclude that in the absence of MMP12, M2c macrophages are the predominant cell population. However, M2a macrophages are prevalent in MMP12 KO instilled with MWCNT at 60D, suggesting that they may be playing a role in the resolution of granulomas.

3.3 M2 immune response is increased in 60D MMP12 KO instilled with MWCNT.

Our previous research found that IFNγ is elevated in wild-type mice instilled with MWCNT at both 10D and 60D (38). In MMP12 KO MWCNT, IFNγ expression is elevated at 10D in macrophages from MWCNT-instilled mice relative to sham, but previous studies showed decreased IFNγ expression at 60D relative to 10D (38). IFNγ is associated with M1 macrophage activation and activity (58). These observations suggest that M2 cytokine release may be elevated in our MWCNT-instilled MMP12 KO mice at 60D, when IFNγ expression is low and there is a high prevalence of M2a macrophages.

M2a macrophages are characterized by the release of cytokines IL-4, IL-10, and IL-13. As M2a macrophages are observed solely in the MMP12 KO mice instilled by MWCNT, we would expect to see an increase in these cytokines. IL-13 levels were measured by ELISA. Our results indicate that the MMP12 KO strain has low basal expression of IL-13 (Figure 3). However, upon challenging MMP12 KO mice with MWCNT, a significant increase in IL-13 relative to sham-instilled was observed ($n \geq 8$, $p \leq 0.05$) (Figure 3).

	Treatment	N	Total cell count ($X10^5$)	AM ($X10^5$)	LYM ($X10^5$)	PMN ($X10^5$)
C57Bl/6	PBS/Surf	9	6.6 ± 1.2	6.6 ± 1.2 [99]	0.04 ± 0.06 [1]	0.0 ± 0.0 [0]
C57Bl/6	MWCNT	10	7.1 ± 1.4	7.0 ± 1.5 [99]	0.04 ± 0.05 [1]	0.01 ± 0.03 [1]
MMP12 KO	PBS/Surf	10	10.1 ± 2.9	9.6 ± 2.7 [97]	0.11 ± 0.14 [1]	0.03 ± 0.1 [2]
MMP12 KO	MWCNT	10	17.7 ± 9.0*^	16.7 ± 7.8 [93]*^	1.1 ± 1.0 [5]*^	0.5 ± 0.7 [2]

Table 1. 60D BAL cell characteristics.

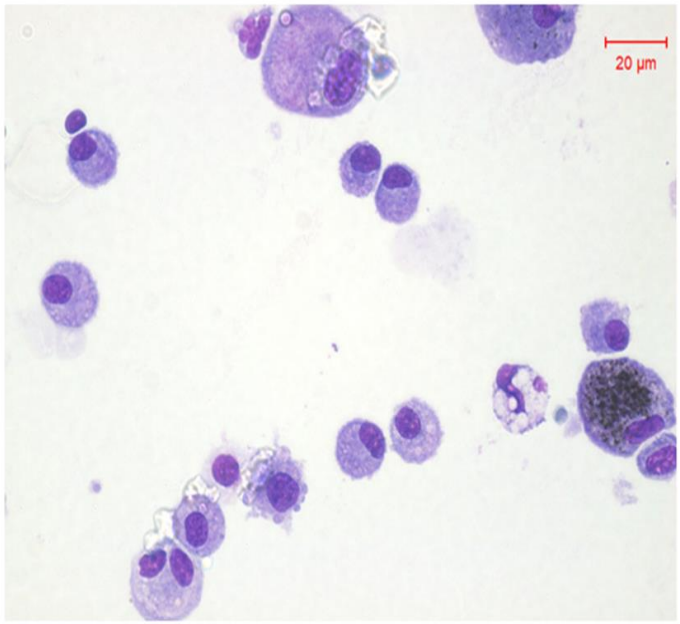
Total cell population and cell subtypes from BAL differentials in 60D C57Bl/6 (wild-type) and MMP12 KO mice.

AM, alveolar macrophages; LYM, lymphocytes; PMN, polymorphonuclear cells.

Means ± SEM, [%], ^ $p \leq 0.05$ relative to MMP12 KO sham, * $p \leq 0.05$ relative to C57Bl/6 MWCNT.

A

C57



MMP12 KO

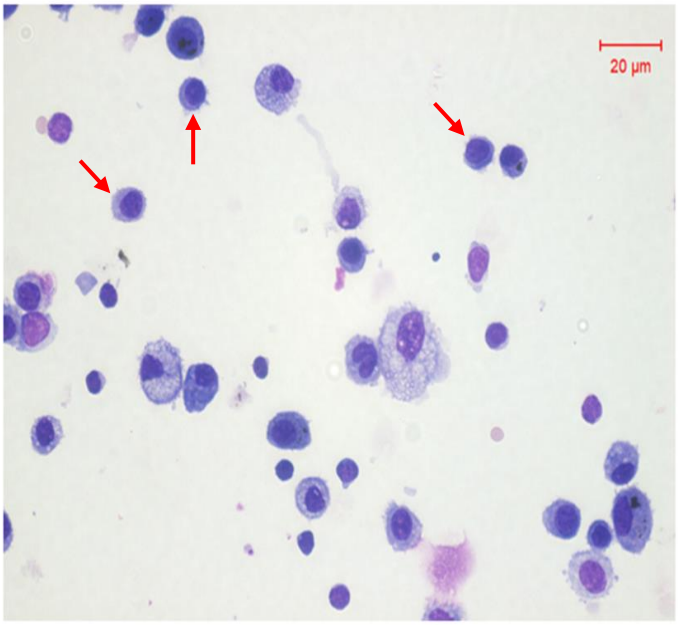
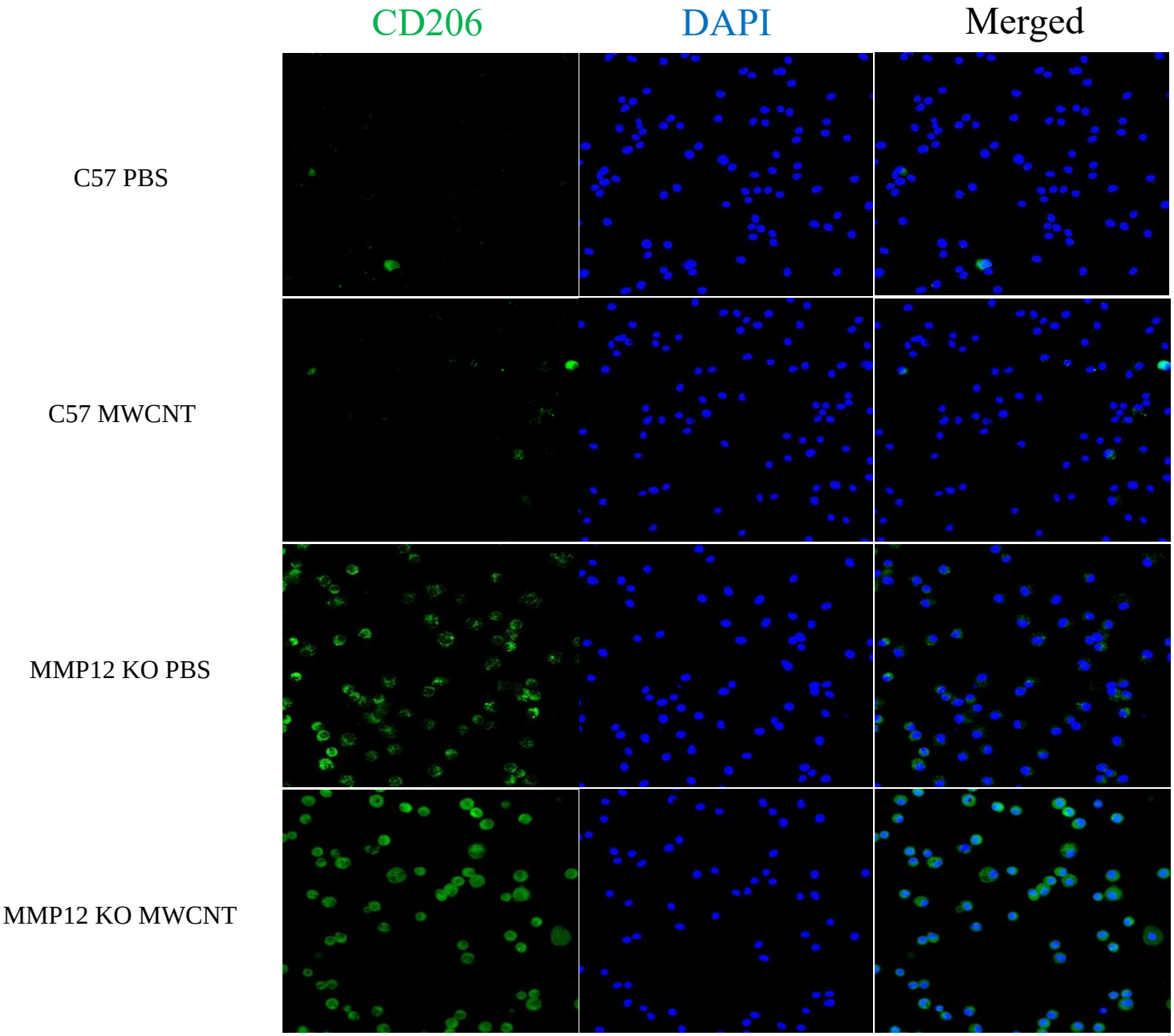


Figure 1. M2 surface marker, CD206, is predominantly expressed in MMP12 KO.

A) Representative 40X images of differential cytospins from 60D post MWCNT instillation wild-type and MMP12 KO mice, $n \geq 6$. A population of smaller alveolar macrophages is observed in the MMP12 KO but is absent in wild-type.

B



B) Representative images of unconjugated CD206 immunocytochemistry staining, n ≥ 3^. Surface expression of CD206, an M2 macrophage marker, is nearly universally expressed in the MMP12 KO sham and MWCNT instilled mice. Few cells stain positive for CD206 in the wild-type strain.

^: 0.1% Tween-20 in 10% goat serum was used to simultaneously block and permeabilize.

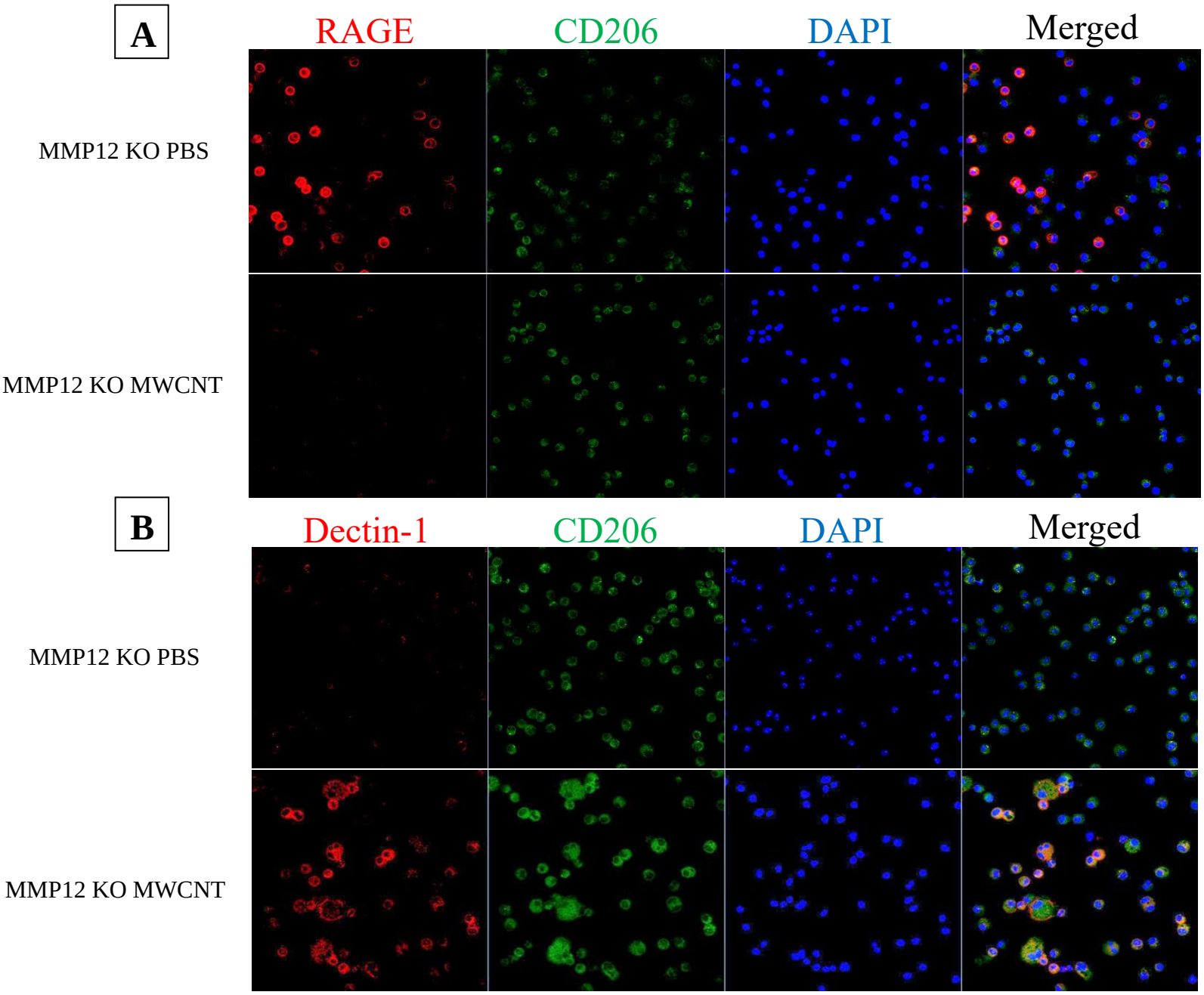


Figure 2. Instillation with MWCNT leads to conversion of macrophage phenotype from M2c to M2a in MMP12 KO.

A) Representative images of RAGE and CD206 immunocytochemistry co-staining^. Surface expression of RAGE, a M2c macrophage marker, is prevalent in the sham-instilled MMP12 KO but is absent in MMP12 KO MWCNT-instilled mice.

B) Representative images of Dectin-1 and CD206 immunocytochemistry co-staining^. Surface expression of Dectin-1, a M2a macrophage marker, was prominent in the MMP12 KO instilled with MWCNT and was low in the sham-instilled MMP12 KO.

^: Conjugated CD206 was added after secondary antibody. $n \geq 3$.

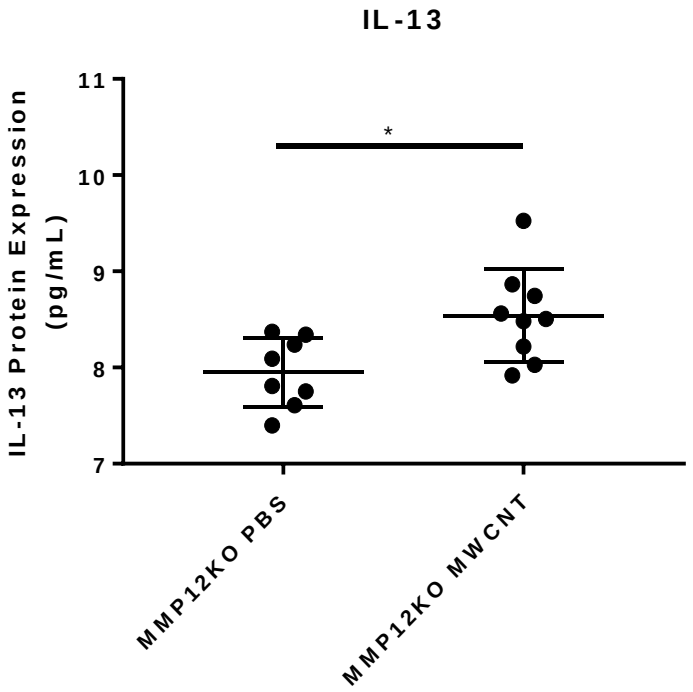


Figure 3. IL-13 is increased in 60D MMP12 KO instilled with MWCNT.

IL-13 protein levels are increased in BAL fluid from MMP12 KO instilled with MWCNT relative to sham. $n \geq 8$.

* $p \leq 0.05$

Discussion:

To summarize, we have been able to delineate that the smaller subpopulation of macrophages seen in the MMP12 KO model are M2 macrophages. While M2 macrophages are observed predominantly in both MMP12 KO instilled sham and MWCNT, a shift is observed within the subtype of M2 macrophages. In the absence of MMP12, M2c macrophages are the predominant immune cells observed. However, when MMP12 KO mice are challenged with MWCNT, a shift in the predominant cell type is observed to the M2a macrophage phenotype. Within our wild-type model where granulomas do not resolve, we observed M1 macrophages as the primary population in the immune response. Thus, the M2a macrophages that are prevalent in MMP12 KO MWCNT-instilled mice at 60D may be involved in the observed granuloma resolution within this model.

In our previous studies of granuloma formation and resolution, we had identified IFNγ as a driver of granuloma formation and persistence, as levels were high within both the MMP12 KO and wild-type MWCNT strains at 10D and remained high in wild-type MWCNT mice at 60D (38). However, IFNγ expression decreases to basal levels in MMP12 KO MWCNT at 60D (38). As IFNγ is indicative of M1 macrophage activation, the decreased levels of IFNγ in MMP12 KO MWCNT at 60D suggest a shift away from the M1 immune response. The prevalence of M2a macrophages and the corresponding increase in the M2 cytokine IL-13 suggest that the M2 immune response is elevated and perhaps counteracting the elevated M1 immune response characterized by IFNγ levels at 10D.

Our current working model is illustrated in Figure 4. The IFNγ elevation seen at the 10D timepoint, suggests that a M1 response may cause the initial formation of granulomas induced by MWCNT in both the wild-type and MMP12 KO models. In the wild-type MWCNT mice, there

is no phenotypic change in the BAL cell population. Due to this, the M1 predominance remains at 60D, and granulomas persist. However, in the MMP12 KO model, we see a M2 predominance and IFNγ expression is correspondingly diminished. In response to MWCNT, we see the macrophage population shift from M2c, as observed in MMP12 KO sham, to M2a. These cells subsequently release M2 cytokines in the form of IL-13, potentiating the M2 immune response. Thus, M2 cytokines increase as M1 cytokines decrease. This enables the immune system to find a balance between the pro-inflammatory and anti-inflammatory responses, which in turn allows for the resolution of granulomas.

We have found the wild-type MWCNT immune response to be M1 dominant and the MMP12 KO MWCNT immune response to be M2 dominant. Optimally, the immune response incorporates a balance of the M1 and M2 immune response, but the pathology of certain diseases, such as Crohn's disease and even sarcoidosis itself, involve a M1-dominant response (59, 60). It is believed that within sarcoidosis, this M1-dominant response to a pathogen or irritant can be attributed to an unknown genetic predisposition (60). Thus, the diminished M1 macrophage response and the potentiation of the M2 immune response by M2a macrophages in the absence of MMP12 may be responsible for granuloma resolution.

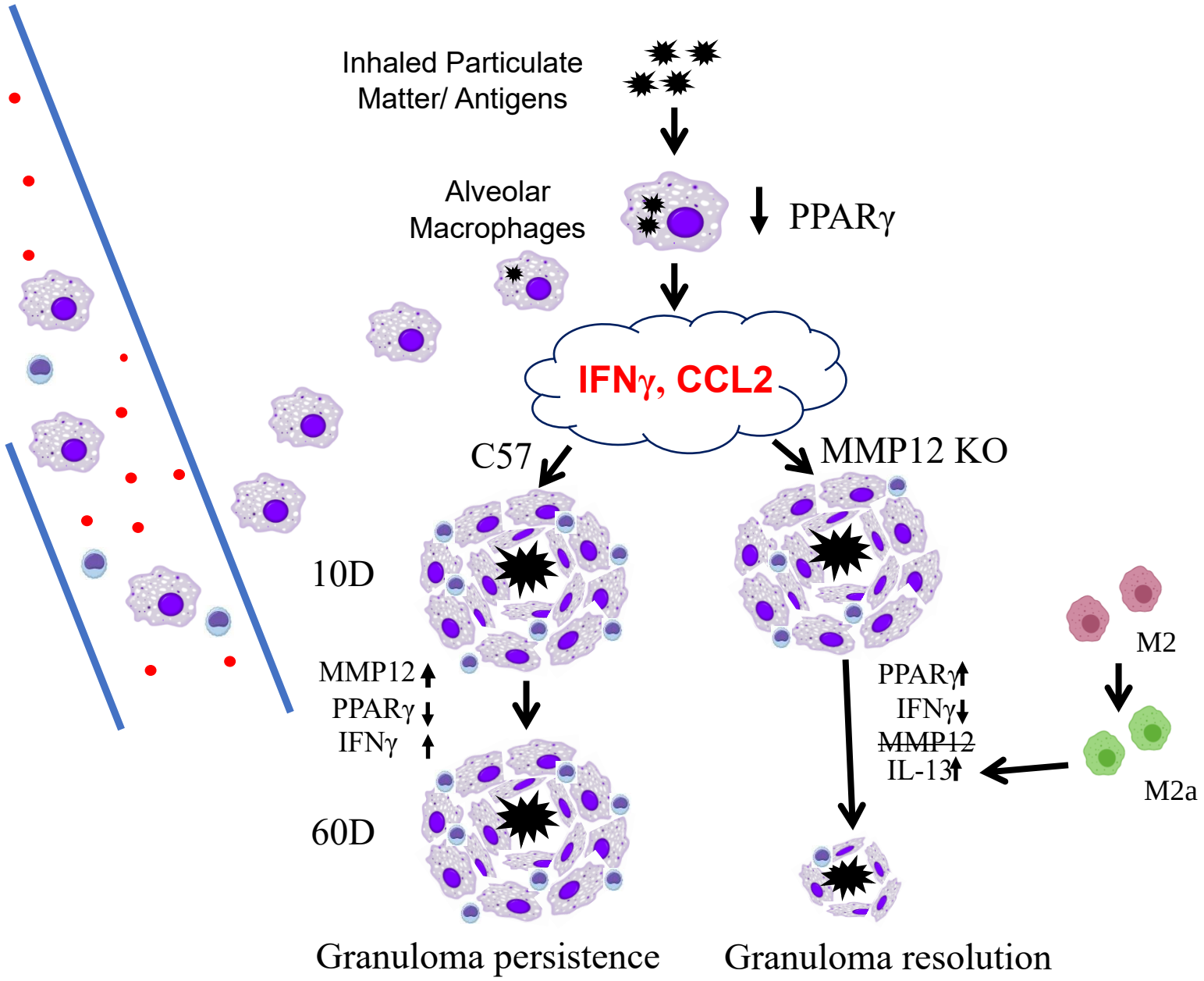


Figure 4. Potential mechanism of granuloma resolution.

The shift in the macrophage population from M2c to M2a causes an increased IL-13 release. This shift corresponds with several other increases that occur between the 10D and 60D timepoint, suggesting that the shift in macrophage population and corresponding increased IL-13 release may be involved in the resolution of granulomatous formations.

CHAPTER 4:

Evaluation of Apolipoprotein E Activity in Granuloma Formation and Resolution.

RESULTS

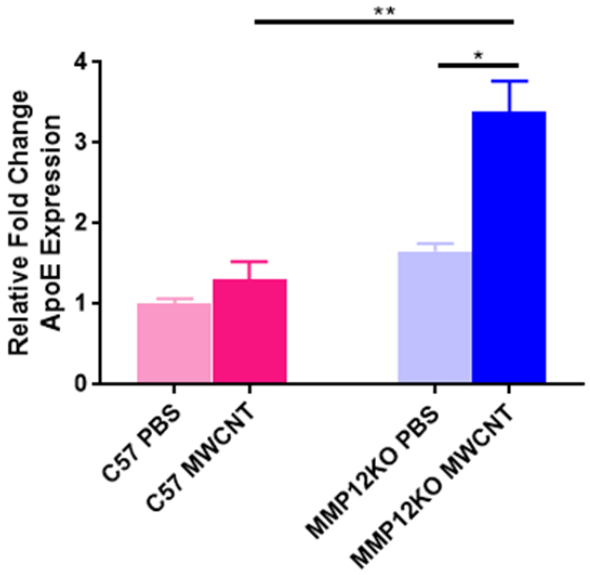
4.1 ApoE gene and protein expression are upregulated in MMP12 KO mice instilled with MWCNT.

Apolipoprotein E is a protein that carries out numerous functions in the body, ranging from lipid transport to cytoskeletal assembly and stability (50). In 2020, macrophage production of ApoE was indicated in the resolution of pulmonary fibrosis (52). More severe cases of sarcoidosis involve lung inflammation that can develop into pulmonary fibrosis, in turn leading to worse patient outcomes (61). Additionally, another group studying sarcoidosis used an ApoE knock out model to induce sarcoidosis-like granulomatous formations, suggesting that decreased ApoE levels could be involved in the pathogenesis of granuloma formation. These links between ApoE and sarcoidosis led us to investigate ApoE expression in both the wild-type and MMP12 KO models. ApoE has previously been linked to macrophage polarization, stimulating the conversion from the M1 phenotype to M2 (55). Additionally, M2 macrophages have been established to have increased ApoE expression (56).

Initially, we explored the gene expression of ApoE in BAL cells through real-time PCR. We found that ApoE expression was significantly elevated in MMP12 KO mice instilled with MWCNT relative to both MMP12 KO sham and wild-type MWCNT at 60D ($n \geq 5$, $p \leq 0.05$) (Figure 5A). Correspondingly, ApoE protein levels in BALF were elevated in MMP12 KO MWCNT relative to MMP12 KO sham and wild-type MWCNT at 60D ($n \geq 4$, $p \leq .05$) (Figure 5B). This observation suggests increased levels of ApoE could be involved in the resolution of granulomas seen in the MMP12 KO strain at the 60D timepoint. Finally, immunocytochemistry

staining yielded that cellular ApoE protein levels are increased within MMP12 KO relative to wild-type (Figure 5C). Within the strains, there is a slight elevation of ApoE in wild-type MWCNT relative to wild-type sham, and an increase in MMP12 KO MWCNT relative to MMP12 KO sham (Figure 5C).

A



B

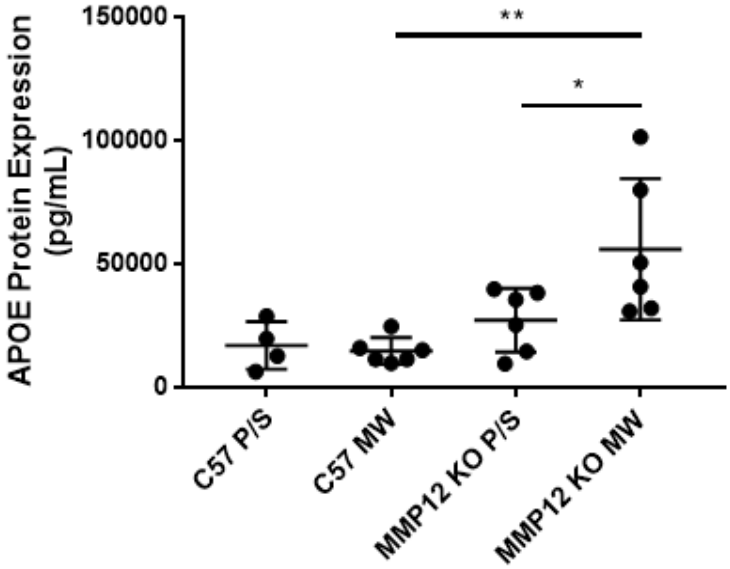
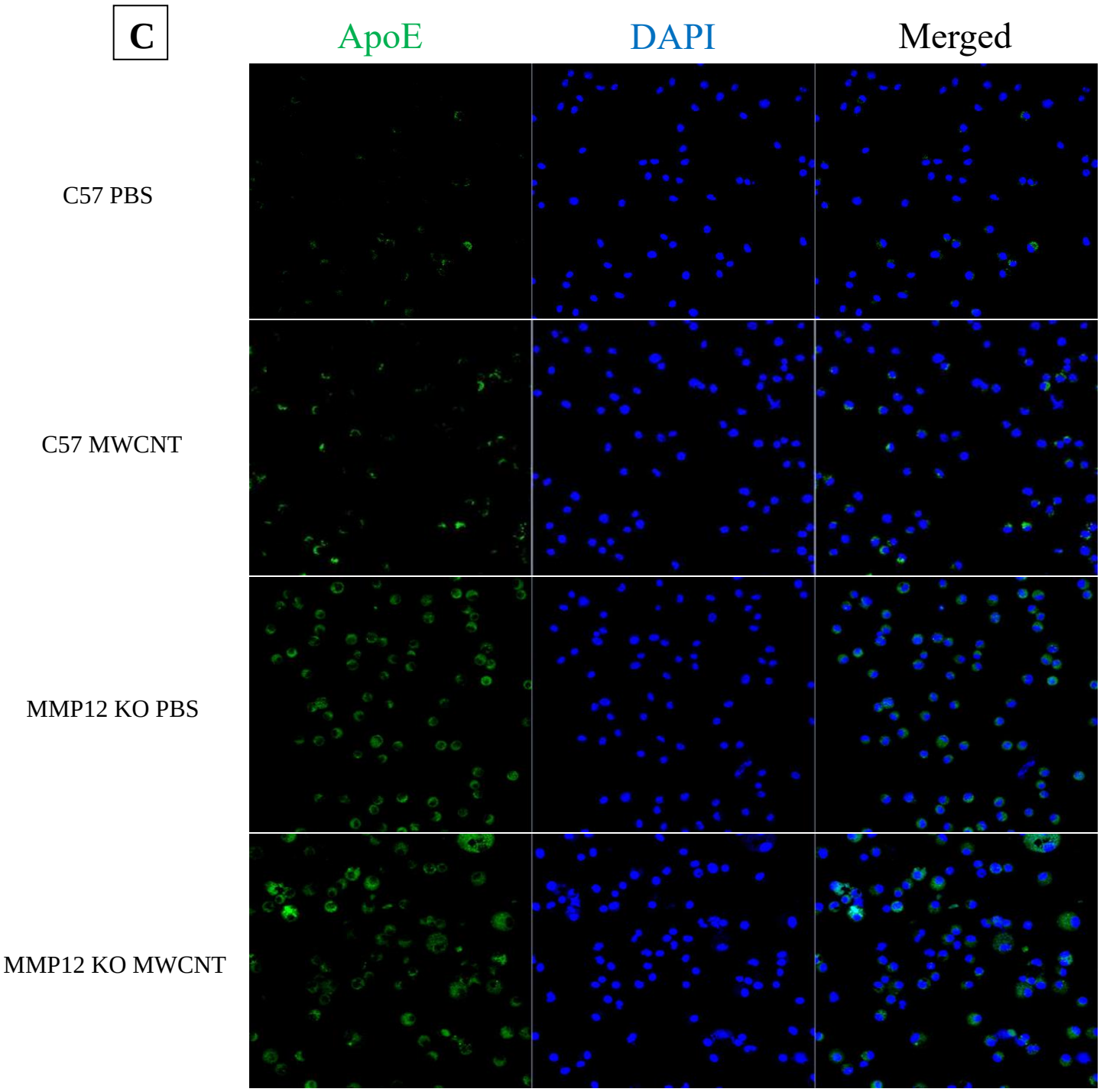


Figure 5. ApoE gene and protein expression are upregulated in MMP12 KO mice instilled with MWCNT.

A) Gene expression of ApoE is elevated in MWCNT-instilled MMP12 KO mice relative to sham-instilled MMP12 KO and MWCNT-instilled wild-type. $n \geq 5$.

B) ApoE protein levels are increased in BAL fluid from MWCNT-instilled MMP12 KO relative to MMP12 KO sham and wild-type instilled with MWCNT. $n \geq 4$.

* $p \leq 0.05$, ** $p \leq 0.01$



C) Representative images of ApoE immunocytochemistry staining, n ≥ 3.^. ApoE expression is low in sham-instilled wild-type mice and slightly elevated in wild-type MWCNT-instilled mice. In the MMP12 KO, expression is higher relative to the wild-type and is increased in the MWCNT-instilled mice relative to sham.

^: Non-permeabilized.

Discussion:

Our results indicate that at 60D, intracellular apolipoprotein E is elevated in the MMP12 KO model relative to our wild-type model, and that the extracellular release of ApoE is increased in MMP12 KO mice instilled with MWCNT relative to sham-instilled. This suggests that in the absence of MMP12, intracellular ApoE levels are high at 60D, and that MWCNT instillation results in its release. A significantly higher level of protein in the BALF of MMP12 KO MWCNT relative to the wild-type MWCNT suggests that high levels of ApoE protein may be involved in granuloma resolution. These observations together with previous studies demonstrating ApoE KO mice form sarcoid-like granulomatous formations support our hypothesis that ApoE is involved in the resolution of granuloma formation (51).

ApoE has previously been indicated in the resolution of pulmonary fibrosis (52). This research found that ApoE carries out this function by binding collagen fragments and carrying them to the LRP1 receptors on macrophages, which will then take up the fragments and degrade them (52). ApoE could be degrading the collagen localized to the periphery of the granuloma that is one of the hallmarks of sarcoidosis (20). Thus, ApoE could potentially decrease the stability of the granuloma itself, allowing it to resolve.

ApoE may be either the cause or an effect of macrophage polarization (55, 56). In wild-type mice, the predominant phenotype of macrophages at 60D is M1. However, in MMP12 KO mice, the macrophages predominantly express the M2 phenotype at 60D. This coincides with high levels of ApoE in BALF at 60D in the MMP12 KO model, suggesting that extracellular ApoE activity may drive the conversion to M2 macrophage phenotype at 60D. This is consistent with previous research indicating that ApoE drives the conversion of M1 macrophages to the M2 phenotype (55). ApoE's activity on M2 macrophage subtype polarization has not been explored,

but we observe higher levels of ApoE in the BALF of MWCNT-instilled MMP12 KO, which predominantly express the M2a phenotype, relative to sham-instilled MMP12 KO, which predominantly express the M2c phenotype. Thus, increased ApoE in BALF could lead to the conversion to the M2a subtype. There is a significantly increased intracellular expression of ApoE gene and protein in the MMP12 KO mice. Correspondingly, the observed M2 macrophage predominance in MMP12 KO mice is associated with increased ApoE expression in our model. This is consistent with previous research (56).

CHAPTER 5:

Evaluation of Macrophage Polarization and Apolipoprotein E Production at 10D in a MMP12 KO Model of Granulomatous Disease.

RESULTS

5.1 CD206 expression is high in both sham and MWCNT-instilled MMP12 KO at 10D.

At the 60D timepoint, we have identified the presence of M2 macrophages in both MMP12 KO sham and MWCNT. Previous studies demonstrated that granulomatous formations at 10D were not different between wild-type and MMP12 KO (38). However, we do not know if there is a shift in the macrophage population between the acute and chronic stages of our model. This led us to attempt to characterize the cell population in 10D MMP12 KO mice. Initially, we attempted to determine whether the macrophages could be characterized as M1 or M2 macrophages by staining for the mannose receptor-1 (CD206). The majority of cells were CD206 positive in both MMP12 KO PBS and MMP12 KO MWCNT (Figure 6; $n \geq 3$). Thus, we determined that M2 macrophages are prevalent in the MMP12 KO strain at 10D, independent of being challenged with MWCNT.

5.2 At 10D, MMP12 KO mice have a high prevalence of M2c macrophages, characterized by surface expression of RAGE and absence of Dectin-1.

We determined M2 macrophages are the predominant population of immune cells at 10D in the MMP12 KO model (Figure 6), just as they are at 60D (Figure 1B). Next, we investigated whether the subpopulation of M2 macrophages shifted from the initial prevalent population to that observed at 60D. The subpopulations were determined through the utilization of Dectin-1 as a marker for M2a macrophages and RAGE as a marker for M2c macrophages. In the co-staining of RAGE and CD206, the macrophages from MMP12 KO PBS are ubiquitously expressing

RAGE, and thus are M2c macrophages (Figure 7A). RAGE expression is also highly prevalent in MMP12 KO MWCNT, rendering it M2c macrophage predominant (Figure 7A). The co-staining of Dectin-1 and CD206 showed no positive cells in the MMP12 KO model at 10D, independent of whether they were sham- or MWCNT-instilled (Figure 7B). Thus, no M2a macrophages were observed at 10D (Figure 7B). However, a subset of the macrophage population from MMP12 KO MWCNT-instilled mice are negative for both M2a and M2c macrophage markers, despite staining positively as M2 macrophages. One potential explanation of this is that this unstained population belong to the M2b subpopulation of macrophages. Alternatively, the unstained macrophages could be in the process of converting from M2c macrophages to the M2a macrophages observed at 60D.

5.3 At 10D, MMP12 KO mice have very low expression of ApoE.

At 60D within the MMP12 KO model, we observe high levels of ApoE. As previously mentioned, the absence of ApoE is associated with sarcoidosis-like granulomatous formations. This led us to hypothesize that at 10D in MMP12 KO mice instilled with MWCNT, when granulomas are forming, that ApoE levels would be low. To test this theory, we utilized immunocytochemistry to stain for ApoE in BAL cells. ApoE was only observed at very low levels intracellularly in MMP12 KO at 10D (Figure 8). Thus, the increased protein expression of ApoE observed at 60 days may be involved in granuloma resolution.

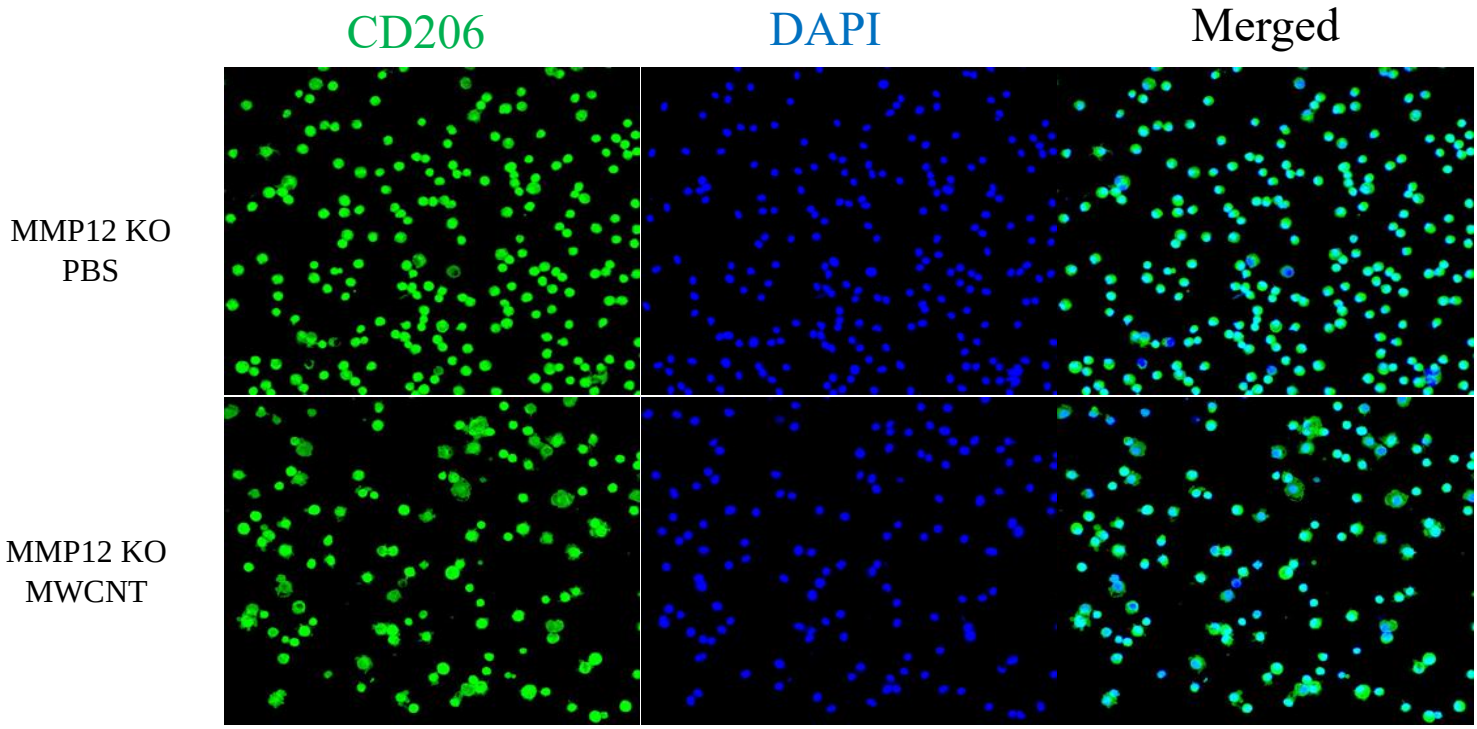


Figure 6. CD206 is universally expressed in both sham and MWCNT-instilled MMP12 KO mice 10 days post-instillation.

Representative images of CD206 immunocytochemistry staining, $n \geq 3$. Surface expression of CD206 was seen in nearly all cells.

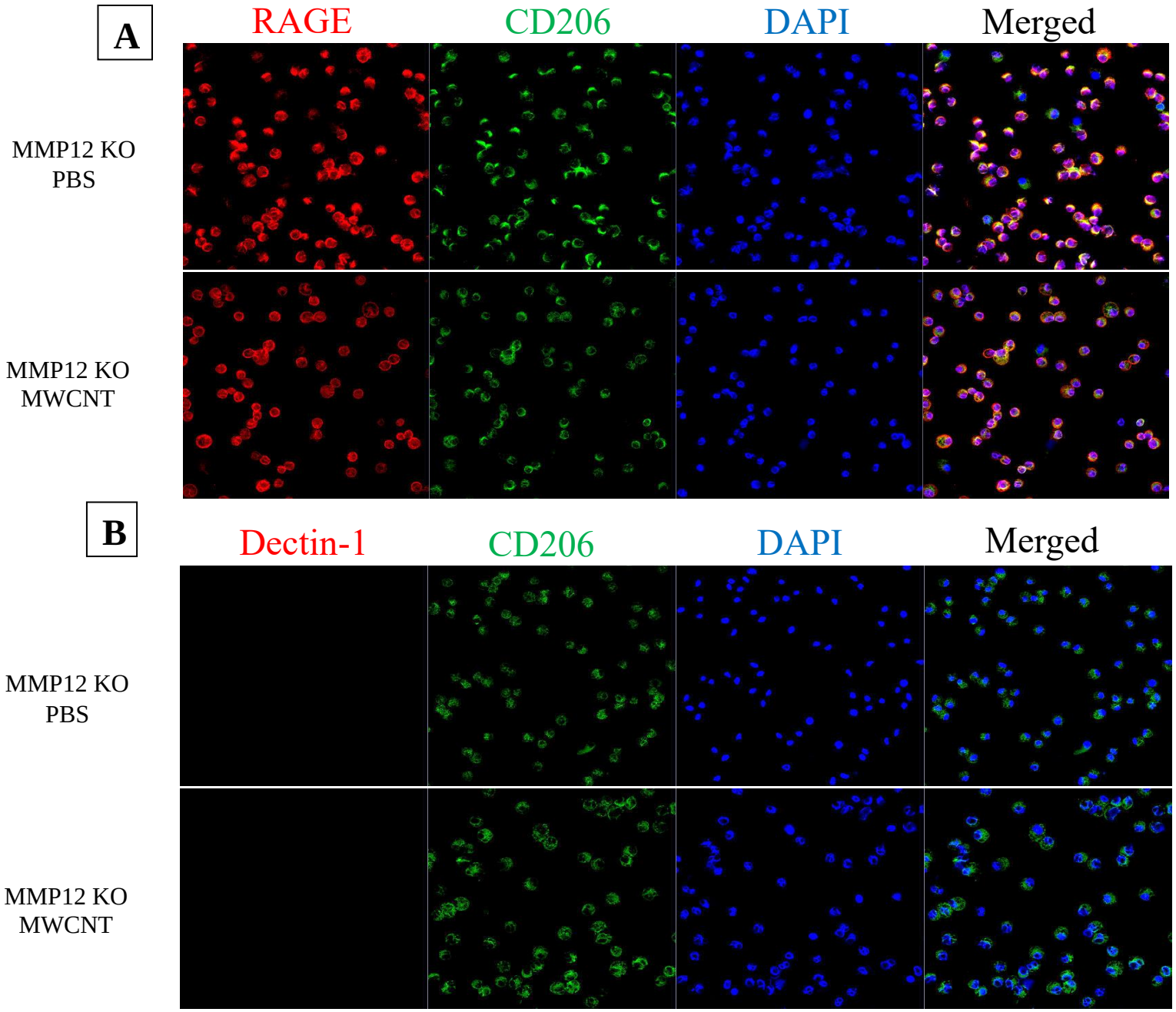


Figure 7. At 10D MMP12 KO mice have a high prevalence of M2c macrophages, characterized by surface expression of RAGE and absence of Dectin-1.

A) Representative images of RAGE and conjugated CD206 immunocytochemistry co-staining, $n \geq 3$. MMP12 KO PBS immune cells had ubiquitous surface expression of RAGE. In MMP12 KO MWCNT, a majority of cells have surface expression of RAGE.

B) Representative images of Dectin-1 and conjugated CD206 immunocytochemistry co-staining, $n \geq 3$. In both MMP12 KO PBS and MWCNT, Dectin-1 is not expressed on the surface of any immune cells.

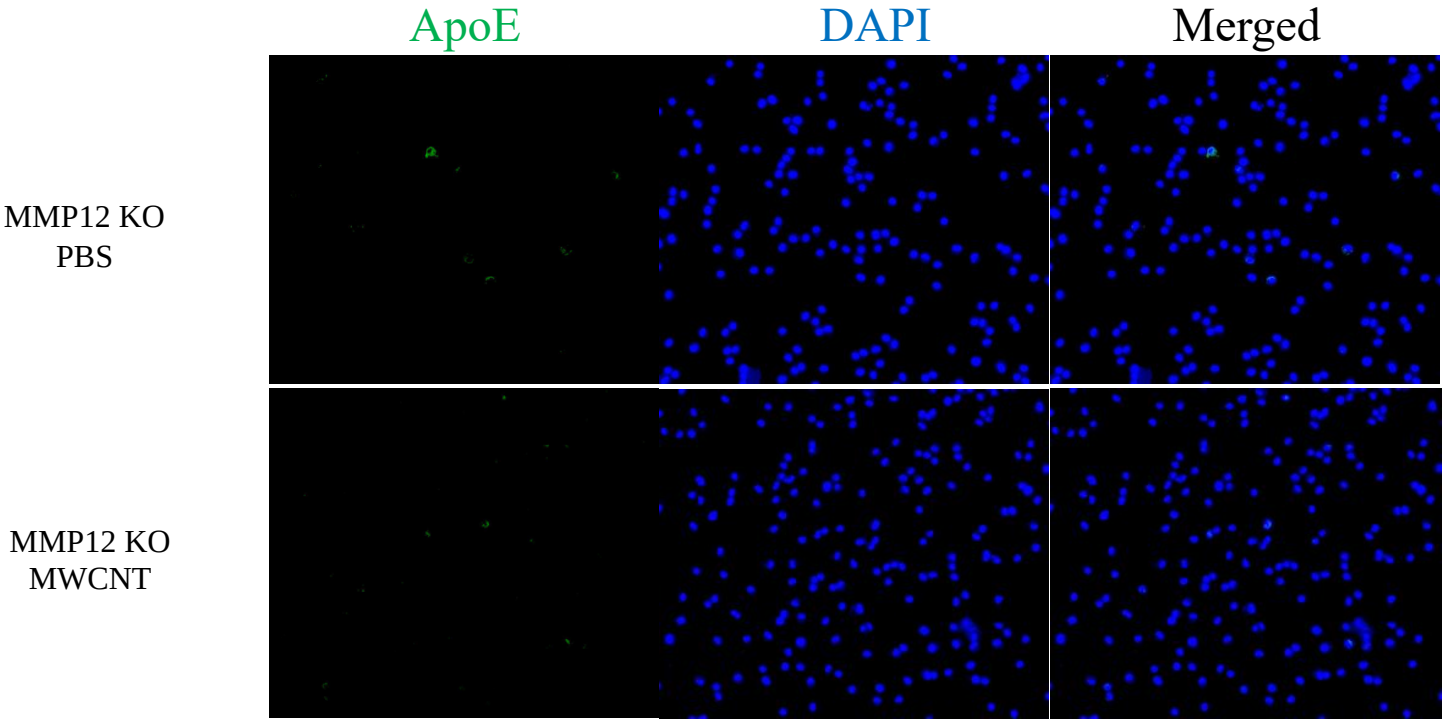


Figure 8. At 10D, MMP12 KO mice have very low expression of ApoE.

Representative images of ApoE immunocytochemistry staining, n ≥ 3. Both MMP12 KO PBS and MMP12 KO MWCNT have few ApoE-expressing cells.

Discussion:

Our lab has previously established that granulomas form in MMP12 KO MWCNT-instilled mice at 10D but are resolving by 60D. By incorporating the same experimentation at the earlier timepoint where granulomas are present, we were able to examine the changes that are occurring between the two timepoints, and thus the changes associated with granuloma resolution.

At the 10D timepoint, there is ubiquitous expression of M2 macrophages in both MMP12 KO PBS and MWCNT. Thus, M1 macrophages, which are typically the first line of defense in the immune system, are not present at the initial formation of the granuloma. M2c macrophages, characterized by RAGE expression, are the predominant immune cell present in the MMP12 KO sham-instilled mice at both the acute and chronic stage of our model, and thus do not undergo a phenotypic transition. However, a change in this population does occur in the MMP12 KO mice instilled with MWCNT. At 10D, the primary population of macrophages are M2c, just as in the sham-instilled mice. However, a shift in this population occurs between 10D and 60D, as the primary macrophage population present in MMP12 KO MWCNT at 60D is M2a. Thus, the presence of these macrophages and their corresponding function in increasing the M2 immune response may play a role in the granuloma resolution observed at 60D.

At 10D, ApoE protein is not expressed at high levels in MMP12 KO sham or MWCNT. Thus, it is not present when granulomas are present in 10D MMP12 KO mice instilled with MWCNT. This supports our hypothesis that elevated levels of ApoE are associated with granuloma resolution, while granuloma formation is associated with low levels of ApoE.

In our model, we have thus established a relationship between increased ApoE levels and the resolution of MWCNT-induced murine sarcoid-like granulomas. ApoE and its isoforms have

been strongly linked to Alzheimer's disease, with literature showing lower levels of ApoE in patients with Alzheimer's disease relative to controls (62, 63). Thus, increasing serum ApoE levels has become a potential therapeutic target in the disease. In mice, administration of the RXR agonist bexarotene, LXR agonists, and the PPARγ agonists rosiglitazone and pioglitazone have been associated with increased serum levels of ApoE (62). In the context of our work, these studies suggest that RXR, LXR, and PPARγ agonists are potential therapeutics that could assist in the resolution of granulomas. Our lab previously utilized rosiglitazone, a PPARγ agonist, on wild-type MWCNT-instilled mice and found that its administration decreased granuloma size (64). The mechanism of ApoE's action in the resolution of granulomas needs to be further explored.

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APPENDIX A:

Animal Use Protocols

ECU

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27354 – 4354
252-744-2436 office | 252-744-2355 fax

October 23, 2020

Mary Jane Thomassen, Ph.D.
Department of Internal Medicine, ECU

Dear Dr. Thomassen:

Your Animal Use Protocol entitled, " Role of MMP-12 in Fibrosis Progression in a Murine Model of Granulomatous Disease" (AUP #J207a) was reviewed by this institution's Animal Care and Use Committee on 10/22/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure

ECU

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27354 - 4354
252-744-2436 office | 252-744-2355 fax

June 23, 2020

Mary Jane Thomassen, Ph.D.
Department of Internal Medicine, ECU

Dear Dr. Thomassen:

Your Animal Use Protocol entitled, "Carbon Nanotubes as a Tool for Generating an Experimental Model of Pulmonary Sarcoidosis" (AUP #J199d) was reviewed by this institution's Animal Care and Use Committee on 06/22/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure

www.ecu.edu

APPENDIX B:

List of qPCR Primers

Target Name	Qiagen Catalog Number
ApoE	PPM04128B
GAPDH	PPM02946E